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THE IN VITRO EFFECTS OF HOMEOPATHICALLY PREPARED RUBUS IDAEUS AND LOW LEVEL LASER THERAPY ON CERVICAL CANCER CELLS

A dissertation submitted to the Faculty of Health Sciences, University of Johannesburg in partial fulfilment of the requirements for the Degree of Masters in Technology, Homoeopathy by

Kelley Sue Joubert
(Student number: 201383285)

Supervisor: Dr Radmila Razlog

Co-Supervisor: Prof Heidi Abrahamse

Co-Supervisor: Dr Blassan P. George
ACKNOWLEDGEMENTS

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- This study was financially supported by the Department of Complementary Medicine, Laser Research Centre, CSIR and the National Laser Centre.
- Fusion Homeopathics Company for supplying the homeopathic remedies.
ABSTRACT

Cervical cancer (CC) is one of the most common cancers in the world, considered to be a direct result from infection with the Human papillomavirus (HPV). Other possible contributing factors towards CC include multiple sexual partners, having sexual activity from a very early age and immunodeficiency. In 2014, it was concluded that 19 098 women in South Africa died as a result of CC. Another report noted that CC is the most common form of cancer amongst black South African women, particularly those over the age of 35 years. Rubus idaeus (commonly referred to as red raspberry), is a plant used most commonly to treat disorders of the female genital tract. It is said to possess high antioxidant properties and has shown many cytotoxic properties in cases of cancer in recent studies. Low level laser therapy (LLLT) makes use of specific wavelengths and fluence to repair damaged tissue. Some in vivo studies done previously suggest that LLLT has the ability to induce apoptosis in cancer cells. No research demonstrating the relationship between Rubus idaeus (R. idaeus) and LLLT on CC has been conducted to date.

The aim of this study was to compare the in vitro effects of R. idaeus (Homeopathic mother tincture, D6 and 30cH) and LLLT on CC cell lines.

This quantitative in vitro research study took place at the Laser Research Centre of the University of Johannesburg. This study made use of HeLa cells (ATCC ccl-2) and R. idaeus (D3, D6 and 30 cH Homeopathic preparations) and LLLT at 680 nm at 5, 10 and 15 J/cm² doses. A HeLa cell culture (ATCC CCL-2) was cultured in Dulbecco’s Modified Eagle Medium and sub-cultured from 75 cm² flasks into 3.3 cm diameter culture plates for experimentation. There were three cell groups in this study with each experiment repeated a total of 3 times in duplicate. Each of the three experimental cell groups were divided as follows; untreated control cells (CC cells not exposed to R. idaeus or LLLT), CC cells treated with R. idaeus (D3, D6 and 30cH), CC cells treated with a combination of the optimal performing potency of R. idaeus and LLLT at 680 nm diode lasers with three fluencies of 5, 10 and 15 J/cm². The effectiveness of these treatments were measured as follows; cell-viability was measured by using Trypan Blue assay, morphology by inverted light microscope and cytotoxicity was measured by observing the different levels of Lactate Dehydrogenase (LDH) present in the culture medium. Apoptosis of the HeLa cell culture was analyzed via Annexin V/PI and Hoechst stain and
proliferation was observed by measuring Adenosine Triphosphate (ATP) levels in the cells.

The experimental procedure was designed as a means to determine if there was a difference in the outcome of CC cell death if the cells were exposed to a single treatment with various potencies of *R. idaeus* or a combined treatment with the optimal potency of *R. idaeus* (D3) with laser 680 nm after a 24 h interval. The single and combined treatment protocol was completed a total of 6 times (n=6) for all experiments except for Annexin V/PI staining where the experiments were completed a total of 3 times (n=3). The data for both experimental procedures were analysed by using Sigma Plot version 14.0 computer software. The t-test was used in order to examine the effects of the various treatment protocols in inducing cancer cell death. In each instance, a statistical difference of *p*<0.05 was identified.

Morphological changes indicated that the single treatment application of *R. idaeus* D3 had the ability to induce CC cell death *in vitro* however this was not the case in the combined treatment protocol of *R. idaeus* D3 and LLLT 680 nm. The combined treatment protocol demonstrated a photobiomodulatory effect which enhanced cancer cell growth.

Based on statistical analysis, the results demonstrated that a single treatment protocol of *R. idaeus* D3 increased apoptosis of CC cells *in vitro*. However, more research needs to be conducted in order to examine the systemic effects of this remedy preparation.
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### LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>nm</td>
<td>Nano meter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>J/cm²</td>
<td>Joules per centimeter squared</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>mW</td>
<td>Milliwatts</td>
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<td>Seconds</td>
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# LIST OF ACRONYMS AND ABBREVIATIONS

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<td>Centesimal Hahnemanni 12</td>
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<tr>
<td>30cH</td>
<td>Centesimal Hahnemanni 30</td>
</tr>
<tr>
<td>AMPK</td>
<td>5'- Adenosine- monophosphate- activated Protein Kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>C. maculatum</td>
<td>Conium maculatum</td>
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<tr>
<td>CC</td>
<td>Cervical Cancer</td>
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<td>Cluster of Differentiation 133</td>
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<td>CD31</td>
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<td>cH</td>
<td>Centesimal Hahnemanni</td>
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<tr>
<td>CIN</td>
<td>Carcinoma in situ</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>D/X</td>
<td>Decimal Ratio</td>
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<tr>
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<tr>
<td>D6</td>
<td>Decimal Hahnemanni 6</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
</tr>
<tr>
<td>Dr</td>
<td>Doctor</td>
</tr>
<tr>
<td>E6</td>
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<tr>
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<td>Endoplasmic Reticulum</td>
</tr>
<tr>
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<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas Cell Surface Death Receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>G- Protein</td>
<td>Guanine Nucleotide-Binding Protein</td>
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<tr>
<td>HAB</td>
<td>Homeopathic Pharmacopoeia</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks Cervical Cancer Cells</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
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<td>HPV E7</td>
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<td>IGF I</td>
<td>Interstitial Growth Factor Type 1</td>
</tr>
<tr>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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LEDs Light Emitting Diodes

LLLT Low Level Laser Therapy

MIKI67 Marker of Proliferation Ki-67

mW/cm² Milliwatts per Centimeter Squared

NAD+ Nicotinamide Adenine Dinucleotide

N-ras Protein Guanosine Nucleotide Binding Protein Type N

P53 Protein 53

Pap smear Papanicolaou smear

PDT Photodynamic Therapy

*R. idaeus* *Rubus idaeus*

RI *Rubus idaeus*

RID3 *Rubus idaeus* D3

RID6 *Rubus idaeus* D6

RID30cH *Rubus idaeus* 30cH

*S. serrulata* *Sabal serrulata*

S2- S4 Sacral Spinal Nerve Roots 2 to 4

*T. occidentalis* *Thuja occidentalis*

TNF-α Tumour Necrosis Factor Alpha

VEGF Vascular Endothelial Growth Factor

XPC Xeroderma Pigmentosum Type C
OUTPUTS OF THE STUDY

This work was presented at the following conferences and workshops:

- Laser Research Centre Journal Club, University of Johannesburg (oral presentation).

The following proceedings emanated from this study:


Awards

- African Laser Centre and Laser Research Centre, Biophotonics in Cancer: Redefining Trends in Cancer, First Place for Best Poster Presentation, September 2019
- Faculty of Health Sciences, Laser Research Centre, Top Achievers Ceremony, Best Performing Student in Research Centre, November 2019
- Laser Research Centre, Journal Club, Best Master’s Presentation, November 2019
CHAPTER 1.
INTRODUCTION

1.1. Foreword

Cancer is a disease which results from the dysregulation of genes leading to a reduction in check-point control accuracy and increased cellular proliferation of abnormal cells (Meyerson et al, 2011). Cervical cancer is one of the most prominent cancers which exists amongst women, and is considered to occur most commonly in women who have low counts of IGF-1 (interstitial growth factor-1) (Serrano et al., 2006). Currently, CC is diagnosed by means of a pap smear test which produces false negative results in 5-10% of cases (Kitchener et al., 2006). The conventional treatment of CC includes full hysterectomy and chemo-radiotherapy (Martin, 2018).

Homeopathy is a form of complementary medicine which has existed since the early 1800s after being discovered by a German physician named Dr Samuel Hahnemann (Kalra, 2016). This form of medicine has slowly increased in popularity due its low number of side effects and affordability. In recent years homeopathic medicine has shown an ability to interact on a genetic level due to its newly found nano-particle structure which leads to the apoptosis of cancer cells (Saha et al., 2013).

*Rubus idaeus* is a plant which contains high levels of antioxidants and polyphenols which are believed to reduce tumour volume and incidence (Giao et al., 2012; de Souza et al., 2014). This plant is typically used as a female tonic in the treatment of female uterine and reproductive disorders and has further shown the ability to induce apoptosis in lung cancer cells by disrupting the ERK1/2 pathways (Hsieh et al., 2013).

Low Level Laser Therapy (LLLT) makes use of light emitted at various wavelengths in order to promote either tissue rejuvenation of diseased normal tissues or to induce cellular apoptosis in cancer cells. The most effective wavelengths observed include those which exist between 600-1070 nm (Chung et al., 2012). LLLT promotes cancer cell apoptosis by activating the release of cytochrome c-oxidase into the cytoplasm of the cancer cell in a non-invasive manner (Bartos et al., 2016; Svanberg et al., 2010).
1.2. Statement of the problem

Cancer has become an incredibly prominent occurrence amongst South Africans, with an estimated 47 350 deaths occurring as a result. This large number of deaths is most likely associated with the prevalence of obesity, smoking and communicable diseases in South Africa. Over time, it has been observed that the mortality rates have increased drastically with the percentage of deaths caused by breast cancer being at 21%, prostate cancer 12% and cervical cancer (CC) 16%. The rapid increase in recorded mortality rates may be attributed to an increase in awareness, diagnosis and improved cancer screenings (Made et al., 2017).

Currently there are Human Papilloma Virus (HPV) vaccinations available, however a vast majority of women (particularly those who are in the poorer rural areas) do not have access to these vaccinations due to insufficient funds and poor access to health clinics. Various imaging technologies are used in the diagnosis of CC, such as computed tomography and ultrasonography. These techniques however are considered to have a low sensitivity and a high occurrence of producing false-positives on screening (Amaro-Filho et al., 2013).

The conventional treatment of CC includes intense therapeutic drugs such as cisplatin which leads to the development of various side effects (Aredes et al., 2018). A comprehensive treatment plan for CC including complementary therapies may increase cancer cell death and decrease treatment time to allow for more comfort for patients. This could potentially reduce the amount of time in hospitals and further reduce nursing time allowing for the patient to potentially return to normal life activities sooner.

1.3. Aim and Objectives

The aim of this study was to evaluate the effectiveness of LLLT (680 nm at fluencies of 5, 10 and15 J/cm²) and Rubus Idaeus homeopathic potencies (D3, D6 and 30cH) on cervical cancer cells, in vitro. In addition, the experimental procedure was designed to determine if there was a difference in outcome if cells were exposed to a single treatment or a combined treatment protocol.

The study was conducted with the following specific objectives:
· Determine the viability and morphology of CC cells using Trypan Blue assay and inverted light microscope after the treatment with *R. idaeus* (30cH, D6 and D3), the optimal potency of *R. idaeus* and LLLT.

· Observe the changes in cellular proliferation and cytotoxicity of CC cells by measuring ATP and LDH levels after the treatment with *R. idaeus* (30cH, D6 and D3) and a combination of the optimal potency of *R. idaeus* and LLLT.

· Study the CC apoptotic cell mechanism using flow cytometry and observe nuclear damage by Hoechst staining after the treatment with a combination of the optimal potency of *R. idaeus* and LLLT and *R. idaeus* (30cH, D6 and D3)

### 1.4. Importance of the study

The importance of this study is to continue to identify new additional treatment options in the treatment of cervical cancer. This study may demonstrate the ability for alternative therapies such as homeopathic medicine and LLLT to treat CC successfully as well as to identify the relationship that *R. idaeus* has with a 680 nm laser when used as a photosensitizer. This study serves as the first step towards establishing whether or not this treatment could be considered a treatment option in the treatment of CC.
CHAPTER 2.
LITERATURE REVIEW

2.1. The Cervix

The cervix or cervix uteri (Latin meaning ‘neck of the uterus’) is the lower part of the uterus in the human female reproductive system. This bulbous, predominantly fibrous organ is of great structural and functional importance to the uterus. It plays an integral role in the maintenance of pregnancy, conception and the deliverance of a baby (Myers et al., 2015).

2.1.1. Anatomical Structure

The cervix is cylindrical in shape and is situated on the uterine corpus at the lower pole. The typical length of the cervix is 25 mm with an anteroposterior diameter measurement of 20-25 mm and a measurement of 25-30 mm transversely (Nott et al., 2016). The cervix is divided into two portions; the protio supravaginalis and the portio vaginalis. The anatomical location of the cervix can be described as anterior to the rectum and posterior to the base of the bladder. A vesicouterine pouch is located between the bladder and superior portion of the uterus. Posterior to the cervix is a ligament known as the rectouterine pouch and is formed as a result of the reflection of the peritoneal tissue inferiorly from the cervix to the posterior vaginal fornix and eventually the rectum. Two ligamentus pairs hold the cervix into position, these ligaments are named the transverse cervical ligaments (Nott et al., 2016). The main blood supply of the cervix arises from the uterine artery with minor contributions from the vaginal arteries which originate from the internal iliac arteries. There are four regions of blood supply at the uterocervical junction; outer region, a venule and arteriole region, endocervical capillary region and pericanalar zone (which contains all small capillaries and veins) (Walocha et al., 2012; Bereza et al., 2012). The uterine cervix is collagenous in structure with muscle contributing to 15% of its structure. The cervix is supplied by nerves which originate from S2-S4 sacral routes. These nerves carry parasympathetic pathways (Abbas et al., 2019).

2.1.2. Histological Structure

The cervix contains no glands, however there are cryp-like structures which exist as a result of columnar epithelial infoldings. These structures secrete a mucous which function
as a means to store spermatozoa after intercourse (Nott et al., 2016). The endocervical canal is lined by columnar epithelium which possesses a mucous-secreting function. This columnar epithelium layer extends towards the squamous epithelium of the vagina and forms an area named the squamocolumnar junction. It is this area which is the most prone to malignancy (Beckmann et al., 2010). The cervical stroma consists of 70% type I collagen and 30% type III collagen. Overall, the subepithelial stroma consists of 80% collagen. The stroma also consists of smooth muscle which has a scattered distribution. This smooth muscle is considered to have no function due to the immaturity of fibre (Nott et al., 2016).

