NELSON MANDELA
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PROFESSORIAL INAUGURAL LECTURE

PROFESSOR MARYNA VAN DE VENTER

Topic:

Exploring plants as medicine: an in vitro approach

Faculty of Science

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Exploring plants as medicine: an *in vitro* approach

Maryna van de Venter

Introduction: Plants as medicine

Archaeological evidence for the use of plants as medicine dates as far back as 60,000 years ago from the area now known as Iraq, and 8,000 years ago from China. The first written records are from the Sumerians from 5,000 BC and the Ancient Egyptians from 1,500 BC. Two well-known plants that were already used in that time are the opium poppy, and cannabis. The first pharmaceutical medicine was only developed in 1804 when the German Friedrich Sertürner isolated morphine from the opium poppy (Pan et al 2014).

Early research career: moving away from animal models of disease

As a biochemistry honours- and master's student I also had the privilege to work as a research assistant for the University of Port Elizabeth's (UPE) biochemistry department. My own research involved exercise biochemistry using Long-Evans rats that were trained to run on a special treadmill to monitor exercise-induced changes in their fat cell metabolism. Others were investigating the effects of cold acclimation on fat development and metabolism. My responsibility was to assist with aspects such as injecting and sacrificing the rats, isolating their fat cells and measuring their responses to different hormones. The work was interesting and I learnt a lot but I don't think anybody ever enjoys having to sacrifice animals.

Then for my PhD which was supervised by Prof Derek Litthauer and the late Prof Willem Oelofsen, I found an alternative source of fat cells; the fat tissue that is removed during surgical procedures such as abdominoplasty, reduction mammoplasty and liposuction of the hip- and thigh areas. This tissue is normally incinerated after the operation, but we obtained permission and patient consent to use it for research. Finally, I could wave animal experimentation goodbye and a new interest was sparked – mammalian cell culture and *in vitro* bioassays.

Diversifying into new areas of research

After a career break of 4 years, I was given the opportunity to return to the university. A few things have changed; the fat cell research at UPE ended because Prof Litthauer has left for the University of the Free State and Prof Oelofsen retired. I was invited to work with Prof Pieter Milne from Pharmacy, investigating the anticancer activity of cyclic dipeptides and I worked with Prof Saartjie Roux. Commercial cell lines were now readily available, and we did not have to work with animals or human donors. I

also started doing anticancer screening of plant extracts for Shimoda Biotech in Plettenberg Bay. A few other opportunities presented themselves in the next year or two which triggered my interest in medicinal plants. A literature search suggested that the only medicinal plant research conducted in South Africa at the time was ethnobotanical surveys, and antimicrobial and antioxidant activity testing. This left the door wide open for anticancer and antidiabetic activities. From humble beginnings in 2000 and with the help of many postgraduate students and colleagues, we have since developed a comprehensive *in vitro* drug screening platform (BioAssaix Screening Services, www.bioassaix.com) that covers not only anticancer and antidiabetic activities, but also anti-inflammatory, antimicrobial and anti-ageing and cosmetic activities and some of the diabetic complications.

How do these bioassays work?

Medicinal plant extracts can be evaluated for toxicity and bioactivity using *in vivo* (animals, for example mice or rats) or *in vitro* models (enzyme assays, cell cultures, etc.). The best way is of course through human clinical trials, but these are only performed if there is enough *in vitro* and *in vivo* evidence of safety and efficacy.

In vitro means outside the body, in an artificial environment. The past few decades have seen vast improvements in the ability of *in vitro* bioassays to accurately predict *in vivo* effects in terms of toxicity and efficacy. The availability of commercial continuous cell lines has greatly contributed to this trend. The cells can be preserved in liquid nitrogen for long periods of time and they are “immortal” which means they can proliferate an unlimited number of times, thus eliminating the need to sacrifice animals or continuously obtaining human biopsy material.

There are many ways to test the safety and potential therapeutic properties of plant extracts or compounds on cells, but for the past 6 years we have been using the ImageXpress Micro XLS high content analysis system which was co-funded by the National Research Foundation and Nelson Mandela University. The ImageXpress Micro XLS is a fluorescence microscope, but it is capable of doing much more than a standard fluorescence microscope. The process will be outlined below.