2.2. HeLa Cells

HeLa cells are named after Henrietta Lacks, who died as a result of cervical adenocarcinoma in the year 1951. Henrietta Lacks had initially sought help from the Johns Hopkins Gynaecology Clinic after experiencing symptoms which included intermenstrual bleeding (Surgical Biopsy number 92498, 1951). Upon examination, a 2.54 cm lesion was observed on the cervix (Jones, 1997). The cervical Biopsy conducted on Henrietta Lacks was given to the Tissue Culture Laboratory in the Department of Surgery at Johns Hopkins Hospital for further research to be conducted. Dr Gey and his wife Margaret Gey worked in association with Dr Lewis during 1922 in a work associated with tissue culture and in vitro investigations (Harvey, 1976). The CC cells of Henrietta Lacks were the first cell specimens to grow exponentially, allowing them to become the first cell line to be immortalised in tissue culture. From this point onwards, the cells have been named HeLa cells in honour of Henrietta Lacks (Lucey et al., 2009).

2.3. Human Papilloma Virus (HPV)

Human papilloma virus is a virus which belongs to the Papilloma viridiae family (Harden & Munger, 2017) and has a prevalence of 24% amongst women in Sub-Saharan Africa (Stewart et al., 2018). Those infected with HPV are usually asymptomatic; however, those who display symptoms show signs of lesions on the cervix which are usually self-limiting (Doorbar, 2017). Viruses in this family consist of non-enveloped DNA which have an affinity for the squamous epithelium (Harden & Munger, 2017). HPV is regulated by various factors such as viral integration, methylation and aberrations of the host’s genes (Baldwin, 2017). HPV E6 and 7 oncoproteins allow for genomes to be amplified in the upper layers of the epithelium, whereby the virus interacts with the adaptive immune
response and keratinocytes of the host. The epithelial cells continually proliferate with a delay in cell differentiation, thus leading to the development of neoplasia and eventually cervical cancer as a result of transformation of atypical cells into cancer cells (Harden & Munger, 2017)

2.4. Cancer

2.4.1. Background

Cancer occurs as a result of abnormal cellular proliferation. There are currently more than one hundred different types of cancer which exist with each cancer type varying in symptomatology and behaviour to therapy. A tumour is considered an abnormal proliferation of cells which can result in either malignant or benign growths. The distinguishing factors between a benign and malignant tumour are simple; a malignant tumour has the ability to metastasise into surrounding tissues whereas a benign tumour typically remains contained within its location. Malignant tumours typically metastasise via lymphatic ducts and the circulatory system. Tumours are classified in accordance with the type of cells that they consist of. Carcinomas are tumours consisting of epithelial cells, sarcomas are tumours consisting of various connective tissues such as fibrous tissue and bones. Lastly lymphomas and leukaemias are cancers arising from cells which exist in the circulatory system (blood cells and immune cells) (Cooper, 2000).

2.4.2. Cancer Development

Tumours arise from a single cell which demonstrates an inactivation of the X-chromosome which is then further converted into heterochromatin. In cancer cells, it has been further observed that only one heterozygous allele of the X-chromosome is expressed as compared to the normal two (Cooper, 2000). This dysregulation and heterogeneity thus result in a disruption in the intrinsic mechanism responsible for cell differentiation and proliferation. Cancer cells have the ability to proliferate rapidly and continually, due to a destruction in check-point controls at a genetic level (Meyerson & Pellman, 2011). The further changes which occur include rearrangement of chromosomes along with point mutations. These changes result in a newly termed phenomenon “chromothripsis”- multiple genomic rearrangements occurring in a once-off cellular crisis (Sephens et al., 2011). Genes which are related to cancer development after undergoing a point mutation include; G-proteins (guanine nucleotide-binding protein), N-ras proteins
and triphosphate-binding proteins (Koeffler et al., 1991). These mechanisms can be observed in infections with Human papillomavirus (HPV).

Cancer cells possess various types of cellular surface markers such as CD44, CD166, CD24 and CD133. These markers allow for each cancer type to be accurately diagnosed from primary tumour tissues, however these markers are considered to be unstable in somatic cell cancers (Madka & Rao, 2011).

2.5. Cervical Cancer

CC is considered the major cause of death amongst women worldwide, with the main aetiology being a history of infection with HPV (Chen et al., 2018) particularly strains 16 and 18 (Markowits et al., 2018). The gene responsible for the formation of CC is named the Sox2 gene which causes tumorigenicity and increased cellular proliferation (Ji & Zeng, 2010).

2.5.1. Risk Factors, Incidence and Symptoms of Cervical Cancer

Various risk factors such as participating in intercourse at an early age, having intercourse with multiple partners, women over the age of 40 and multiple pregnancies are said to play a major role in the development of CC (Patil et al., 2018). Some research has observed that women with low levels of Insulin like growth factor (IGF)- IGF-I and IGF-II: Insulin like factor binding protein (IGFBP)-3 had a significantly higher risk of developing cervical cancer (Serrano et al., 2006). CC is more prevalent in women who seldom go for cervical screening, or who have avoided screening for five or more years (Williamson & Snyder, 2015). Made (2017) observed statistics from 2014 and discovered that in South Africa alone, there is an estimated 47 350 deaths which occur as a result of CC. As of 2017, there is an estimated 500 000 cases of CC reported worldwide each year with 50% of those who are diagnosed succumbing to the disease (Harden & Munger, 2017). It has been observed that of the women diagnosed with CC, 43% were under the age of 45 and 20-28% were under the age of 40 years (Wang & Chen, 2018).

The most common sites of cancer formation after HPV infection include the vagina and vulva (Doorbar, 2017), with symptoms ranging from intermenstrual bleeding, foul-smelling vaginal discharge, lower abdominal pain and haematuria (Stewart et al., 2018). Some women may be asymptomatic, or present with other symptoms such as lower or pelvic back pain (Williamson & Snyder, 2015).
2.5.2. Pathophysiology

CC is usually a result of recurrent infection with HPV, particularly strains 16 and 18. Infection with HPV results in a change of epithelial cells at the squamocolumnar junction (Williamson & Snyder, 2015) leading to invasive CC by driving cells into the synthesis-phase (Amaro-Filho et al., 2013). E2F proteins, a transcriptase factor, is responsible for the transactivation of cellular cyclin-dependant kinases (CDKs) proteins which are responsible for viral DNA replication taking place. This DNA replication is responsible for the development of cancer. E6 is responsible for the degradation and ubiquitination of p53, thus resulting in a decreased effectiveness of the damage response of the cellular DNA and therefore leading to secondary mutation accumulation. Nuclear protein MKI67 is associated with cellular proliferation and is present during the active phases of cell cycles. MKI67 is used as a predictive factor for the development of tumours (Amaro-Filho et al., 2013). Infection with HPV effects the cell-cycle controls when the viral oncoproteins produce perturbation of the cellular cycle controls, resulting in a mild carcinoma in-situ (CIN) (Kitchener et al., 2006). The latest findings suggest that a genetic susceptibility is of major influence in the development of CC. In fact, the most noticeable observations note that a variation in Xeroderma pigmentosum C (XPC) contributes the most to a woman’s susceptibility to CC (Patil et al., 2018).

2.5.3. Screening and Treatment

The most common form of CC screening is a Pap (Papanicolaou) smear test. This test is usually performed in a doctor’s office and may either be completed by the traditional smear test or the liquid (ThinPrep pap smear test or SurePAth Liquid-based pap smear) test (Williamson & Snyder, 2015). In order for the monitoring of any changes in the epithelium to be optimal, the patient should be screened annually for a period of 5 to 10 years (Kitchener et al., 2006). The physician will send the sample of tissue to a laboratory, whereby results are reported as follows- no abnormalities detected, atypical squamous cells, low-grade squamous epithelial lesion, high-grade squamous intraepithelial lesion, squamous cell carcinoma or atypical glandular cells (Williamson & Snyder, 2015). In South Africa, various guidelines have been put into place with regards to cervical cancer screening, which acknowledges the unique health conditions of South African women with regards to a high prevalence of both HPV and HIV infections, the existence of both private and public healthcare systems and health laboratories. The primary screening for HPV is more sensitive towards the detection of pre-cancerous changes and is said to
possess a negative predictive value which is better than cytological tests. This specificity allows for longer intervals to occur between screenings (Botha & Dreyer, 2017).

2.5.3.1. Criteria considered for HPV and CC screening

Screening aims at detecting the unsuspected cancer risk in women who are asymptomatic (Botha & Dreyer, 2017). There are various criteria which must be considered before screening can take place. Women who are to be screened should be no younger than the age of 21, women between the ages of 30 and 65 should have both HPV and cytology testing, women over the age of 65 no longer need to be screened if they have had 3 negative cytology results or two negative dual screenings. In the case of women who have a previous history of stage 2 and 3 cervical cancer, screening should be done for at least 20 years after diagnosis (Williamson & Snyder, 2015). In South Africa, further criteria suggest that women who test seropositive for HIV should undergo cervical screening at the time of diagnosis with HIV. It has been further suggested that women who are HIV positive should undergo screening at least every 3-5 years (Botha & Dreyer, 2017).

Botha (2017) further explains the management of patients when abnormal results occur. If abnormal results are found upon visual inspection using Lugol’s iodine, they recommend a biopsy in the case of a possibly invasive form of lesion, chemo therapeutic treatment when a small lesion is identified and a large loop excision of the transformation zone in the case of a large lesion.

2.5.3.2. Limitations of Pap smear

With every test, there are various limitations related. With regards to the pap smear test, approximately 5-10% of test results produce a false-negative, an insufficient cellularity can occur whereby there are less than 5000 well-visualised cells (Kitchener et al., 2006), integrity issues in approximately 10% of samples received by the laboratories and human error occurs in 3% of cases. Perhaps the most concerning factor of all, is that the pap smear test was designed for the sole purpose of screening of squamous cell tumours, thus resulting in an inability to identify other concerning tumours such as adenocarcinoma or sarcoma (Williamson & Snyder, 2015). Despite the limitations of the pap smear test, it has still managed to significantly decrease the incidence of cervical cancer due to its ability to aid practitioners in detecting CC, benign cellular morphology or HPV infection (Klobocista et al., 2018).
2.5.3.3. Conventional Treatment

The most common form of treatment for CC (particularly in the case of carcinoma \textit{in situ} stages 2 and 3) is chemo- radiation therapy and in cases of advanced carcinoma a hysterectomy is performed (Martin, 2018). Chemotherapy is usually used to treat more advanced stages of cancer when tumour sizes exceed 4 cm. The most cytotoxic agent used to treat cervical cancer, cisplatin, is usually used in combination with daily pelvic radiotherapy which is given as 25 fractions (Aredes \textit{et al}., 2018). Cisplatin is a chemotherapeutic agent which is platinum-based. This chemotherapeutic drug has previously shown the ability to enhance the expression of Fas (Fas cell surface death receptor) which is a gene responsible for apoptosis (Duenas- Gonzalez \textit{et al}., 2014). Cisplatin is usually used in combination with radiotherapy, however survival rates of patients receiving this treatment combination is usually low and brings with it many unwanted and serious side-effects (Milrot \textit{et al}., 2013).

2.6. Homeopathy

2.6.1. History of Homeopathy

Homeopathy was discovered in the 1800s by a German physician Samuel Hahnemann, born in the year 1755 (Yadav \textit{et al}., 2018). Hahnemann studied towards a degree in medicine in Erlangen, Germany whereby he would translate books in his spare time. In the year of 1789, Hahnemann was translating a book written by W. Cullen about the use of Cinchona bark as a means of treating malaria. Hahnemann decided to test the effects of the Cinchona bark in a healthy individual, and thus decided to ingest the bark. During the period, he noticed a strange development of symptoms which were closely related to those experienced by individuals infected with malaria (Vigano \textit{et al}., 2015). This strange development of symptoms led him to develop the hypothesis of a particular healing principle stating that, “that which can produce a set of symptoms in a healthy individual, has the ability to treat and cure the same set of symptoms produced in a diseased individual”. This finding led to the development of a very important homeopathic law known as the “Law of Similars” (Ernst, 2016). In the year 1796, Hahnemann wrote and published an article titled “Essay on a new principle for ascertaining the curative powers of drugs and some examinations of the previous principles”. Later, in 1810, Hahnemann wrote his first book titled “Organon of Rational Art of Healing”. It is in this book that all
homeopathic principles are listed and explained in exceptional detail, and by which all homeopathic practitioners base their method of practice on (Vigano et al., 2015).

2.6.2. Homeopathic Philosophy and Principles

Homeopathy is practiced by following several laws and principles which were written by the founder Samuel Hahnemann. Perhaps the most important is the Law of Similars and second is the Law of Minimal Dose of a remedy in order to increase the therapeutic effects of the particular remedy in diseased individuals (Ernst, 2016).

2.6.2.1. The Law of Similars

Fisher and others (2015) explain the Law of Similars in a rather scientific fashion, by comparing it to Newton’s first law: Every action is matched by an equal and opposite reaction. Fisher also mentions another infamous scientific law which states that energy cannot be created nor destroyed but can constantly change form as with our health. It is this energy that has the ability to effect and either improve or worsen an individual’s health. The best way in which to explain the use of the Law of Similars in homeopathic practice, would be by making use of the remedy Allium cepa, made from the red onion. This remedy is commonly used to treat the symptoms of hay fever, as it produces a set of symptoms similar to those experienced in cases of hay fever, such as a running nose which burns and becomes red and painful and is associated with bland lachrymation from the eyes (Ernst, 2016).

2.6.2.2. The Law of Minimal Dose

Homeopathic practice is aware of the toxic side effects which can be elicited onto patients when a medicinal substance is taken in its crude and undiluted form. Hahnemann believed that the more dilute a substance, the less toxic the substance. However, by diluting the substance and succussing (shaking) the remedy, it results in a stronger healing energy (Ernst, 2016). A therapeutic effect is established by using the smallest amount possible (Callinan, 1996).

2.6.2.3. Remedy Preparation

Homeopathic remedies are prepared most commonly in accordance with the methods laid out in the German Homeopathic Pharmacopoeia (Bozzuto & Buzzuto, 2000) however other methods such as the American, French and British pharmacopoeia also exist. The
preparation of a homeopathic remedy starts with a mother tincture (MT), which is a solution produced by macerating a particular substance (plant, animal or mineral) in alcohol and diluting this maceration using either a centisimal (cH; 1:100) ratio or a decimal (D/X; 1:10) ratio. A homeopathic mother tincture is often further diluted according to the previously mentioned ratios, until the desired “potency” is acquired. This series of diluting the desired substance is referred to as a “serial dilution”. Between each dilution, the remedy is “potentised” and “dynamised”. What this means is that the remedy is “succussed” against a hard service either 100 or 10 times (depending on the ratio used in dilution) between each dilution (Ernst, 2016). As an example of this, the remedy which will be used in this experiment, *R. idaeus*, was diluted 3 and 6 times using the decimal ratio in order to create a D3 and D6 and 30 times using the centisimal ratio in order to make a 30 cH. The mother tincture of *R. idaeus* is prepared by using the leaves of the plant. Some vehicles used include ethanol from concentrations of 20% to 90%, distilled water, glycerol or lactose (Bozzuto & Buzzuto, 2000).

![Diagram of the process of producing a homeopathic remedy](image)

Figure 1: Process of producing a Homeopathic remedy (Ernst, 2016)

### 2.6.2.4. The Use of a Single Remedy

When prescribing a remedy, the homeopath aims to match the symptoms of the remedy to those of the patient. This is done by reading the symptoms in the homeopathic materia medica. The Concordant Reference written by Frans Vermeulen has two symptom...
categories; the first category consists of symptoms which were obtained during provings (as described below). The second category of symptoms includes symptoms obtained from clinical cases (Vermeulen, 2015). The use of a single remedy is optimal, as it is still unclear as to how different remedies react when taken in unison. Hahnemann also believed that using a single remedy prevents confusion with regards to which remedy in a complex was responsible for a cure or aggravation of symptoms (Fisher et al., 2015).

2.6.2.5. Potency Selection

The use of a particular potency (strength) of a remedy is dependent on several things; the stage of the disease, the intellectual state of the individual, how sensitive the individual is to the remedy and what condition the homeopath is treating. In the case of the stage of the disease, the homeopath would use a medium potency (usually a strength of 30CH) to treat an acute or reversible disease. In cases of chronic or irreversible disease, the patient will be treated with a low potency (usually a 6CH or lower). When treating purely physical symptoms, lower potencies are used and when the patient is experiencing symptoms of an emotional or mental nature higher potencies are typically used (Kansal & Kaushal, 2017).

2.6.3. Homeopathic Provings

Homeopathic remedies are made by using animals, plants and minerals. As previously mentioned, homeopathy is based on the practice of the law of similars, therefore it would only be beneficial for the homeopath to examine the effects of homeopathic medicines on the healthy individual (van Haselen & Hoover, 2018).

For a proving to take place, the host has to follow a set of drug-proving protocols (Kaur, 2018). Homeopathic provings make use of a group of homeopaths who are in an optimum state of health (Renoux, 2018), who are over the age of 18 and are intelligent enough to consent to the practice of a homeopathic proving. During a homeopathic proving, it is expected of the participants to make note of the physical, emotional and mental symptoms they develop during the course of taking the homeopathic remedy (Fisher et al., 2015). It is important for these guidelines to be adhered to, as provings are the main source of guidance in the remedy selection process (van Haselen & Hoover, 2018).

Once the proving has successfully taken place and all participants have stopped experiencing symptoms of the remedy, the individual responsible for hosting the proving
gathers all the symptoms experienced by the participants. The host will then organise the symptoms according to those which appeared the most amongst participants (Teut et al., 2010), to less common symptoms and then very unusual (referred to as “strange, rare and peculiar”) symptoms. These symptoms are then placed in the homeopathic materia medica. The symptoms are graded in accordance with their appearance amongst individuals in the proving, as follows; most common scores a 4, less common but still quite a prevalent symptom scores a 3, a symptom only seen amongst a small number of participants scores a two and lastly, a very rare symptom scores a 1. These scores according to symptoms experienced by participants are placed in the homeopathic repertory for the homeopath to use as a means to “score” and compare symptoms of various remedies, before prescribing the remedy which scores the highest for the particular patient (Fisher et al., 2015).

2.6.4. Mechanisms Involved

It has been observed in recent studies that homeopathic remedies act on the cytosol of cells (Beeraka, 2009). Homeopathy is based on the Arndt-Shultz law in which low doses stimulate cellular activity, medium doses inhibit cellular activity and high doses destroy this activity. In doing so, it enables the body to heal itself via its own capacity (Turner & Hode, 2002). Latest research has found the ability for homeopathic remedies to influence various genetic expressions of cells. It is believed that homeopathic remedies function by either upregulating or downregulating various genes which leads to a correction of genetic expression when it has been altered by disease (Das et al., 2011). A study conducted by Saha (2013) found that different homeopathic remedies alter different genes.

2.6.5. The Nanoparticle Structure of Homeopathic Medicines

Nanomedicine is the practice of medicine which makes use of materials of a nano-scale in order to improve both diagnostic and therapeutic outcomes (Feng et al., 2019). Nanomedicine in homeopathy can be best explained by examining the relationship of hydrogen protons and their spin. Hydrogen protons are the only protons that possess a spin which has the ability to align itself when yielded to B0- a magnetic field. In relation to quantum theory, there are two energy levels which correspond to a nuclear moment. These energy levels are named parallel and anti-parallel. The parallel energy level is considered to be a low-energy level. When energy equivalent to these two energy levels is applied (in the case of homeopathic succussion), the atoms transition to an excited
state which is then closely followed by a relaxed state. It is during the relaxed state that the pre-existing energy of the nucleus is exchanged to surrounding molecules. With this theory of nanomedicine, it has been observed that homeopathic remedies contain nanoparticles resembling the original starting substances in potencies as diluted as a 12cH (Demangeat, 2013). In accordance with the above nature of protons, it has been noted that nanoparticle structure (most specifically in homeopathic remedies made from metal materials) is the same amongst different potencies (Upadhyay & Nayak, 2011). A few of the remedies which have been analysed for nanoparticle include Natrum muriaticum and Aurum metallicum in potencies diluted at ratios of 1:1000, and Hypericum perforatum 6cH and CM. Nanoparticles were found to be present in all potencies of the afore-mentioned remedies. It is this theory of nanoparticles present in homeopathic medicine that could explain how homeopathic remedies have the ability to alter the genetic expression of cells and disturb the membrane potential of cells. These findings on nanoparticles in homeopathic medicines are relatively new with various theories arising to attempt to scientifically explain the mechanism of homeopathic medicines. One particular theory is that the nanoparticles interact with cellular membranes resulting in a change in the membrane potential of cells via a process known as stochastic resonance (Rajendran, 2018; Bellavite et al., 2014). More research needs to be conducted however to truly understand the effect that homeopathic nanoparticles have on various cellular functions and immune responses.

2.6.6. Homeopathic Remedies in the Treatment of Cancer

Homeopathic remedies are one of the safest and most cost-effective therapies which exist and have been used as a means to reduce the side-effects experienced during chemotherapeutic treatment, and recently even as a means to treat various cancers (Yadav et al., 2018). Evidence has observed that homeopathic remedies (particularly Conium maculatum, Sabal serrulata, Thuja occidentalis and Carcinosin) have the ability to reduce tumour incidence, progression, weight and volume of tumours (Jonas et al., 2004). Thuja occidentalis has been shown to possess the ability to suppress vascular endothelial growth factor (VEGF), angiogenin-4 and CD31 (platelet endothelial cell adhesion molecule) expression in cancer cells (Torres et al, 2016). A study conducted in 2006 observed the anti-neoplastic effects of homeopathic remedies (T. occidentalis (1000cH), C. maculatum (1000cH), S. serrulata (200cH), Carcinosin (1000cH)) in rats which were injected with MAT-LyLu prostate cells. The study found that in 23% of the rats, there was a significant reduction in tumour incidence and tumour volume in rats
treated with the homeopathic remedies. The study thus concluded that homeopathic remedies have the ability to increase apoptosis in cancer cells (Jonas et al., 2006).

Remedies have further shown the ability to inhibit the development of melanoma and metastasis in mice which were injected with B16F10 melanoma cells (Ferrari et al., 2016). Homeopathic treatment in breast cancer has shown commendable results where the homeopathic remedies used were Carcinosin 30cH, Phytolacca decandra 200cH, C. maculatum 3CH and T. occidentalis 30cH, 200cH and 1M (Frenkel et al., 2010). Further research into the effect of homeopathic remedies on the gene expression in HeLa cervical cancer cells has shown the ability of homeopathic medicine to completely change the expression of 6024 genes in these cells (Saha et al., 2013).

2.7. Rubus idaeus L.

Rubus idaeus L. species, commonly known as Red Raspberry, is a plant belonging to the Rosaceae family, under the subgenus Idaeobatus with biennial stems and fruit (Tobyn et al., 2011). R. idaeus contains a large number of antioxidants, particularly anthocyanin and ascorbic acid (de Souza, 2014). A study completed in 2005 by Anttonen & Karjalainen, found that R. idaeus contained high levels of quercetin and ellagic acid (Tobyn et al., 2011). The polyphenols from R. idaeus are absorbed by the body in various as degradants. The most common polyphenols found to exist in R. idaeus include cyaniding-3-O-sophoroside, cyaniding 3-O- glucoside, lambertianin C and sanguiin H6 (Zhang, 2018).

2.7.1. Clinical uses of Rubus idaeus

The polyphenols in R. idaeus possess many antioxidant, anti-tumour, antimicrobial and anti-inflammatory properties (Giao et al., 2012), along with the ability to induce metabolic detoxification (Milbury, 2009). R. idaeus has the ability to decrease risk biomarkers for obesity and diabetes, by reducing the levels of plasma interleukin (IL)-6 by enhancing the activity of glutathione peroxidase in the liver (Noratto et al., 2017). It has also been found that R. idaeus has the ability to decrease inflammatory and oxidative stress involved in the promotion of heart morphological changes in the elderly. R. Idaeus does so by downregulating proteins such as natriuretic peptide precursor type A and glutathione S-transferase A4 (Noratto et al., 2016). New research has found that R. idaeus extract has the ability to reduce signs and symptoms related to arthritis, by reducing soft tissue swelling, formation of osteophytes, reduction in particular destruction and bone resorption.
Impressive neuroprotective properties are also produced by its fruit, whereby *R. idaeus* has the ability to increase the activity of catalase in human neuroblastoma SH-SY5Y cells (Zhou *et al*., 2018).

### 2.7.2. *R. idaeus* and Cancer Cells

No research has been done with regards to the effect of *R. idaeus* as a homeopathic preparation on CC cells, however there is substantial evidence showing the anti-neoplastic effects of herbal preparations of *R. idaeus*. A study conducted in 2013 observed that herbal preparations of *R. idaeus* has the ability to reverse epithelial to mesenchymal transition in cases of lung cancer. The study noted that *R. idaeus* did so by targeting the FAK (focal adhesion kinase) and ERK1/2 pathways (Hsieh *et al*., 2013). Further research identified the ability of *R. idaeus* to destabilise cancer cells by penetrating the cell membranes (Cho *et al*., 2016).

### 2.8. Low Level Laser Therapy (LLLT)

A laser is a device which emits light via a process involving optic amplification as a result of emission of photons. LLLT is a form of complementary therapy (Chung-Sik *et al*., 2004), which was discovered in the 1960s by Endre Mester. Cells are exposed to low levels of near infrared or red light at low energy densities in order to promote healing. Another term given to LLLT is “cold laser therapy”, due to the low power densities used to prevent the heating of tissue (Chung *et al*., 2012).

#### 2.8.1. History of Low Level Laser Therapy

Lasers were discovered in the early 1960s, after Townes observed that it was possible to create short-waves by manipulating ordinary light. However, it was Arthur Schawlow who created the way in which light waves could be emitted effectively. He noticed that the best way to emit waves would be to place the atoms in a narrow and long cavity with mirrors attached at each end, so as to allow for the waves to radiate. Gordon Gould further added to the development of the laser by investigating ways in which to “pump” atoms to higher energy states to emit light. It was Gould who developed the name “Laser” which stands for “Light Amplification by Stimulated Emission of Radiation”. The trio of scientists further studied the benefits of using various gases and crystals as a means of increasing the energy of atoms (Weart, 2019). The use of low level laser began after the development of the ruby laser in 1960 along with the Helium-neon laser which was created in 1961.
Endre Mester, who worked at Semmelweis University in Budapest, was the first to observe the benefits of laser in a therapeutic setting. He observed this when he applied laser light to hairless mice, who began to grow hair after treatment with a Helium-Neon (HeNe) laser. After his discovery, Mester treated patients with skin ulcers which did not heal (Chung et al., 2012).

2.8.2. Sources of Light

There are currently several different sources of light which are used in LLLT. Light emitting diodes (LEDs) have currently become a more popular source of light for lasers, along with gallium arsenide (GaAs) semi-conductor diode lasers. Another common source of light used in lasers is the He-Ne, a gas which emits light at a wavelength of 632.8 nm. The increased popularity of light emitting diodes (LEDs) has developed due to the fact that LEDs have the ability to emit light over a much wider range of wavelengths than any of the other lasers. With new technology becoming available, the latest source of light currently under investigation is the organic LEDs with an electroluminescent layer which emits light in response to an electrical current (Agostinis et al., 2011)

2.8.3. Mechanisms and Laser Parameters

There are three components which are important to the functioning of a laser; oxygen, light and photosensitizers (Agostinis et al., 2011).

2.8.3.1. Photosensitizers

Photosensitizers are otherwise referred to as dyes which can absorb, reflect and transfer the energy of the light emitted by the laser into other molecules in order to bring about photochemical reactions (Wainright, 2004). These photosensitizers function by absorbing light at various wavelengths and further converting the light into fluorescence and different atomic arrangements. This conversion process leads to a transference of energy across oxygen molecules and further leads to the formation of reactive oxygen species (Dougherty et al., 1998). Photosensitizers are generally placed into two groups, namely those which are non-porphyrin based or porphyrin based. Photosensitizers which fall into the porphyrin-based category include chlorins, phthalocyanines and bacteriochlorin. Photosensitizers such as cyanines, psoralens, anthracyclines and hypericin fall into the non-porphyrin-based category (Ormond, 2013; Wainright, 2004). In order for a photosensitizer to be considered "ideal" in nature, various characteristics need to be met.
These characteristics include the ability for a photosensitizer to be water-soluble, chemically stable, possess a high yield of oxygen generation, be able to accumulate rapidly in target tissues and possess a molar coefficient anywhere between 600- 900 nm (Yano et al., 2011; Mehraban et al., 2015).