We grow the cells in 96-well plates, add plant extracts for a certain amount of time and then use fluorescent dyes to detect changes that have occurred in the cells. We can stain things like organelles, DNA, a specific protein of interest, lipids and much more. After staining the cells, the plate is placed in the instrument and fluorescence photographs are taken of the cells in each well of the 96-well plate. In Figure 1, the instrument was set up to take 9 images in each well. In this example we used two dyes to measure oxidative stress: the blue is a dye that binds to DNA and therefore labels all the nuclei, and the yellow dye is CellRox, which only fluoresces when it reacts with reactive oxygen species inside the cell. It takes about 40-45 minutes to take all these images – 1728 in total. Taking a closer look at one image site, you can see the

high resolution image of the nuclei and the corresponding image for CellRox. The last image shows an overlay of these two. A higher intensity of CellRox staining indicates a higher level of oxidative stress.

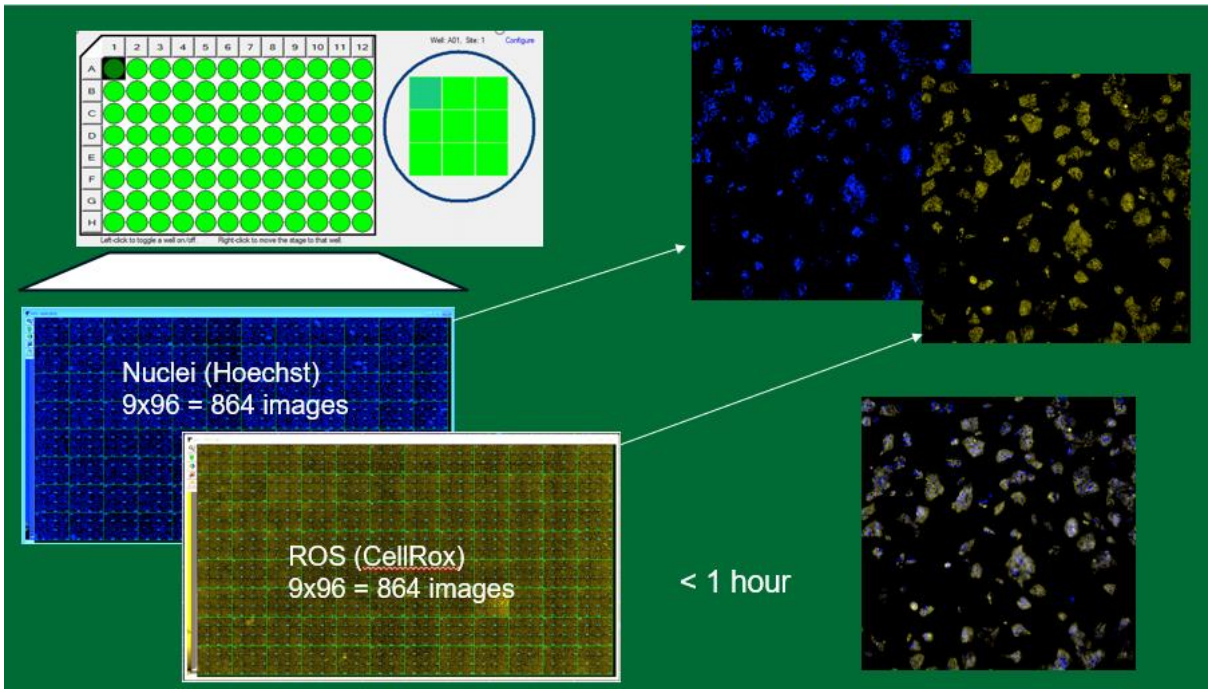
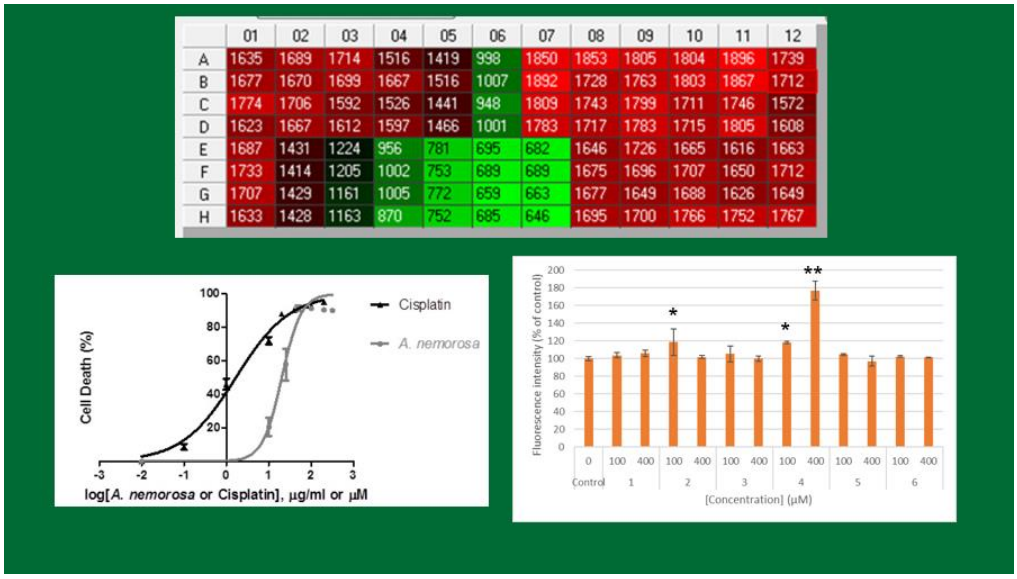