2.8.3.2. Dose

The dose of a laser, also referred to as the fluency, is defined as the energy of light which is directed at a particular unit of area during a given period of time. The unit of measurement used is joules per centimetre squared (J/cm²). The dose is calculated by taking in consideration the power of the laser's output (measured in watts) and multiplying this measurement by the duration of treatment (in seconds). This is summarised in the formula below:

\[ \text{Dose} = \frac{\text{Power (W) x time (s)}}{\text{area treated (cm}^2\text{)}} \] (Turner & Hode, 2002)

2.8.3.3. Power Density

The power density, also referred to as the density of light emitted by the laser, indicates the power output concentration. This power output is calculated by taking into account the area (cm²) of target tissue. The area of treatment and the power density have an inverse relationship, meaning that the larger the treatment area, the lower the power intensity will be (Turner & Hode, 2002).

2.8.3.4. Mechanisms

LLLT follows the principles of Arndt-Schultz law, in which weak stimuli activate cellular activity, moderate stimuli inhibit this activity and intense/high stimuli destroy cellular activity (Bresler, 2012). LLLT is believed to induce photochemical reactions in the cells via a process known as photobiomodulation (Chung & Dai, 2012). LLLT consists of very slow irradiation intensities which stimulate healing of damaged cells (Chun-sik et al., 2004). LLLT has an effect on tissues due to a phenomenon whereby air and tissue refractive indexes change as a result to light being reflected. This can be summarised by the law of Snellius (Chung et al., 2012);

\[ \frac{\sin \theta_1}{\sin \theta_2} = \frac{n_2}{n_1} \]
$\theta_1$ represents the angle between normal air surface and light, $\theta_2$ represents the angle between normal tissue surface and laser ray, $n_1$ represents air refraction index and $n_2$ represents tissue refraction index. This calculation notes that light has a “scattering behaviour” in tissue which effects the light intensity and volume distribution in tissues (Chung et al., 2012). LLLT makes use of infrared (700 - 1000 nm) and visible radiation (380 - 700 nm), as well as fluence- the level of energy per area of a particular size. Fluence is expressed by Joules per centimetre squared. Another inclusion in laser parameters is the power density expressed in Watts per centimetre squared (Vero, 2013).

The light wavelengths which are typically used in LLLT fall into a range of red and near-infrared wavelengths of 600-1070 nm (Chung & Dai, 2012).

2.8.4. Laser Interaction With Tissue

The most effective wavelengths used to penetrate tissue are between 600-1070 nm, with haemoglobin and melanin absorbing wavelength bands shorter than 600 nm. Superficial conditions are usually treated at wavelengths of 600-700 nm and deeper tissue is penetrated with wavelengths ranging between 780-950 nm. Recently it has been understood that LLLT has an effect on the mitochondria of cells, resulting in an decreased level of adenosine triphosphate (ATP), increased level of reactive oxygen species and transcription factor induction (Chung et al., 2012). Laser also has a large influence on the endoplasmic reticulum (ER) of cells (Dewaele et al., 2011).

LLLT affects the ER by causing an activation of the caspase-12 dependant pathway (Dewaele et al., 2011) by cleaving caspase-12 with protease m-Calpain (Rao et al., 2002; Nakagawa et al., 2000). Photodynamic therapy (PDT) (the use of LLLT in vitro to promote tissue repair) effects mitochondria resulting in an increase in cell proliferation and migration along with modulation of growth factors, cytokines and increased oxygenation of tissue (Chung et al., 2012). Cytokine expression has also been shown to reduce in the presence of a laser, by reducing levels of IL-1$\beta$ and IL-6 (Bartos, 2016). Further studies have shown that different cells react differently to LLLT. One study observed that fibroblast cells have a lower sensitivity to laser irradiation than keratinocyte cells. The level of reactive oxygen species in keratinocytes was significantly higher than that found in fibroblast cells, whereas fibroblasts had a higher basal catalase expression when treated with laser (Engel, 2016).
Further research into the effect of LLLT’s relationship with immune cells such as mast cells and leukocytes have shown that certain wavelengths have the ability to cause degranulation of mast cells, which thus results in the release of cytokine TNF-α. With this action taking place, there is an increase in the number of leukocytes infiltrated into the tissues. LLLT has also shown the ability to increase epithelial cell motility and proliferation, which thus increases the rate of wound healing. When laser is applied to cells, it results in a photon of light to be absorbed by chromophores. Once the photon is absorbed, an electron will jump from an orbit of low energy, to an orbit of high energy. This movement of electrons promotes the activation of various cellular tasks (Chung & Dai, 2012). Currently, LLLT is used as a means of treatment for the side effects experienced by patients undergoing chemotherapy and radiation therapy. Such side effects include lymphadenopathy in women with breast cancer and oral mucositis (Ferreira et al., 2016). In the case of lymphoedema in patients with breast cancer, the use of LLLT at an energy density of 1-2 J/cm² allowed for a large reduction in arm circumference (Omar et al., 2012).

LLLT can be used in conjunction with a photosensitizer, resulting in a therapeutic approach known as photodynamic therapy (PDT). A large amount of research has been conducted in order to observe how the addition of a photosensitizer to LLLT may effect cells in vitro and how this PDT may induce apoptosis in cancer cells.

Apoptosis is a term applied to the morphological component of cell death. This process is typically recognized by nuclear fragmentation, cell volume reduction and chromatin condensation. Unprogrammed apoptosis occurs in the presence of an external influence (for example LLLT or PDT when applied to cells) and programmed apoptosis occurs when cells fail to meet normal chromosomal structure during the proliferation process, and therefore die as a result (Kroemer et al., 2009). LLLT and PDT are both cost-effective and non-invasive in their therapeutic approach, which has led to increased use and popularity of the years (Svanberg et al., 2010). The main mechanism of cell death as a result of PDT is the formation of ROS via two pathways- Type I (produces free radicals and radical ions) and Type II (a singlet oxygen is produced due to energy transference from the photosensitizer to a triplet oxygen) (Kim et al., 2017). The formation of these ROS leads to cyto-damage of carcinogenic cells and further autophagy, apoptosis and cellular necrosis (Robertson et al., 2009).
A photosensitizer named pheophorbide has demonstrated the ability to induce apoptosis in androgen-insensitive prostate cancer cells (Lin et al., 2015) when combined with a laser of 670 nm wavelength at a fluency of 5 J/cm² (Xu et al., 2011). Research has further shown that this combination of laser and pheophorbide prevents prostate cancer cells from forming colonies (Gheewala et al., 2018). With regards to MCF-7 breast cancer cells, PDT has demonstrated a remarkable ability to produce a cytotoxic effect when used at a fluency of 15 J/cm² in combination with phthalocyanines (Horne et al., 2012). A chlorin-based photosensitizer named chlorin-bexarotene has shown a tremendous ability to induce cytotoxicity in triple-negative breast cancer cells by targeting vitamin A nuclear receptors (Isaac-Lam et al., 2019). Research has further supported the ability of a 680 nm laser, when combined with ZnPcS mix, to induce cancer cell death in human colon (DLD-1) and lung (A549) cells by localising in the lysosomes and mitochondria (Manoto et al., 2012). This research has been further concluded with the addition that the best performing dose at 680 nm wavelength is 5 J/cm² (Manoto et al., 2013). In the case of CC, a study conducted by Chizenga (2019) found that PDT at a wavelength of 673.2 nm in combination with AlPcS mix proved effective in inducing apoptosis in cervical cancer stem cells in vitro.
CHAPTER 3.
METHODOLOGY

3.1. Research Procedure and Design

This quantitative *in vitro* research study, took place at the Laser Research Centre of the University of Johannesburg. This research study made use of HeLa cells, *R. idaeus* (D3, D6 and 30cH homeopathic preparations) and LLLT at 680 nm with 5, 10 and 15 J/cm² fluencies. A flow diagram showing the summary of the research process is shown in Appendix A.

3.1.1. Chemicals and Media Used

Refer to Appendix B, C and D for a table demonstrating the list of chemicals, media and products used for this study.

3.1.2. HeLa Cell Culture

CC HeLa cells (ATCC CCL-2) were grown in Dulbecco’s Modified Eagle Medium (DMEM) complemented with Fetal Bovine Serum (FBS) (10%) (FBS; Gibco 306.00301) and penicillin/streptomycin (1%) (PAA Laboratories GmbH, P11-010). Cells were then cultured at a temperature of 37°C with 5% CO₂ and 80% humidity. CC cells were sub-cultured on a weekly basis. Once cells had become confluent, they were washed with Hank’s Balanced Salt Solution (HBSS, Invitrogen, 10-543F) and dissociated using TrypLE (1 ml/25 cm²) (Gibco, 12604). Cells were then seeded in culture plates (1x10⁶) with a diameter of 3.5 cm² and allowed to incubate for 6 h in order to become attached before experiments.

3.2. *Rubus idaeus* Remedy Preparation and Administration

Fusion Homeopathics homeopharmaceutics company used *R. idaeus* MT, prepared by a company named Herbamed by using the fresh leaves of the plant, in order to create the D3, D6 and 30cH potencies required for experiments. The Homeopathic Pharmacopoeia (HAB) 3a method was followed for mother tincture preparation and plant used contained 60% moisture. This dilution method is conducted by using a ratio of 3:7 (mother tincture:
ethanol 62%) for the creation of the D3 and D6 potencies and 3:97 (mother tincture: ethanol 62%) for the creation of the 30cH potency. Once remedies had been created in their relevant potencies, they were stored in 20 mL amber glass bottles which had been previously sterilized and placed in a temperature controlled room of 20°C with no sunlight. Each potency was administered at a dose of 40 μL before incubating at 37°C with 5% CO₂ and 80% humidity for 24 h (Appendix E).

3.3. Cell Treatment

There were three experimental cell groups in this study, whereby each group underwent experiments which were repeated three times in duplicate. The layout of the experimental cell groups were completed in accordance with the table below:

Table 1. A Table Summarizing the Layout of Experimental Cell Groups used in Experimental Procedures

<table>
<thead>
<tr>
<th>Group number</th>
<th>Description of Experimental Cell Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CC cells not exposed to <em>R. idaeus</em> or a combination of <em>R. idaeus</em> (optimal potency) and LLLT-untreated control</td>
</tr>
<tr>
<td>2</td>
<td>CC cells treated with <em>R. idaeus</em> (D3, D6 and 30cH)</td>
</tr>
<tr>
<td>3</td>
<td>CC cells treated with a combination of the optimal potency of <em>R. idaeus</em> and 680 nm diode laser at three fluencies of 5, 10 and 15 J/cm²</td>
</tr>
</tbody>
</table>

The different cell groups were monitored with regards to their levels of lactate dehydrogenase (LDH) release. Adenosine triphosphate (ATP) levels were measured as a means of assessing cellular proliferation. Trypan Blue assay and an inverted light microscope were used to assess changes in cell viability and morphology. Lastly, the apoptosis of the HeLa cell culture was measured via Annexin V/PI and Hoechst staining.

3.4. Laser Irradiation

A diode laser (Oriel Corporation, USA, LREBT00-ROITHI) emitting at a wavelength of 680 nm supplied by the National Laser Centre of South Africa was used for this study.
Before cell irradiation, a power meter (FieldMate) was used to determine the power output of the laser. The power output of the meter measured at 201 mW. The intensity of the laser was calculated and the measurement was 22.15 mW/cm² for cells that were irradiated for ATP, LDH, morphology and viability assays. The cells were then irradiated for 3 min 47 sec, 7 min 34 sec, 11 min 22 sec to provide an energy of 5, 10 and 15 J/cm². For cells irradiated for the Hoechst and Annexin V/PI experiments the laser output was measured at 205 mW and a laser intensity of 22.59 mW/cm² was attained. For this experiment cells were exposed to laser at times of 3 min 37 sec, 7 min 14 sec and 10 min 52 sec to provide an energy of 5, 10 and 15 J/cm² respectively. A Table summarising these laser parameters used can be found in appendix F and the calculations used to work out laser parameters can be found in Appendix G. Culture plates containing cells and the optimal potency of *R. idaeus* were irradiated in the dark at 5, 10 and 15 J/cm² fluencies whereas irradiated cells without the *R. idaeus* were used as laser control.

Dosage calculations were determined as follows:

\[
\text{Irradiance (J/cm}^2\text{)} = \text{time (s)} \times \left[ \text{power (W)/ surface (cm}^2\right].
\]

Post irradiation assays were then carried out after 24 h of incubation at 37°C. Appendix F demonstrates the laser parameters used in this study.
3.5. Cellular Dose Response Assays

3.5.1. Cellular Viability

The trypan blue assay was used as a means of evaluating the percentage of viable cells after treatment with *R. idaeus* (RI) and a combination of RID3 and 680 nm laser diode at doses of 5, 10 and 15 J/cm². An equal volume (10 μL) of suspended cells and 0.4% Trypan blue were mixed before being transferred to a Neubauer haemocytometer (Countess™ Automated Cell Counter, Invitrogen) in order to count the number of living and dead cells.

3.5.2. Cellular Proliferation

Twenty four hours after administration of a single treatment of RI30cH, D6 and D3 and then a combination of the optimal potency of RI (D3) with 680 nm laser at doses of 5, 10 and 15 J/cm², CellTiter-Glo luminescent (Promega G7573) assays were used as a means of evaluating cellular proliferation via ATP quantification. An equal volume of ATP reagent (50 μL) was added to cells suspended in HBSS and mixed well. The mixture was then incubated in dark for 10 min. The luminescence signal was measured using a 1420 Multilabel Counter Victor3 (Perkin-Elmer, VICTOR™).

3.5.3. Cellular Cytotoxicity

Cell cytotoxicity was measured 24 h after administration of RI30cH, D6 and D3 and a combination of the optimal potency of RI (D3) and 680 nm laser at doses of 5, 10 and 15 J/cm². The LDH assay measured the integrity of the cellular membranes. An equal volume (50 μL) of Cyto-TOx96 (Anatech Promega G400) was added to cells suspended in DMEM and incubated for 30 min. After incubation LDH levels in the culture media were captured at 490 nm (Perkin-Elmer VICTOR™).