Figure 1. ImageXpress Micro XLS workflow.

The most impressive part of this instrument is its onboard analysis software that can analyse the images to give us quantitative data to work with. It counts the cells, measures the fluorescence intensity in each cell and also the average intensity of all the cells in each well.



	01	02	03	04	05	06	07	08	09	10	11	12
A	1635	1689	1714	1516	1419	998	1850	1853	1805	1804	1896	1739
B	1677	1670	1699	1667	1516	1007	1892	1728	1763	1803	1867	1712
C	1774	1706	1592	1526	1441	948	1809	1743	1799	1711	1746	1572
D	1623	1667	1612	1597	1466	1001	1783	1717	1783	1715	1805	1608
E	1687	1431	1224	956	781	695	682	1646	1726	1665	1616	1663
F	1733	1414	1205	1002	753	689	689	1675	1696	1707	1650	1712
G	1707	1429	1161	1005	772	659	663	1677	1649	1688	1626	1649
H	1633	1428	1163	870	752	685	646	1695	1700	1766	1752	1767

Figure 2. After image analysis, data is presented in various formats like the heat map shown here (top). The data can be logged and used to prepare graphs and to perform statistical analysis (bottom).

That is how we do many of our *in vitro* bioassays. Cancer and diabetes will be used as examples to explain how plant extracts or chemical compounds can be tested *in vitro* for potential therapeutic properties.

Anticancer activity

I want to stress that we are not advocating the use of herbal remedies as cancer treatments at all. Not on their own or in combination with cancer chemotherapy. The aim of this research is to identify plants that produce compounds that may one day lead to the development of new anticancer chemotherapies. Cancer patients should discuss all alternative medicine or supplements taken while they undergoing treatment with their Oncologists because there is always potential for herb-drug interactions that may affect the outcome of the treatment (Lam et al 2022).

Cancer is one of the leading causes of death worldwide. In 2020, nearly 10 million people died of cancer. About one third of cancer deaths are preventable because the cancer was caused by bad lifestyle choices. Tobacco use, alcohol consumption, being overweight, not consuming enough fruit and vegetables in the diet and a lack of physical activity can all increase the risk of developing certain types of cancer. https://www.who.int/news-room/fact-sheets/detail/cancer

There is a general perception that tumours grow fast because cancer cells divide faster than normal cells, but this is not the case. Cell division is required to allow tissue growth or to replace old cells. About 1% of all the cells in an adult's body are replaced daily (Fischetti & Christiansen 2021). That means that as you are sitting there, 1% of your cells are dying, 1% are dividing to replace the dead ones, while the rest are in a so-called resting phase. The result is that the total cell number remains more or less constant (Figure 3). Cancer cells do not divide any faster than normal cells, but they do so more often. In a tumour, almost all the cells are continuously diving. If 90% of the cells in a tumour are dividing, the cell number will be almost double by day 2, and that number will again double by day 3. A cancer cell just doesn't know when to stop dividing, and they also have methods to evade cell death.

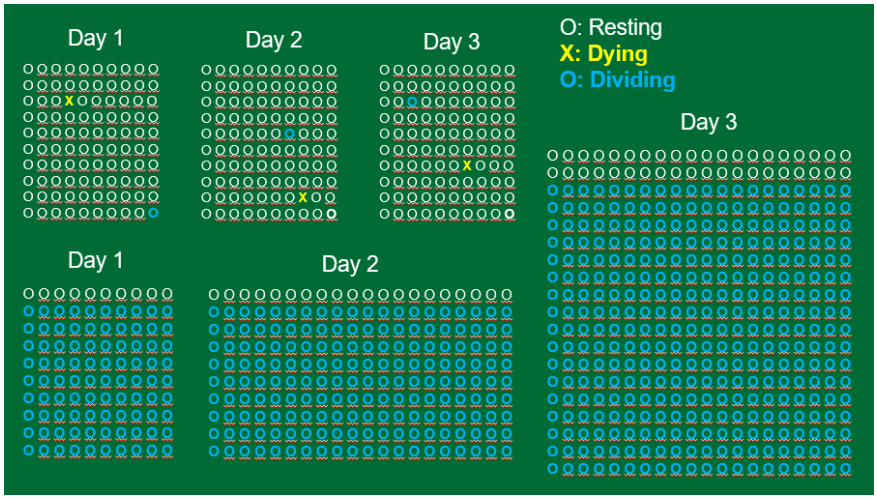


Figure 3. The difference in growth rate between normal cells (Days 1-3, top) and cancer cells (Days 1-3, bottom).

A normal cell spends most of its time in a non-proliferating state and will only enter the cell cycle to produce two daughter cells when it receives a signal from a growth factor. At numerous points throughout the cell cycle are checkpoints to ensure that the two daughter cells do not have any defects, including genetic mutations (Figure 4). The damage will be corrected before the cell cycle will continue, or if it cannot be corrected the cell will be programmed to die (Yan et al 2020). The most well-known form of programmed cell death is known as apoptosis, where cellular pathways are activated to break down the cell, its organelles and its macromolecules in an orderly fashion that does not lead to inflammation. This is different from accidental cell death where the cell membrane bursts and the cellular content spills into the surrounding tissue, causing inflammation and pain (Yan et al 2020).

Cancer cells have genetic mutations that enable them to proliferate uncontrollably. They proliferate even in the absence of growth factors and they have the ability to evade all the cell cycle checkpoints and programmed cell death, thereby producing generation after generation of genetically mutated cells (Matthews et al 2022).

Most anticancer chemotherapies target the cell cycle and cell death pathways to stop cancer cell proliferation and induce programmed cell death. This is also the approach that we follow when screening plant extracts for potential anticancer activity.

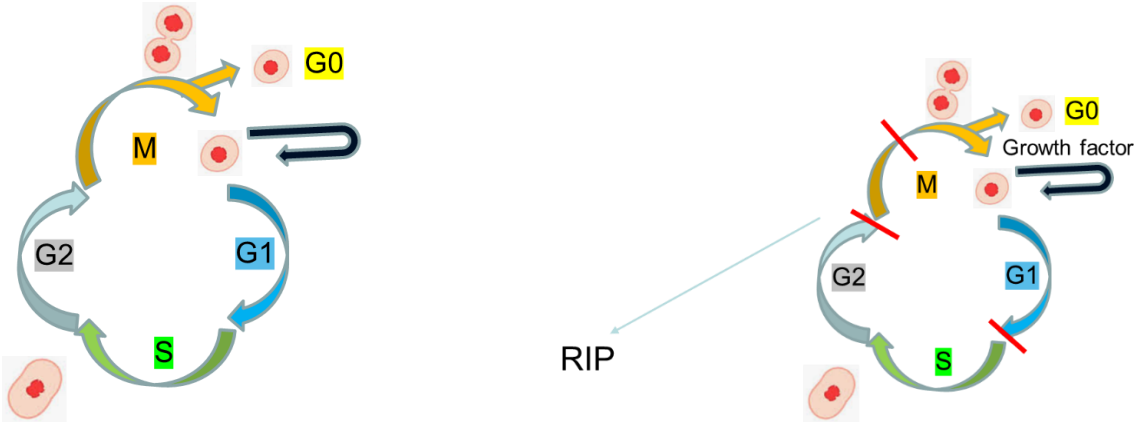


Figure 4. Phases of the cell cycle. Left: The cell cycle consists of four phases (G1, S, G2 and M). Normal cells "rest" outside the cell cycle in G0 phase until growth factors instruct them to enter the cycle at G1 to divide, forming two identical daughter cells. Right: Restriction points throughout the cell cycle (red lines) will halt the cell cycle if any defects such as DNA damage are detected, allowing the cell to correct the defects or if the damage cannot be corrected, the cell will be programmed to die.

Cells can die in many ways. Slamming your finger in a door or spilling acid on your skin causes accidental cell death. The cell membranes burst, the cellular contents spill into the surrounding tissue, leading to inflammation and pain.

Then there is programmed cell death. There are different forms of programmed cell death, the most well-known is apoptosis, where cellular pathways are activated to break down the cell, its organelles and its macromolecules in an orderly fashion that does not lead to inflammation (Yan et al 2020). The cell basically commits suicide without you even knowing about it. This is ideally how cancer cells should be killed. When we screen plant extracts for potential anticancer activity, we look for typical morphological and biochemical processes that occur in different forms of cell death.