3.6. Cell Death Analysis

3.6.1. Analysis of Apoptosis (Annexin V/PI)

The optimal potency of RI (D3) individually and in combination with LLLT 680 nm at doses of 5, 10 and 15 J/cm² were used to study the cell death analysis using Annexin V/PI
staining. Untreated control cells were also analysed using Annexin V/PI staining in order to compare findings from the two treated cell groups. Flow cytometric analysis (BD Accuri™ C6 Cytometer) was used to analyse the relative size, internal density and fluorescence intensity of cells as they flowed in a liquid passing through a beam of light. Before experimentation, cells were centrifuged at 300 x g for 5 min at a temperature of 20°C. After being centrifuged, the TE/HBSS mixture was pipetted out before cells were aspirated in 10 mL of cold PBS. Once cells were re-suspended in PBS, they were centrifuged again at a speed of 300 x g for 5 min at a temperature of 20°C. This process was repeated twice. Annexin V- fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, 556570, Scientific Group, South Africa) was used to detect the percentage of cells which underwent apoptosis. FITC is a fluorochrome attached to Annexin V, a protein complex with high affinity for phosphatidylserine. Externalization of phosphatidylserine to the plasma membrane occurred in early apoptosis that facilitated the binding of FITC Annexin V. Propidium iodide (PI) is a vital dye used in conjunction with FITC Annexin V to differentiate viable cells (FITC Annexin V negative and PI negative) from non-viable cells. Cells were incubated with FITC Annexin V and PI at room temperature for 10 min and analysed by flow cytometry, which detected a green fluorescence for FITC Annexin V and red fluorescence for PI.

3.6.2. Nuclear Damage Using Hoechst Stain

Once the optimal potency of RI had been established (D3), it was combined with 680 nm laser at doses of 5, 10 and 15 J/cm². RID3 as a single treatment protocol was compared to cells irradiated at 5, 10 and 15 J/cm² in combination with RID3 and compared to untreated control cells. After the treatment of HeLa cells, the nucleus was stained with Hoechst dye (33342/33258) (10 mg/mL). A mixture of Hoechst dye with Phosphate-buffered saline (PBS) in a dilution of 1:5000 was then incubated for 30 min at room temperature. Thereafter, the PBS was replaced immediately before the fluorescence was measured at 355 nm excitation and 455 nm emission from Carl Zieiss Axio Z1 Observed with DAPI Filter set. The nuclei of various cell groups were then analysed for any appearances of nuclear shrinking or degradation as compared to the untreated control groups of cells. Live cells’ nuclei absorb the Hoechst dye whereas dead cell nuclei do not.
3.7. Reliability and Validity Measure

Optimum temperatures were considered with regards to cellular incubation (37°C at 5% CO₂) and cellular stocks in liquid nitrogen and in -80°C deep freezer. During passaging of cells, flasks were labeled according to passage number and date of passage so as to allow for accurate identification. Petri dishes were labeled according to the subculture group numbers. Cell lines were handled with appropriate care, with use of gloves and mouth masks so as to prevent any forms of contamination.

3.8. Data Collection and Analysis

The experiments were repeated three times in duplicate. The untreated cells formed the control, which were then used to compare the treated cells using ANOVA to determine the statistical difference. The data was statistically analysed by using software SigmaPlot version 14. The significance between the groups is shown as $p<0.05(*)$, $p<0.01(**)$ and $p<0.001(***)$.

3.9. Ethical Consideration

A commercial cell line procured from ATCC (HeLa cells CCL-2) was utilised to conduct the in vitro studies.

Cells are readily available and kept in liquid nitrogen stocks in the LRC laboratory. Guidelines on the ethics standards for obtaining human materials from ATCC can be viewed on their website (https://www.lgcstandards-atcc.org/About/About_ATCC/Ethical_Standards_for_Obtaining_Human_Materials.aspx), and all ethical standards have been met by ATCC when isolating these cells. Donation of the tissue is anonymous, and the cell lines cannot be traced back to the donors (Appendix H). The certificate of analysis can be found in Appendix I. The LRC has also received the necessary biosafety clearance required from ATCC when ordering such cells (Appendix J). A letter from the research ethics committee as well as a letter from the higher degrees committee authorising the commencement of this study are found in Appendices K and L. Plagiarism report can be found in Appendix M.
CHAPTER 4.
RESULTS

This *in vitro* study aimed to identify the effects of homeopathically prepared *R. idaeus* (RI) on CC HeLa cells by observing the effects of this homeopathic medicine individually and in conjunction with the 680 nm diode laser. The first set of experiments were conducted as a means of identifying the best performing potency of the homeopathically prepared RI. After identifying that, the optimal potency was RID3, this potency was then used in conjunction with the 680 nm laser at doses of 5, 10 and 15 J/cm\(^2\) in order to identify the synergistic effects.

In both treatments, cells were examined using an inverted light microscope in order to observe the morphological changes of CC after treatment. The cytotoxic effects of both treatment protocols were examined using Trypan blue assay as explained previously and measuring the LDH released by dead cells after treatment were also measured. ATP levels were measured as a means to see whether the treatment protocols were able to decrease production, therefore indicating unfavourable cellular environments for survival.

Results were further scrutinized by using the Annexin V/PI staining in order to confirm the findings observed in the above-mentioned experiments, and finally Hoechst staining was completed as a means of observing the effects of treatment protocols on the cellular nuclei.

4.1. Dose Response Studies

4.1.1. Cellular Morphology

The morphological changes of cells treated with RI in potencies of 30cH, D6 and D3 as well as cells treated with a combination of the optimal potency of RI (D3) and 680 nm laser at doses of 5, 10 and 15 J/cm\(^2\) were compared to untreated control cells as shown in Figure 3. The morphological features of CC HeLa cells were determined using inverted light microscopy to analyse the ability of RI and a combination of the optimal potency of RI the 680 nm laser diode to induce apoptosis after 24 h incubation.

With the experiment conducted to identify the optimal potency of RI, the D3 potency demonstrated an ability to induce blebbing of the CC HeLa cells. With blebbing the cellular
membranes seemed to show a loss of integrity as compared to the membrane of control cells. Dead cells could be observed floating in the medium. With regards to the D6 potency, there seemed to be no significant changes with regards to the cellular membranes when compared to the control. Fewer dead cells could be observed floating in the medium as compared to those seen in the cells treated with D3. Lastly, cell treated with the 30cH potency appeared to have become overly confluent on the plate as compared to control cells. Membrane integrity seemed intact and no dead cells could be observed floating in the medium. The laser irradiated cells were combined with the optimal potency of RI (D3 potency). CC HeLa cells irradiated with a combination of the optimal potency of RI (D3) (RID3) and 680 nm laser at 5 J/cm$^2$ appeared to have no changes in cellular membrane integrity or nuclei shape when compared to untreated control cells. No dead cells could be observed floating in the medium along with no blebbing of cells. Cells irradiated with a combination of RID3 and 680 nm laser at 10 J/cm$^2$ also seemed to demonstrate an no changes in cellular structure when compared to control cells. There appeared to be no blebbing or dead cells floating in the medium. Lastly, cells irradiated with a combination of RID3 and 680 nm laser at 15 J/cm$^2$ appeared to demonstrate a decreased number of viable cells as compared to the untreated control cells. Cellular structure and membrane integrity, however, did not appear to be significantly different when compared to untreated control cells. Few dead cells could be seen floating in the medium.

Figure 3: Morphological analysis of CC cells. Fig 2(a) and 2(e) represents the untreated control cells, (b) represents D3 potency, (c) D6 potency and (d) 30cH potency, (f) RID3 + 5 J/cm$^2$, (g) RID3 + 10 J/cm$^2$ and (h) RID3 +15 J/cm$^2$. The black arrows demonstrate dead cells.
4.1.2. Cellular Viability Using Trypan Blue Assay

The percentage of viable cells were compared to that of control cells. Trypan blue dye is absorbed by dead cells and not viable cells, allowing for a count to be conducted on the percentage of viable cells. The control cells demonstrated a viability of 74%. CC HeLa cells treated with RI30cH had an average increase in viability of 82% ($p<0.186$), whereas cells treated with RID6 showed a reduction in the cellular viability, with an average percentage of 61% ($p<0.283$). Cells treated with RID3 showed a significant reduction in cellular viability with an average viability of 59% ($p<0.292$). The results depicted in the Figure 4 demonstrate the average viability results obtained per group. In the experiment comparing the combined effects of RI in a D3 potency and 680 nm laser, cellular viability of treated cells were compared to the cellular viability of control cells (75%). Cells treated with a combination of RID3 at 680 nm laser irradiation at a dose of 15 J/cm$^2$ demonstrated an average viability of 59%. Cells treated at RID3 + 10 J/cm$^2$ showed a viability of 53%. Lastly, cells treated at a dose of RID3 + 5 J/cm$^2$ depicted a viability of 60% respectively. The results of the combined experiments in 5 J/cm$^2$ ($p<0.88$), 10 J/cm$^2$ ($p<0.41$) or 15 J/cm$^2$ ($p<0.80$) did not show any statistical significance. These results are shown in Figure 5.
Figure 4: Viability of CC cells after the treatment with RI. Percentage of Viable cells after 24 h treatment with RI30cH, D6 and D3. The mean percentage value has been depicted with the control demonstrating a viability of 74%. Cells treated with RI30cH demonstrated an average viability of 82%, cells treated with RID6 showed 61% and D3 showed 59% respectively.
Viability of Cervical Cancer Cells After the Treatment With *R. idaeus* D3 and 680 nm Laser

![Viability of Cervical Cancer Cells](image)

Figure 5: Viability of CC cells after the treatment with RID3 and 680 nm laser. Cells treated with RID3+ 15 J/cm² had a viability of 59% whereas cells treated with RID3 + 10 J/cm² had a viability of 53% and cells treated with RID3 + 5 J/cm² showed 60% respectively.

### 4.1.3. Adenosine Triphosphate (ATP) Proliferation Assay

ATP levels of treated groups were compared to those of the control groups in order to determine a statistical significance. The methodology of this experiment can be found under 2.5. Cells treated with RI30cH resulted in an increase in ATP levels (*p*<0.945) whereas cells treated with RID6 did not show much difference in ATP levels (*p*<0.411) as compared to the control cells. Cells treated with RID3 demonstrated a decrease in ATP levels as compared to the other two treatment groups and the control (*p*<0.194).

Cells treated with a combination of RID3 and 680 nm Laser at 5, 10 and 15 J/cm² showed a remarkable increase in ATP levels as compared to untreated cells. With the significance of cells treated at RID3 + 5, RID3 + 10 and RID3 + 15 J/cm² showing significance of *p*<0.001, 0.004 and 0.001 respectively. All results are demonstrated in Figure 6 and 7.
ATP Proliferation After Treatment With *R. idaeus*

Figure 6: ATP proliferation after treatment with RI. Cells treated with the 30cH potency demonstrated no difference in ATP levels as compared to the control, the D6 treatment group demonstrated an increase in ATP levels and the D3 treatment group demonstrated a decrease in ATP levels (*p*<0.194).
Figure 7: ATP proliferation after combined treatment with 680 nm laser and RID3. The results demonstrate an increase in ATP levels in cells treated with RID3 + 5, 10 and 15 J/cm². *p*<0.001 *** and *p*<0.01 **

4.1.4. Lactate Dehydrogenase (LDH) Assay

LDH levels were measured 24 h after treatment with RI30cH, D6 and D3. All treatment groups were compared to the control group. All potencies of RI had an effect on the levels of LDH. RI30cH (*p*<0.11) and D6 (*p*<0.08) decreased the levels of LDH released by cells whereas, RID3 increased the levels of LDH (*p*<0.212) as compared to the control group (Figure 8).
In the combined treatment of RID3 with 680 nm laser at 5, 10 and 15 J/cm². LDH levels increased in all treatment groups, with LDH levels appearing highest in the cell group exposed to RID3 + 5 J/cm² ($p<0.472$) and increased in cells treated at 10 J/cm² ($p<0.065$) and 15 J/cm² ($p<0.174$) (Figure 9).

Figure 8: Cytotoxicity of CC cells after treatment with RI. Cells treated with RI30cH and D6 demonstrated a slight decrease in LDH levels as compared to the control, whereas cells treated with RID3 demonstrated an increase in LDH ($p<0.212$).
Figure 9: Cytotoxicity of CC cells after treatment with a combination of RID3 and 680 nm laser. All combined treatment groups demonstrated an increase in LDH levels as compared to the control, with cells irradiated with RID3 + 5 J/cm² demonstrating the highest increase in LDH respectively.

4.2. Cell Death Analysis

4.2.1. Annexin V/PI Staining

Annexins are calcium-regulated proteins which bind to phospholipids and play a large role in the life cycle of cells with regards to apoptosis (Mirsaeidi et al., 2016). An Annexin kit makes use of fluorescent dye in order to detect the externalization of phosphatidylserine in cells which are apoptotic. Cells which are dead conjugate to propidium iodide (PI) which stains dead cells with a red fluorescence. Live cells show no
fluorescence (Invitrogen Catalogue V13242). Flow cytometers typically consist of a light source, an electronic network which detects various light signals at various intensities and a computer which records the results derived from the electronic detectors (Givan, 2001).

The percentage of various cell populations were analysed using Annexin V/PI staining. The treatment groups studied in this experiment included the combination of RI (D3 potency) with laser doses of 5, 10 and 15 J/cm$^2$. Three fluencies of the 680 nm laser were used to find out the optimal fluency. Each treatment group was statistically analysed against the laser control group. These results have been summarised in Table 2.