Figure 5 shows some examples of signs of apoptosis that we look for. The membrane phospholipid phosphatidylserine is only exposed on the outside of the cell membrane when a cell is undergoing apoptosis (Yan et al 2020). This is a signal for immune cells to remove and degrade the dying cell. We can detect this with a fluorescently labelled protein that binds to phosphatidylserine. Healthy cells show no green fluorescence, while apoptotic cells will fluoresce green.

Healthy mitochondria have a strong electrochemical potential across their membranes to generate energy. In apoptotic cells, this potential collapses and the effect can be seen with fluorescent dyes that accumulate in the mitochondria according to the strength of the membrane potential (Hariharan et al 2021). Healthy cells stain much brighter than apoptotic cells.

Caspases are enzymes that are activated during apoptosis to degrade cellular proteins (Hariharan et al 2021). For this, fluorescent antibodies are used to detect the active form of the enzyme, with an increase in intensity indicating apoptosis.

One of the final steps of apoptosis involves condensation and degradation of the DNA (Hariharan et al 2021), which can be seen with DNA-binding dyes. There is an increase in fluorescence intensity and clear evidence of fragmented DNA.

Results obtained from anticancer research done by our research group for the medicinal plants *Artemisia afra* and *Anemone nemorosa* will be briefly described in the next section.

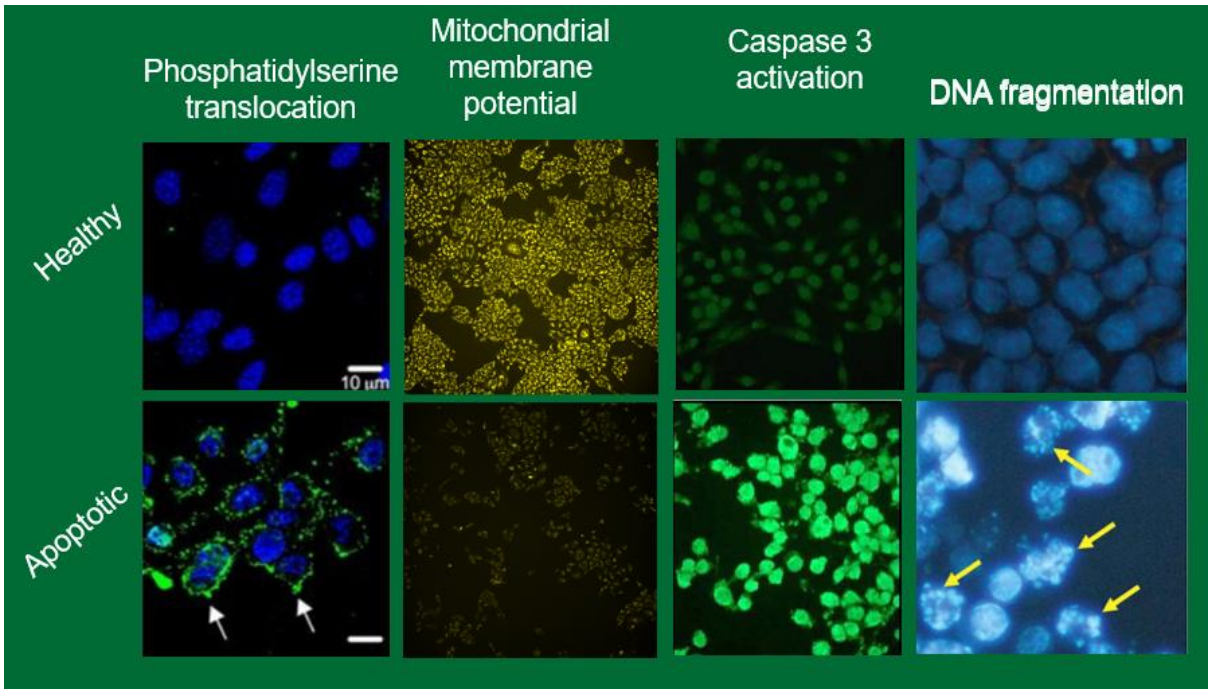


Figure 5. Examples of morphological and biochemical changes that can be detected in apoptotic cells using fluorescence image analysis.

Artemisia afra

This plant is commonly known as umhlonyane (Xhosa, Zulu), wormwood (English) or wildeals (Afrikaans) and is one of South Africa's most widely used medicinal plants (Liu et al 2009). Although it is used for a wide variety of conditions, especially but not exclusively respiratory and inflammatory conditions, literature has not reported its use for the treatment of cancer (Spies et al 2013).

When we tested aqueous and ethanol extracts of the leaves of *A. afra*, the aqueous extract had no effect on cancer cell growth, while the ethanol extract inhibited HeLa

cervical cancer and U937 leukaemia cell growth at IC50 values of 18.21 and 31.88 µg/mL, respectively. Inhibition of the cell cycle occurred in the mitotic phase. This was caused by tubulin hyperpolarization which induced a process known as mitotic catastrophe. The cells were unable to overcome this inhibition and apoptotic cell death was induced (Spies et al 2013). Dying cells showed all the classical signs of apoptosis, including membrane blebbing, a collapse of the mitochondrial membrane potential, phosphatidylserine translocation, caspase activation and DNA fragmentation. A new sesquiterpene lactone named isoalantolactone, was isolated which was partly responsible for the effects seen with the crude extract (Figure 3) (Venables et al 2016).

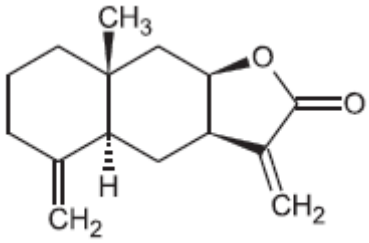


Figure 6. A new sesquiterpene lactone named isoalantolactone, was isolated from the ethanol extract of *A. afra.* This compound was partly responsible for the antiproliferative effects seen with the crude extract (Spies et al 2013).

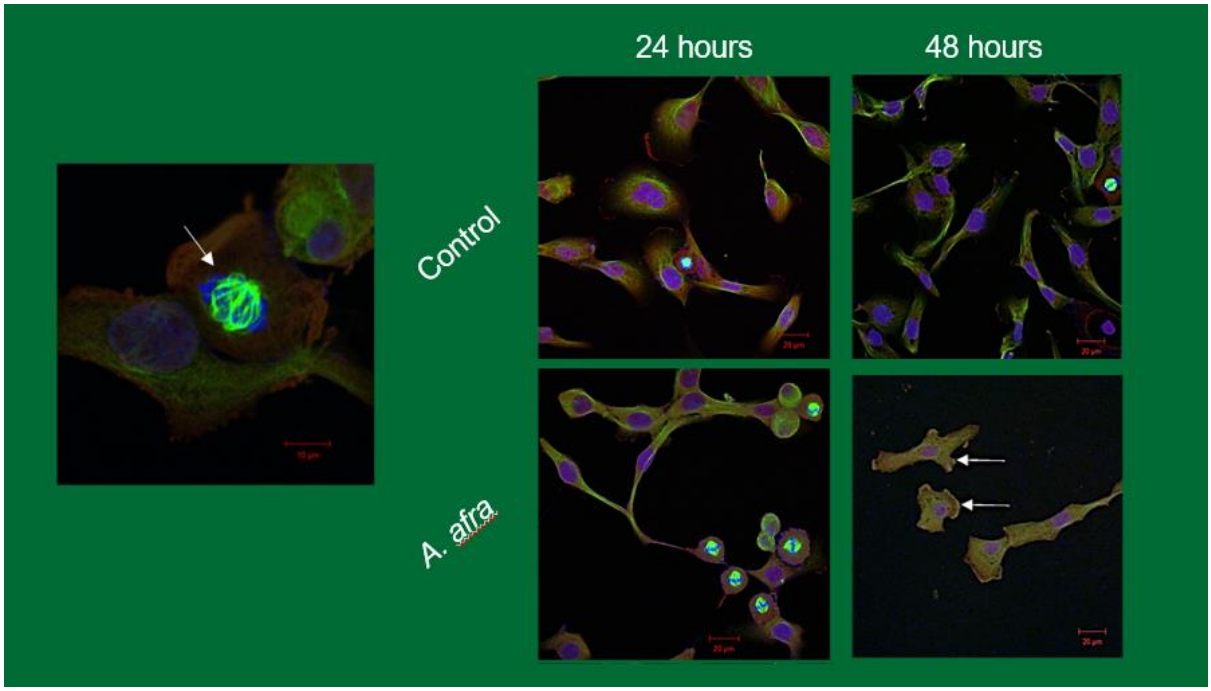


Figure 7. Confocal micrographs where an increase in the number of mitotic cells can be seen in cells treated with *A. afra* ethanol extract for 24 hours, and membrane blebbing (a sign of apoptosis) after 48 hours. Green fluorescence is tubulin and blue is DNA – hyperpolarization of tubulin prevented the cells from completing mitosis, leading to apoptosis.