Cells treated with RID3 had a lower percentage of non-apoptotic cells (UL) (0.14%) compared to the control group ($p<0.05$). Surprisingly, the number of live cells (LL) was higher (74%) than the control group ($p<0.01$). There was a 21.36% of early apoptotic cells (LR) and 4.31% late apoptotic cells (UR). Cells exposed to a combination of *R. idaeus* D3 and 680 nm laser at 5 J/cm$^2$ showed only 12% of UL as compared to the 20% of control cells. There was an increased number of LL in all three fluencies combined with RI/D3 with 74.3%, 74.3 and 76.3 for RID3 +5, RID3 + 10 and RID3 + 15 J/cm$^2$ as compared to the control (72%). The combined treatment groups showed lower percentage of early apoptotic cells (LR) such as RID3+ 5 J/cm$^2$ (21.28%), RID3 +10 J/cm$^2$ (20.78%) and RID3+ 15 J/cm$^2$ (19.1%) compared to the control group (23.02%). Cells treated with RI D3+ 5J/cm$^2$ (4.28%) and RID3 + 15 J/cm$^2$ (4.32%) showed a slight increase in the number of late apoptotic cells. Cells treated with a combination of RID3+ 10J/cm$^2$ (4.78%) demonstrated a large increase in the number of late apoptotic cells ($p<0.001$). RID3 and 680 nm at 15 J/cm$^2$ produced the most statistically significant results out of the entire group, with $p$ values of $p<0.05$ in UL, LL and LR. Combined treatment of RID3 and 680 nm at a fluency of 10 J/cm$^2$ produced the most statistically significant result in UR with a $p$ value of $<0.001$. The dot plot graphical images of the results from the BD C- Sampler software can be found in Figure 10 and results are summarised in Table 2.
Table 2: Percentage of various cell populations after Annexin V/PI staining. The data depicted in the table was extracted by using the mean values of all percentages obtained from different treatment groups plus or minus the standard deviation (n=3). The lowest percentage of cellular apoptosis was obtained using the control and HeLa cells. (p<0.05 *; p<0.01 **; p< 0.001 ***).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Upper Left (UL)- non-apoptotic</th>
<th>Lower Left (LL)- live Cells</th>
<th>Lower Right (LR) - Early Apoptotic cells</th>
<th>Upper Right (UR)- Late Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.20 ± 0.01</td>
<td>72.70 ± 0.57</td>
<td>23.02 ± 0.94</td>
<td>4.25 ± 0.06</td>
</tr>
<tr>
<td>RID3</td>
<td>0.14 ± 2.88 **</td>
<td>74.00 ± 0.58**</td>
<td>21.36 ± 0.09</td>
<td>4.31 ± 0.39</td>
</tr>
<tr>
<td>RID3 + 5 J/cm²</td>
<td>0.12 ± 0.04</td>
<td>74.30 ± 0.33</td>
<td>21.28 ± 0.16</td>
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<td>RID3 + 10 J/cm²</td>
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<td>74.30 ± 0.88</td>
<td>20.78 ± 0.95</td>
<td>4.78 ± 0.001 ***</td>
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<tr>
<td>RID3 + 15 J/cm²</td>
<td>0.11 ± 0.02 *</td>
<td>76.30 ± 0.88 *</td>
<td>19.10 ± 0.96 *</td>
<td>4.32 ± 0.03</td>
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Figure 10: Examples Annexin V/PI dot plots. One out of the three dot plots obtained per cell group are depicted in the above image. Fig. 10a) Untreated control cells demonstrating 74% viable cells with 4.4% late apoptotic cells, b) Cells treated with *R. idaeus* D3 demonstrating 73.3% live cells and 5% late apoptotic cells, c) RID3 + 15 J/cm² demonstrating 78.1% live cells and 4.3% late apoptotic cells, d) RID3 + 10 J/cm² demonstrating 74.8% live cells and 4.2% late apoptotic cells and e) RID3 + 75.9% demonstrating 75.9% live cells and 4.8% late apoptotic cells.

4.2.2. Nuclear Damage Analysis Using Hoechst Staining

The nuclear changes of cells treated with RID3 as well as cells treated with a combination of RID3 and 680 nm laser at doses of 5, 10 and 15 J/cm² were compared to cells as shown in figure 10.
In the control group, nuclei appeared spherical and evenly distributed. There were a large number of cells present in the sample which demonstrated good cellular viability. When analysing the group of cells treated with RID3, there appeared to be a significantly reduced number of cells which had been stained by the Hoechst dye which indicated a reduction in living cells. There also appeared to be irregularly shaped nuclei with more shrunk appearance as compared to those seen in the control group, which further confirmed the possibility of cellular death.

With the combined treatment groups, the cells treated with RID3 and 680 nm laser at a dose of 5 J/cm² showed a reduced number of viable cells as compared to the control group. There were no significant differences in the shape of the nuclei in this treatment group when compared to the control cells. Cells treated with RID3 and 680 nm at a dose of 10 J/cm² showed a significant reduction in viable cells with few cellular nuclei which appeared to have a more rounded shape. The final treatment group of RID3 and 680 nm at a dose of 15 J/cm² showed a slight decrease in cell number when compared to the control group. There was evidence of degrading nuclei seen with very low fluorescence uptake. Multiple nuclei showed a change in shape and became more rounded or shrunk in appearance as compared to the shape of the nuclei seen in the control group. The images obtained can be observed in Figure 11.

![Figure 11: Hoechst Staining of CC HeLa cells. Cytotoxic changes in CC HeLa cells were observed after 24 h of treatment with R. idaeus D3 and combined treatment of R. idaeus D3 and 680 nm laser diode (5, 10 and 15 J/cm²). Fig 7a represents the control cells, (b) R. idaeus D3, (c) R.](image-url)
idaeus D3 + 5 J/cm², (d) R. idaeus D3 + 10 J/cm² and (e) R. idaeus D3 + 15 J/cm². White arrows indicate dying cells with rounded/ shrunked nuclei.
CHAPTER 5.
DISCUSSION

5.1. Contextualising Cervical Cancer and the Research Study

HPV rates are a cause for concern in Sub-Saharan Africa, with a minimum rate of 24% of women diagnosed with the infection annually. With this rise of HPV it is further a concern that CC rates will also increase due to the direct relationship between HPV strains 16 and 18, and the development of CC (Stewart et al., 2018; Williamson & Snyder, 2015). Currently the treatments for CC possess numerous side effects, which cause discomfort in patients (Aredes et al., 2018). Both homeopathic medicine and LLLT have recently shown promise over the last few years, by inducing cell death and reducing cellular proliferation in cancer cells through various metabolic pathways (Bartos et al., 2016) which interfere with cellular mitochondria, endoplasmic reticulum (Dewaele et al., 2011) and various genes (Saha et al., 2013).

Previous studies have shown an ability for R. idaeus in herbal preparations to possess high antioxidant content (De Souza, 2014) as well as an ability to improve absorption of chemotherapeutic drugs into cancer cells, further inducing apoptosis (Cho et al., 2016).

The results observed in this study demonstrated antagonistic as well as complimentary results when compared to previous studies of a similar nature.

5.2. Cellular Morphology

Cellular morphology was observed by identifying the typical morphological features present in cellular apoptosis. Nuclear fragmentation, reduction in cell volume and chromatin condensation were considered in the evaluation (Kroemer et al., 2009).

Morphological features of apoptosis were not present in cells treated with RI30cH and control cells. The cells were proliferating and were more confluent. When observing the morphological feature of RID6, there were several dead cells observed floating in the medium. The RID3 potency demonstrated the best results out of all the homeopathic preparations of RI. The morphological results demonstrated cellular shrinking and blebbing of CC in vitro, which are typical indications of cellular apoptosis.
Results obtained from the combined treatment were almost identical despite the fluence of laser used at this wavelength. The morphological changes which could be observed in the findings demonstrated no signs of blebbing in any of the groups of a combination of RID3 and 680 nm laser at doses of 5, 10 or 15 J/cm². There were also no signs of loss of membrane integrity and cell structure changes with all cell groups demonstrating a good consistency.

The low potency performance of RID3 goes against a previous study which made use of *T. occidentalis*, *C. maculatum*, *Carcinosin* and *S. serrulata* in potencies ranging from 12cH to 1000cK *in vitro* and *in vivo*. This study found that none of the remedies had the ability to induce apoptosis of breast cancer cells *in vitro* or *in vivo*. The RI30cH results conflict with a study conducted by Samidha and colleagues (2017) which made use of remedies in a 30cH potency and observed the ability for this potency to induce apoptosis in melanoma (B16F10) cells.

The laser results conflict with other research findings conducted by Allison *et al.* (2011) whereby laser had the ability to induce apoptosis in lung cancer cells at a wavelength of 630 nm at a dose of 5 J/cm². It is possible that the results obtained in this study conflict with Allison *et al.* (2011)’s findings due to the difference in wavelength used.

More research needs to be conducted in order to investigate the mechanism behind these results.

### 5.3. Cellular Viability Using Trypan Blue Assay

CC cells treated with RI30cH demonstrated an increased cellular viability of 82% (*p*<0.186) as compared to control cells which has a viability of 74%. This finding contradicts previous findings which made use of highly diluted homeopathic remedies in *in vitro* studies. One particular study made use of various remedies in dilutions of 200cH and 1000cH in the treatment of prostate cancer cells lines *in vitro* and observed a decrease in the number of viable cells (Jonas *et al.*, 2006). *Ruta graveolens* 30cH demonstrated an ability to reduce colon cancer cell proliferation (Arora & Tandon, 2015).

Homeopathic aggravations occur in cases whereby there is an initial worsening of symptoms which had existed at the time of administering the remedy. Aggravations are believed to occur to induce the body’s own healing potential by activating immune-system
cells. However, it is not possible for this to be the case in this study, as no immunological response could have occurred due to the *in vitro* nature of this study.

CC cells treated with RID6 and D3 demonstrated a reduced cellular viability to 61% (*p*<0.283) and 59% (*p*<0.292) respectively. These findings correlate to previous studies which made use of low potencies of homeopathic remedies to induce apoptosis in cancer cells and increase cytotoxicity (Arora *et al*., 2013; Mondal *et al*., 2016; Arora *et al*., 2015).

The combined treatment protocol also demonstrated a decreased cellular viability 60% in cells treated with a dose of RID3 + 5 J/cm² (*p*<0.88), 53% in cells treated at a dose of RID3 +10 J/cm² (*p*<0.41) and 59% in cells treated at a dose of RID3 + 15 J/cm² (*p*<0.80). These results compliment results from previous studies whereby laser had the ability to reduce the viability of MCF-7 breast cancer cells at a dose of 15 J/cm² (Horne *et al*., 2012). These results also correlate with a previous study which demonstrated anti-proliferative effects in prostate cancer cells at a dose of 5 J/cm² with a wavelength of 670 nm (Gheewala *et al*., 2018).

5.4. **Adenosine triphosphate (ATP)**

ATP is a nucleotide found in all living cells and its main function is to carry energy in cells. This nucleotide consists of three different structures; ribose, adenine and a triphosphate chain (Augustyn *et al*., 2019). ATP has been used extensively as a means to measure cellular viability. When cells lose membrane integrity, the ability for them to synthesize ATP resulting in a decrease in ATP levels in cellular cytoplasm. The ATP assay is also considered one of the fastest means of measuring cellular viability (Riss *et al*., 2004).

ATP levels increased in cells treated with RI30cH (*p*<0.945) and decreased in cells treated with RID3 (*p*<0.194). The increased ATP production after treatment with RI 30cH contradicts previously conducted studies which used highly diluted homeopathic remedies (Jonas *et al*., 2004; Torres *et al*., 2016; Noratto *et al*., 2016). One theory to this increase could be related to a promotion of cellular proliferation to improve CC cell viability. More research should be conducted in order to understand the mechanism behind this increase in ATP after treatment with RI30cH. RID6 demonstrated an increase in ATP suggesting the CC cell proliferation. The decreased ATP levels after the treatment with RID3 further relates to the blebbing observed in the morphological results, allowing
for assumptions to be drawn towards the ability for this potency of RI to induce cell death in CC cells.

CC cells treated with laser in combination with RID3 demonstrated an increase in ATP production at all doses of RID3 +5, RID3 +10 and RID3+ 15 J/cm² (p<0.001, <0.01 and <0.001). As mentioned previously, LLLT possesses not only an anti-carcinogenic and pro-apoptotic function but also has the ability to induce photobiomodulatory effects; a process which is believed to induce photochemical reactions in cells in order to stimulate the healing of damaged cells (Chung & Dai, 2012). When photobiomodulation occurs, there is an increase in ATP levels due to the stimulation of the mitochondria of the cell (Chung et al., 2012). CC is also considered an aggressive form of cancer which utilizes glycolysis in order to produce high levels of ATP (Weinber & Chandel, 2015). This further relates to how a treatment protocol favours CC cell proliferation could result in an increased level of ATP. This finding further correlates to the morphological changes in CC cells.

5.5. Lactate Dehydrogenase (LDH)

LDH is an enzyme which is produced by metabolically active cells and is released into the medium when a cell loses its membrane integrity (Liu et al., 2016). LDH is considered an accurate means of establishing the number of dead cells present in the culture plate. This is due to the fact that LDH leaks out of cells into the medium when cells undergo apoptosis and loss of membrane integrity (Legrand et al., 1992).

Both RI30cH (p<0.11) and RID6 (p<0.08) demonstrated a decrease in LDH levels. These findings in conjunction with the increase in ATP levels further confirm the favourable proliferative effect of these two potency preparations of RI on CC HeLa cells in vitro. LDH levels increased in cells treated with RID3 (p<0.212) which complimented the morphological results of blebbing and changes in membrane structure of the CC cells, decreased ATP levels and decreased cellular viability. This further enhances the proposed theory of the ability for RID3 to possibly induce apoptosis in CC cells.

The cells treated with 680 nm laser combined with RID3 demonstrated a large decrease in LDH levels upon analysis at 5 (p<0.472), 10 (p<0.065) and 15 (p<0.174) J/cm². These findings along with the findings of increased ATP and decreased viability are quite conflicting and go against the initial hypothesis that this particular wavelength of laser would increase cellular apoptosis in cancer cells when combined with RID3. However,
these results can be explained by making use of a phenomenon, observed in aggressive cancer cells, known as the Warburg effect.

The Warburg effect refers to an increased uptake of glucose by cancer cells when in an optimal environment leading to an increase in the amount of LDH produced (Flores et al., 2019) and is considered a major metabolic feature which exists in cancer cells (Zheng et al., 2018). A theory exists whereby LDH is considered a necessary acid in the proliferation of cancer cells. The reason behind this is that LDH is considered an important factor for angiogenesis and migration of cancer cells as well as the generation of Nicotinamide Adenine Dinucleotide (NAD+) (Husain et al., 2013; Bensinger & Christofk, 2012; Doherty & Cleveland, 2013).

Research pertaining to the metabolic processes of CC has been conducted and demonstrated the oncogenic effects of microRNAs with the Warburg effect. MicroRNAs are considered to be responsible for the utilization of glucose in aerobic and anaerobic environments with LDH-A being the LDH responsible for an increase in CC cells proliferation (Zhang et al., 2016). With this knowledge, it can be hypothesized that the RID3 potency possibly interfered with cell cycle checkpoints in CC cells by interacting the 5'- adenosine-monophosphate-activated protein kinase (AMPK) metabolic pathway (Marin- Aguilar et al., 2009). However, in the case of the combined treatment protocol of RID3 and the 680 nm laser, it can be theorised that an optimal environment was created for the CC cells to thrive and proliferate. This increased proliferation possibly resulted in an increased release of LDH-A, which was observed upon the LDH analysis. CC is considered an aggressive cancer which utilizes a process known as glycolysis in order to produce high ATP levels (Weinber & Chandel, 2015). This could further explain how a treatment protocol which favours CC proliferation could result in increased ATP levels.

With an understanding of the various metabolic processes utilized by cancer cells and the need for substantial amounts of glucose for the glycolytic pathways, a further theory which could be deducted from the above-mentioned findings can be derived. Assuming that the decreased cellular viability observed in the viability results may be a result of the cells depleting their glucose stores in the media. This suggests that this particular wavelength should not be used in the treatment of CC cells and further research needs to be conducted in order to identify the best wavelength to induce apoptosis in CC cells.
5.6. Cell Death Analysis

5.6.1. Annexin V/PI Staining

Upon analysis of results obtained from the Annexin V/PI staining, it appeared that the results were conflicting when compared to other experimental analyses (ATP, LDH and Trypan blue).