Anemone nemorosa

This work was done in collaboration with researchers from the Carol Davila University of Medicine and Pharmacy, Bucharest, Romania. *A. nemorosa*, a European medicinal plant, is used for a variety of health conditions, including malignant ulcers. The plant material was collected and extracted in Romania and tested at Nelson Mandela University against HeLa cervical cancer cells. The aqueous extract inhibited cervical cancer proliferation with an IC50 value of 20.33 µg/mL. This was achieved through cell cycle arrest in the M-phase, accompanied by a significant increase in oxidative stress and induction of apoptosis (PS translocation, decreased mitochondrial membrane potential, caspase activation) (Swanepoel et al 2019).

Antidiabetic activity

Type 2 diabetes is a lifestyle disease and, in many cases, preventable through lifestyle changes (Bellou et al 2018). The most common risk factors are:

- Sedentary lifestyle
- Overweight and obesity (53% of South Africans are overweight; 28% obese)
- Unhealthy diet
- Stress (overproduction of cortisol, the “stress hormone”)
- Family history
- Age

There are risk factors like a family history of type 2 diabetes and age that we have no control over, but even those individuals can lower their risk by adopting a healthy lifestyle.

To understand diabetes, you have to first understand the effect of insulin on blood glucose levels. If you eat carbohydrates, starch is digested to glucose, this is absorbed and blood glucose levels increase within a few minutes. The pancreas detects this increase in blood glucose and responds by secreting insulin. The insulin travels through your bloodstream to muscle, liver and fat tissue where it stimulates the cells to take up glucose, which lowers blood glucose levels back to normal within an hour or two. In an individual with type 2 diabetes, fasting glucose levels are elevated, and the decrease after ingestion of carbohydrates is much slower. This is because insulin is not working as it should. So why doesn’t it work as it should?

Pre-diabetes is characterized by chronic inflammation and oxidative stress that leads to insulin resistance. Insulin resistance is then what causes high blood glucose levels

and diabetic complications. Insulin resistance is a situation where insulin is secreted, but it does not have the expected effect on the target cells (Yaribeygi et al 2019).

To understand how insulin and other hormones works, picture a domino cascade effect. Once the first domino has been knocked over, it starts a chain reaction of events that will continue until the last one has fallen. So the hormone knocks over the first domino, and the last domino is the final reaction that the hormone triggers inside the cell. Figure 8 gives a simplified illustration of the effect of insulin on one of its target cells. In the top figure, there are insulin receptors in the membrane and other proteins inside the cell that won't do anything until insulin initiates this domino effect. After a meal, blood glucose levels are high, but the glucose cannot get over the membrane without assistance.

In a normal cell where there is no insulin resistance, insulin is released by the pancreas, and it binds to insulin receptors on the muscle cell as shown in Figure 8 (middle). This binding activates the receptor and that initiates a complex cascade of events that ultimately opens glucose transporters in the membrane to enable the cell to take up the glucose and return blood glucose levels to normal. The glucose in the cell can be used for energy or stored as glycogen or fat, depending on the cell type (Biglou et al 2021).

If one of these dominoes doesn't fall, the rest of the pathway will remain inactive and the glucose will stay in the bloodstream. This is what happens in insulin resistance, as illustrated in Figure 8 (bottom).

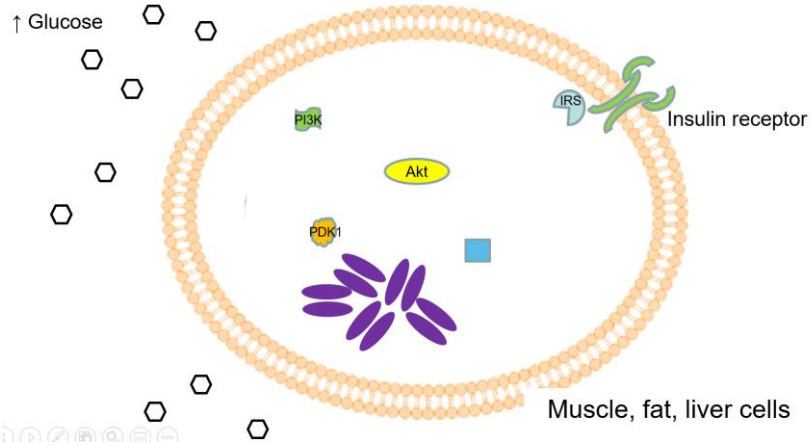
Lifestyle interventions can improve insulin resistance and type 2 diabetes. Improving your diet and exercise both increase glucose uptake, while reducing stress will decrease cortisol levels which will stop the liver from overproducing glucose. All of these will improve insulin sensitivity, they don't have to cost you anything and they have no detrimental side effects.