The most concerning results obtained were those pertaining to the untreated control cells. There were high levels of both Late and early apoptotic cells (4.25% and 23.02%) with only 72.7% viable cells. These results seemed to be closely related to other results obtained in cells treated with RID3 + 5, RID3 + 10 and RID3 + 15 J/cm² which demonstrated 4.28%, 4.78% and 4.32% of late apoptotic cells and 21.28%, 20.78% and 19.10% of early apoptotic cells. These results were conflicting when the high ATP levels and low LDH levels are taken into account from the previous analyses. Cells treated with a single treatment of RID3 demonstrated similar findings with 4.31% late apoptotic cells and 21.36% early apoptotic cells.

Annexins are calcium-regulated proteins which bind to phospholipids (Mirsaeidi et al., 2016). An Annexin kit makes use of fluorescent dye in order to detect the externalization of phosphatidylserine in cells, which is a measure of apoptosis in cells. Cells which are dead conjugate to propidium iodide (PI) which stains dead cells with a red fluorescence (Givan, 2001).

It is reported that certain cell lines seem to have phosphatidylserine which exists naturally on the surface of cell membranes. This is particularly prominent in cell lines which are tumorigenic in nature (De et al., 2018). Phosphatidylserine typically exists on the internal membrane of cells due to aminophospholipidtranslocase (Kenis et al., 2009; Davis et al., 2019). However, cancer cells lose the ability to translocate phosphatidylserine, resulting in its presence on the external surface of the cell membrane (Vallabhapurapu et al., 2015) without any signs of apoptosis (De et al., 2018). This is a result of an enzyme known as scramblase (Beck et al., 2006).

With this, it can be theorized that the Annexin V and PI dyes bound to the naturally occurring externalized phosphatidylserine of the highly viable and apoptotic CC cells and this could be the reason why the Annexin V and PI results were conflicting when compared to other analyses.
It can further be suggested that further research into the use of Annexin V/PI staining in cancer cell lines with high expression of phosphatidylserine should be conducted in order to identify which cancer cell lines should not be assessed using flow-cytometry.

5.6.2. Cell Death Results using Hoechst Staining

Hoechst dye is considered as a very popular fluorophore used to stain the DNA of cells, as it has a specificity towards DNA (Bučevičius et al., 2018). Hoechst dyes are excited by a UV light or laser which emits a blue light with a maximum wavelength of 460 nm. An advantage of Hoechst dyes is that they do not induce cytotoxicity, allowing for objective results to be obtained when examining cell death (Watkins et al., 1996).

Hoechst dye was used as a means of staining nuclei of CC cells in order to observe if any changes had occurred to the cellular nuclei after being treated with RID3 and a combination of LLLT 680 nm with RID3 at doses of 5, 10 and 15 J/cm².

Hoechst staining complimented other findings observed in ATP, LDH and viability assays. Cells treated with RID3 demonstrated a loss of nuclear integrity and a decreased number of viable cells as compared to untreated control cells. Cells treated with a combination of 680 nm laser and RID3 at doses of 5, 10 and 15 J/cm² demonstrated few changes with regards to shape and number when compared to the control cells. These findings further confirmed the theory that RID3 as a single treatment protocol has the ability to induce CC cell apoptosis whereas a combined treatment of RID3 and 680 nm laser enhances CC cell growth in vitro.

The findings observed with RID3 compliment previous research studies which used lower potencies such as mother tinctures and 6cH. One such study found the ability for *Ruta graveolens* mother tincture to induce apoptosis in colon cancer cells by condensing the chromatin and releasing cytochrome c (Arora, 2015). The results obtained from RI30cH however conflict other research findings. Another study observed the ability for both *Phosphorous* and *Arsenicum sulphuratum flavum* in 30cH potencies to induce apoptosis in melanoma cancer cells (Samidha et al., 2017).

No research has been conducted on a combination of 680 nm laser and RID3 or homeopathic remedies. Therefore more research needs to be conducted on the combined effects of homeopathic remedies and LLLT at a wavelength of 680 nm in order to further understand the mechanism behind these results.
6.1. Conclusion

Cervical Cancer is one of the leading causes of death amongst women worldwide (Chen., et al, 2018). Conventional therapies currently used induce unfavourable side-effects in patients resulting in undesired discomfort. Recently, homeopathic medicine has become a popular form of therapy and has shown promise in previous in vitro studies to induce apoptosis in cancer cells. Low level laser therapy has also shown promise in its ability to induce apoptosis in cancer cells by targeting the mitochondria of cancer cells.

This study aimed to investigate the use of *Rubus idaeus* in three homeopathic dilutions (30cH, D6 and D3) as a single treatment protocol and then in conjunction with 680 nm laser at doses of 5, 10 and 15 J/cm².

After the analysis of the results obtained from this study, there is an indication that *R. idaeus* D3 can initiate apoptosis of CC HeLa cells *in vitro* in the following respects; morphological changes depicted a fewer number of live cells as compared to untreated cells, decreased percentage of viable cells (59%), a decrease in ATP levels, increase in LDH levels, and nuclear and cellular changes typical of cells undergoing apoptosis (blebbing and shrinking) were evident from Hoechst staining. These results may indicate the beneficial effect of this homeopathic preparation of CC *in vitro*. The results could be more evident in an *in vivo* model.

Further results established indicated the ability of *R. idaeus* 30cH to accelerate CC HeLa cell growth in the following respects; little changes were observed upon cellular morphology, significantly increased percentage of viable cells (82%) with an increase in ATP levels and decrease in LDH levels. This suggests that this potency of *R. idaeus* should be avoided in patients with CC.

The combined treatment protocol of *R. idaeus* D3 with the 680 nm demonstrated an ability to activate the Warburg effect which is seen in highly viable tumorigenic cancer cells. This conclusion was drawn after careful analysis of the results obtained, demonstrating an increase in both LDH and ATP levels in cells treated with *Rubus idaeus* and 680 nm laser at all three doses of 5, 10 and 15 J/cm². This further denotes that the 680 nm laser
wavelength in combination with *R. idaeus* D3 is an unfavourable combination treatment for cervical cancer.

It is advisable that further *in vitro* and *in vivo* studies be conducted in order for a conclusion to be drawn with regards to the exact mechanisms of action of *R. idaeus* D3 on CC HeLa cells and a combination of homeopathic remedies and LLLT. This research further exemplifies that homeopathically prepared solutions cannot be regarded as placebo.

### 6.1.1. Future Perspectives

- Experiments were conducted 24 h after the treatment with homeopathically prepared *R. idaeus* and a combination of *R. idaeus* D3 and 680 nm laser. This could be a plausible explanation behind why certain results were insignificant in nature. It is recommended that future studies conduct experiments at various time lapses in order to observe if any changes in results occur.
- Further research into the investigation of which cancer cell lines exhibit higher levels of phosphatidylinerine on the cell membrane should be conducted with careful consideration into which cell death analyses should be used for future research.
- Further research into the use of combined treatment protocols of homeopathic medicine and LLLT should be conducted to develop novel CC treatment strategies.
CHAPTER 7.
REFERENCES


Models of Acute Inflammation and Collagen-Induced Arthritis. *Food Functions*, 5(12):3241-3251


Protease Activities by Targeting ERK1/2 and FAK Pathways in Human Lung Cancer Cells. *Food and Chemical Toxicology*, 62: 908-918


Surgical Biopsy Number 92498. (1951). MD: Department of Pathology, Johns Hopkins Hospital, Baltimore.


APPENDIX A

The *in vitro* Effects of Homeopathically Prepared *Rubus idaeus* and Low Level Laser Therapy on Cervical Cancer Cells

Cervical Cancer HeLa Cells (CCL-2) Cultured at 37°C with 5% CO₂ and 80% humidity

Cell Treatment Groups

- **Group 1**: Untreated Control Cells
- **Group 2**: *Rubus idaeus* (30cH, D6 and D3)
- **Group 3**: Optimal potency of *R. idaeus* and 680 nm laser (5, 10 and 15 J/cm²)

Cellular Response Assays after 24 hours

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<th>Morphology: inverted light microscope</th>
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<td>Viability: Trypan Blue</td>
<td>Apoptosis: Annexin V/PI Staining</td>
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<td>Cytotoxicity: LDH</td>
<td>Nuclear Damage: Hoechst Stain</td>
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<td>Proliferation: ATP</td>
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### APPENDIX B

**List of Media and Solutions Used**

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**Products used for the Process of Sterilization**

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**Products used for Experiments Conducted**

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</tr>
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**APPENDIX C**

List of Equipment Used for Irradiation, Morphological Analysis and Cell Counting of HeLa (CCL-2) Cells.

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<tr>
<th>Product name</th>
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## APPENDIX D

### List of Chemicals and Kits Used

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<td>Z666505</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Eppendorf®, Microtubes</td>
<td>Z666515</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Falcon (5mL) Polystyrene round bottom tube (flow cytometry tubes)</td>
<td>BD352054</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>Foetal Bovine Serum</td>
<td>10499-044</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Hanks Balances Salt Solution</td>
<td>H9394</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Heavy tin foil 5 m x 45 cm</td>
<td>6001007162603</td>
<td>Pick n’ Pay</td>
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<tr>
<td>Hoechst 33258 Pentahydrate (bis-benzimide) 10 mg</td>
<td>H1398</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Latex gloves small powder free</td>
<td>EV/40511</td>
<td>Scientific group</td>
</tr>
<tr>
<td>HeLa cell line</td>
<td>ATCC: CCL-2</td>
<td>ATCC</td>
</tr>
<tr>
<td>Microplate, 96 well, F-Botton clear, sterile 2pcs/bag</td>
<td>655161</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Item</td>
<td>Code</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>Microplate, 96 well, F-Botton (Chimney well), white, lumitrac, high binding, sterile 10pcs/bag</td>
<td>655074</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>P4333</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tissue culture flask 25 cm², angled neck, vent, sterile</td>
<td>CR/423052</td>
<td>Corning</td>
</tr>
<tr>
<td>Tissue culture flask 75 cm², canted neck, anti-tip, vent, sterile</td>
<td>CR/430641U</td>
<td>Corning</td>
</tr>
<tr>
<td>Tissue culture flask 175 cm², canted neck, vent, sterile</td>
<td>CR/431080</td>
<td>Corning</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>T8154</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Universal fit pipette tips, 100-1000 µL Bulk</td>
<td>CR4868</td>
<td>Corning</td>
</tr>
<tr>
<td>Rubus idaeus 30Ch, D6 and D3 30 ml Amber Glass Bottle</td>
<td></td>
<td>Fusion Homeopathics</td>
</tr>
</tbody>
</table>
APPENDIX E

Outline on the calculations conducted in order to create various potencies of *R. idaeus*

Decimal Ratio Mathematical Formula to produce *R. idaeus* 20 ml of D3 and 20 ml of D6

3/10 x 10 = 3 mL of mother tincture

10 mL – 3 mL = 7 mL 62%

3 mL of mother tincture will then be added to 7 mL of 62% EtOH to make *Rubus idaeus* D1.

1/10 x 10 = 1 mL of *Rubus idaeus* D1

10 mL- 1 mL = 9 mL of distilled water

Therefore, 1 mL of *Rubus idaeus* D1 will be added to 9 mL of distilled water.

Remedy will then be succussed 10 times in order to create *Rubus idaeus* D2.

1/10 x 20 = 2 mL of *Rubus idaeus* D2

20 mL – 2 mL = 18 mL of Distilled water

Therefore, to create *Rubus idaeus* D3, 2 mL of D2 will be added to 18 mL of distilled water and succussed 10 times.

- Further dilutions following the same process as above, will be done until a D5 potency has been achieved.

In order to create 20 mL of *Rubus idaeus* D6:

1/10 x 20 mL = 2 mL of *Rubus Idaeus* D5

20 mL – 2 mL = 18 mL of distilled water

Therefore 2 mL of *Rubus idaeus* D5 will be added to 18 mL of distilled water
This will then be succussed 10 times in order to create the final product of 20 mL of *Rubus idaeus* D6

**Centesimal Mathematical Formula for the production of *R. idaeus* 30cH**

**Mathematical Formula for the Calculation of Amount of *R. idaeus* Mother Tincture Needed to Make 10 mL of *Rubus idaeus* 1cH:**

\[
\frac{3}{100} \times 10 = 0.3 \text{ mL of mother tincture}
\]

10-0.3 mL = 9.7 mL of 62% alcohol

0.3 mL of *R. idaeus* mother tincture will be added to 9.7 mL of 62% EtOH to make *Rubus idaeus* 1cH

\[
\frac{1}{100} \times 10 = 0.1 \text{ mL of *Rubus idaeus* 1cH}
\]

10 mL – 0.1 mL = 9.9 mL of distilled water

Therefore, 0.1 mL of *Rubus idaeus* 1cH will be added to 9.9 mL of distilled water

Remedy will then be succussed 100 times in order to create *Rubus Idaeus* 2cH

This process will be repeated until a 29cH potency has been created.

In order to create 20 mL of *Rubus idaeus* 30cH:

\[
\frac{1}{100} \times 20 \text{ mL} = 0.2 \text{ mL of *Rubus Idaeus* 29cH}
\]

20 mL – 0.2 mL = 19.8 mL of distilled water

Therefore 0.2 mL of *Rubus idaeus* 29cH will be added to 18 mL of distilled water

This will then be succussed 100 times in order to create the final product of 20 mL of *Rubus idaeus* 30cH
APPENDIX F

Laser Parameters Used in This Study

a) Parameters for Cells irradiated with a Combination of *r. idaeus* D3 and LLLT 680 nm for ATP, LDH and ATP Assay Results

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Oriel Corporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name and Type</td>
<td>Diode Semiconductor Laser</td>
</tr>
<tr>
<td>Wavelength</td>
<td>680 nm</td>
</tr>
<tr>
<td>Spectrum</td>
<td>Visible (Red)</td>
</tr>
<tr>
<td>Wave Emission</td>
<td>Continuous</td>
</tr>
<tr>
<td>Spot Size</td>
<td>9.1 cm²</td>
</tr>
<tr>
<td>Power Output</td>
<td>201 mW</td>
</tr>
<tr>
<td>Power Density</td>
<td>22.15 mW/cm²</td>
</tr>
</tbody>
</table>

Fluence and Irradiation time:
- 5 J/cm² (3 min 47 sec)
- 10 J/cm² (7 min 34 sec)
- 15 J/cm² (11 min 22 sec)
b) Parameters for Cells Irradiated with a Combination of *R. idaeus* D3 and LLLT 680 nm for Cytotoxicity Assays

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Oriel Corporation</td>
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<tr>
<td>Name and Type</td>
<td>Diode Semiconductor Laser</td>
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<tr>
<td>Wavelength</td>
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<tr>
<td>Wave Emission</td>
<td>Continuous</td>
</tr>
<tr>
<td>Spot Size</td>
<td>9.1 cm²</td>
</tr>
<tr>
<td>Power Output</td>
<td>205 mW</td>
</tr>
<tr>
<td>Power Density</td>
<td>22.59 mW/cm²</td>
</tr>
<tr>
<td>Fluence and Irradiation time</td>
<td>5 J/cm² (3 min 37 sec)</td>
</tr>
<tr>
<td></td>
<td>10 J/cm² (7 min 14 sec)</td>
</tr>
<tr>
<td></td>
<td>15 J/cm² (10 min 52 sec)</td>
</tr>
</tbody>
</table>
APPENDIX G

Laser Calculations

The power output of the 680 nm laser diode was measured before treatment by using a laser power meter which measures in mW. The energy of the laser decreases as the light moves through air. The dosage of each treatment was expressed as J/cm², with fluencies delivered at 5, 10 and 15 J/cm².

The below image demonstrates the formulae used for the laser calculations:

Calculation for first cell group irradiated for viability, morphology, ATP and LDH experiments:

\[ \frac{(201 \text{mW} \times 4)}{(3.14 \times 3.4^2)} = 22.15 \text{mW/cm}^2 \]

\[ 22.15 \text{mW/cm}^2 / 1000 = 0.022 \text{ W/cm}^2 \]

Dose = 0.022 x time (s)

5 J/cm²

Time (s) = 5 J/cm² / 0.022

= 227.27 sec
Therefore for an energy of 5 J/cm² cells must be irradiated for 3 min 47 sec.

10 J/cm²

Time (s) = 10 J/cm² / 0.022

= 454.55 sec

= 454.55 / 60

= 7,575833333333333

= 7 min

7,575833333333333 – 7

= 0,575833333333333

0,575833333333333 x 60

= 34 sec

Therefore, for an energy of 10 J/cm² cells must be irradiated for 7 min 34 sec.

15 J/cm²

Time (s) = 15 J/cm² / 0.022

= 681.82 sec
Therefore, for an energy of 15 J/cm² cells must be irradiated for 11 min 22 sec.

Calculation for second cell group irradiated for Hoechst staining and Annexin V/PI staining experiments

\[
\frac{205 \text{ mW} \times 4}{(3.14 \times 3.4^2)} = 22.59 \text{ mW/cm}^2
\]

\[
22.59 \text{ mW} / 1000 = 0.023 \text{ W/cm}^2
\]

Dose = 0.023 x time (s)

\[
5 \text{ J/cm}^2
\]

Time (s) = \frac{5 \text{ J/cm}^2}{0.023}

\[
= 217.39 \text{ sec}
\]

\[
= 217.39 / 60
\]

\[
= 3.62316666666667
\]

\[
= 3 \text{ min}
\]

\[
3.62316666666667 - 3
\]

\[
= 0.62316666666667
\]

\[
0.62316666666667 \times 60
\]

\[
= 37 \text{ sec}
\]
Therefore, for an energy of 5 J/cm\(^2\) cells must be irradiated for 3 min 37 sec.

10 J/cm\(^2\)

Time (s) = \(10 \text{ J/cm}^2 / 0.023\)

\[= 434.78 \text{ sec}\]
\[= 434.78 / 60\]
\[= 7.246333333333333\]
\[= 7 \text{ min}\]

7.246333333333333 – 7

\[= 0.246333333333333\]

0.246333333333333 x 60

\[= 14 \text{ sec}\]

Therefore, for an energy of 10 J/cm\(^2\) cells must be irradiated for 7 min 14 sec.

15 J/cm\(^2\)

Time (s) = \(15 \text{ J/cm}^2 / 0.023\)

\[= 652.17 \text{ sec}\]
\[= 652.17 / 60\]
\[= 10.8695\]
\[= 10 \text{ min}\]

10.8695 – 10

\[= 0.8695\]

0.8695 x 60

\[= 52 \text{ sec}\]

Therefore, for an energy of 15 J/cm\(^2\) cells must be irradiated for 10 min 52 sec.
APPENDIX H

HeLa Cells (ATCC CCL-2) Product Information

ATCC Product Sheet
HeLa (ATCC® CCL-2™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
2

Intended Use

This product is intended for research use only. It is not intended for use in any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-Available Eagle’s Minimum Essential Medium, Catalog No. 30-2033. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: HeLa (ATCC® CCL-2™)

Description

Organism: Homo sapiens, human
Tissue: cervix
Disease: adenocarcinoma
Cell Type: epithelial
Age: 31 years old
Gender: female
Morphology: epithelial
Hela Markers: Y
Growth Properties: adherent
Virus Susceptibility:
Viral Testing: ATCC confirmed this cell line is positive for the presence of human papilloma (HPV) viral DNA sequences via PCR.
Bioenzymes:
GEPD, A
ENA Profile:
Aneuploidy: X
C3F/PO: 9.10
D13S317: 12,13,3
D16S539: 9.10
D5S818: 11,12
D7S820: 8.12
THO: 7
TPCO: 8.12
VWA: 16.18

Cytogenetic Analysis: Modal number = 82; range = 70 to 194.
There is a small telocentric chromosome in 10% of cells. 100% aneuploidy in 1305 cells examined. Four typical HeLa marker chromosomes have been reported in the literature. HeLa Marker Chromosomes. One copy of M1, one copy of M2, four copies of M3, and two copies of M4 as revealed by G-banding patterns. M1 is a rearranged long arm and centromere of chromosome 1 and the long arm of chromosome 3. M2 is a combination of short arm of chromosome 3 and long arm of chromosome 1. M3 is an isochromosome of the short arm of chromosome 5. M4 consists of the long arm of chromosome 11 and an arm of chromosome 19. Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.

Batch-Specific Information

Refer to the Certificate of Analysis for batch-specific test results.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials; this is important to note that some vials may become submerged in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force causing flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from dry ice packaging and immediately place the vials at a temperature below -150°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the cultures as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.
1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the cap off and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL complete culture medium and spin at
# APPENDIX I

## HeLa Cells (ATCC CCL-2) Certificate of Analysis

### Certificate of Analysis

<table>
<thead>
<tr>
<th>Test / Method</th>
<th>Specification</th>
<th>Result</th>
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<tbody>
<tr>
<td>Ampule passage number</td>
<td>Report results</td>
<td>Unknown ± 3</td>
</tr>
<tr>
<td>Population doubling level (PDL)</td>
<td>Report results</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Total cells/ampule</td>
<td>Report results</td>
<td>7.7 x 10^6 total cells/ampule</td>
</tr>
<tr>
<td>Post-freeze viability</td>
<td></td>
<td>93.8%</td>
</tr>
<tr>
<td>Growth properties</td>
<td>Adherent</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Epithelial-like</td>
<td>Epithelial-like</td>
</tr>
<tr>
<td>Test for mycoplasma contamination</td>
<td>None detected</td>
<td>None detected</td>
</tr>
<tr>
<td>Hemoctat DNA stain (indirect method)</td>
<td>None detected</td>
<td>None detected</td>
</tr>
<tr>
<td>Apurcature (direct method)</td>
<td>None detected</td>
<td>None detected</td>
</tr>
<tr>
<td>PCR-based assay</td>
<td>None detected</td>
<td>None detected</td>
</tr>
<tr>
<td>Species determination: C01 assay (interspecies)</td>
<td>Human</td>
<td>Human</td>
</tr>
</tbody>
</table>

---

**ATCC**

10831 University Boulevard
Manassas, VA 20110-3208 USA

www.atcc.org

900-926-8507 or 703-365-2700
Fax: 703-505-2750
E-mail: whcl@atcc.org
or contact your local distributor
## CERTIFICATE OF ANALYSIS

**ATCC® Number:** CCL-2™

**Lot Number:** 7000153

<table>
<thead>
<tr>
<th>Species determination: STR analysis (intraspecies)</th>
<th>Human (Unique DNA Profile)</th>
<th>Human (Unique DNA Profile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH01: 7</td>
<td>D5S818: 11, 12</td>
<td>D5S818: 11, 12</td>
</tr>
<tr>
<td>D5S818: 11, 12</td>
<td>D7SS800: 8, 12</td>
<td>D7SS800: 9, 12</td>
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<tr>
<td>D13S317: 12, 13</td>
<td>D16S539: 6, 10</td>
<td>D16S539: 6, 10</td>
</tr>
<tr>
<td>Amelogenin: X</td>
<td>CSF1PO: 9, 20</td>
<td>CSF1PO: 9, 10</td>
</tr>
<tr>
<td>vWA: 18, 18</td>
<td>TPOX: 8, 12</td>
<td>TPOX: 8, 12</td>
</tr>
</tbody>
</table>

| Sterility test (Bact/ALERT 3D)                    | No growth                  | No growth                  |
| (AST bottle (aerobic) at 32°C)                    |                            |                            |
| (NST bottle (anaerobic) at 32°C)                  |                            |                            |

| Human pathogenic virus testing                     | Report results             | HIV — None detected        |
| (PCR-based assay for HIV, HepB, HPV, EBV, and CMV) |                            | HepB — None detected       |
|                                                   |                            | HPV — Detected             |
|                                                   |                            | EBV — None detected        |
|                                                   |                            | CMV — None detected        |

*Epithelioid-like: Any adherent cells of a polygonal shape with clear, sharp boundaries between them.*

---

**Robbin L Smith**

Quality Assurance Specialist, Quality Assurance

ATCC hereby represents and warrants that the material provided under this certificate is pure and has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company’s knowledge and belief. This certificate does not extend to the growth and/or passage of any living organism or cell line beyond what is supplied within the container received from ATCC.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics. Appropriate Biosafety Level (BSL) practices should always be used with this material. Refer to the Product Information Sheet for instructions on the correct use of this product.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

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APPENDIX J

ATCC Laboratory Biosafety Clearance

-----Original Message-----
From: ATCC Sales [mailto:Sales@atcc.org]
Sent: 14 September 2010 10:46 PM
To: Houreld, Nicolette <nhoureld@uj.ac.za>; Abrahamse, Heidi <habrahamse@uj.ac.za>
Subject: ATCC Accounts LKS: BSL2 Upgrade Complete/184983

Good Afternoon,

This is to confirm that your request to have Cnr Siemert & Beit Streets be able to receive BSL-2 materials, has been approved and completed. Your order has been sent for processing. If we can be of further assistance please don't hesitate to contact us.

Regards,

Lauren
New Accounts
sales@atcc.org
APPENDIX K

Research Ethics Committee Clearance Renewal Letter

<table>
<thead>
<tr>
<th>Student/Researcher Name</th>
<th>Joubert, K</th>
<th>Student Number</th>
<th>20133285</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supervisor Name</td>
<td>Dr R Rading</td>
<td>Co-Supervisor Name</td>
<td>Dr G Blaisse</td>
</tr>
<tr>
<td>Department</td>
<td>Homoeopathy</td>
<td></td>
<td></td>
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<tr>
<td>Qualification</td>
<td>368</td>
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<tr>
<td>Research Title</td>
<td>The In Vitro Effects of Homoeopathically Prepared Rhus Toxus and Low Level Laser Therapy on Cervical Cancer Cells</td>
<td></td>
<td></td>
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<tr>
<td>Previous Clearance Date</td>
<td>30 October 2018</td>
<td>Clearance Number</td>
<td>REC 01 155 2018</td>
</tr>
<tr>
<td>Date</td>
<td>18 February 2019</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Approval of the research with details given above is renewed and is valid until 31 January 2020.

1. Conditions:

2. Renewal:
   It is required that the ethical clearance is renewed annually, within two weeks of the date indicated above. Renewal must be done using the Ethical Clearance Renewal Form (REC 10.0), to be completed and submitted to the Faculty Administration office. See Section 15 of the REC Standard Operating Procedures.

3. Amendments:
   Any envisaged amendments to the research proposal that has been granted ethical clearance must be submitted to the REC using the Research Proposal Amendment Application Form (REC 8.0) prior to the research being amended. Amendments to research may only be carried out once a new ethical clearance letter is issued. See Section 13 of the REC Standard Operating Procedures.

4. Adverse Events, Deviations or Non-compliance:
   Adverse events, research proposal deviations or non-compliance must be reported within the stipulated time-frames using the Adverse Event Reporting Form (REC 9.0). See Section 14 of the REC Standard Operating Procedures.

The REC wishes you all the best for your studies.

Yours sincerely,

Prof. Christopher Stein
Chairperson: REC
Tel 011 529 8034
Email: christ@uj.ac.za

RECX 3.0 – Faculty of Health Sciences
Research Ethics Committee

Secretariat: Ms Raisa Botes
Tel 011 529 8034 email: rbotes@uj.ac.za
APPENDIX L

Higher Degrees Committee Approval Letter

FACULTY OF HEALTH SCIENCES
HIGHER DEGREES COMMITTEE

HDC-01-105-2018
29 October 2018

TO WHOM IT MAY CONCERN:

STUDENT: JOUBERT, K
STUDENT NUMBER: 201363285

TITLE OF RESEARCH PROJECT: The In Vitro Effects of Homoeopathically Prepared *Rubus idaeus* and Low Level Laser Therapy on Cervical Cancer Cells

DEPARTMENT OR PROGRAMME: HOMOEOPATHY
SUPERVISOR: Dr R Retzlag
CO-SUPERVISOR: Prof H Abrahams
CO-SUPERVISOR: Dr G Blasman

The Faculty Higher Degrees Committee has scrutinised your research proposal and concluded that it complies with the approved research standards of the Faculty of Health Sciences; University of Johannesburg.

The HDC would like to extend their best wishes to you with your postgraduate studies.

Prof H Abrahams
Acting Chair: Faculty of Health Sciences HDC
Tel: 011 559 6550
Email: habrahams@uj.ac.za
## APPENDIX M

### Turnitin Plagiarism Report

**thesis report**

<table>
<thead>
<tr>
<th>ORIGI-NALITY REPORT</th>
</tr>
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<td>STUDENT PAPERS</td>
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### PRIMARY SOURCES

1. **Submitted to University of Johannesburg**
   - Student Paper
   
   - Publication