Pharmaceutical intervention is another approach to treat type 2 diabetes. There are many types of antidiabetic drugs on the market, targeting one of the following:

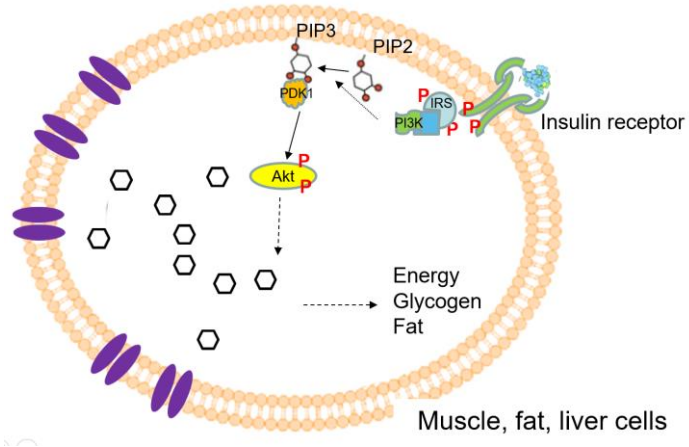
- Increase glucose uptake in muscle and fat cells;
- Reduce glucose production by the liver;
- Reduce the digestion of starch in the digestive system.

These approaches have the same overall effect as lifestyle changes, but they can be expensive and all drugs have side effects.

Target cell in the absence of insulin



Target cell (not insulin resistant) after activation by insulin.



Insulin resistant target cell in the presence of insulin.

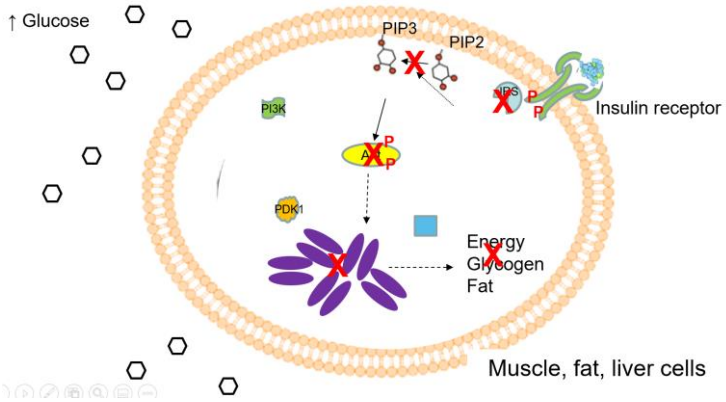


Figure 8. The insulin signal transduction pathway. Top: Normal insulin signalling pathway in a muscle cell. Insulin binds to insulin receptors on the cell membrane to activate a series of events that lead to glucose being taken up by the cell via glucose transporters in the membrane. This reduces blood glucose levels. Bottom: In insulin resistant cells, the signalling pathway is defective. One or more of the signalling intermediates is not activated in response to insulin and glucose cannot be removed from the blood.

Plants produce secondary metabolites that can be developed into pharmaceutical drugs. Metformin, the most widely used drug for the treatment of type 2 diabetes, is based on chemical compounds produced by the plant goat's rue. Plant extracts may contain more than one compound that work at different antidiabetic targets to improve efficacy. These can be taken as supplements or as herbal teas.

Two examples of plants that we have tested extensively for *in vitro* antidiabetic activity are *Aspalathus linearis* and *Sutherlandia frutescens.*

Aspalathus linearis **(Rooibos)**

Rooibos is a popular and well-known South African herbal tea. Its traditional uses include the following (Chaudhary et al 2021):

- Soothe digestion
- Stomach cramps
- Colic (babies)
- Diarrhea
- Allergies & eczema
- Slow aging

Quite of lot of research has been done on Rooibos confirming its health benefits. Rooibos is known for its exceptional antioxidant activity. We have shown potential anti-aging effects through reversal of mitochondrial dysfunction (Hattingh et al 2019). Its wound healing effects have been shown *in vitro* (Pringle et al 2018) and confirmed *in vivo* by Dr Abimbola Sowemimo, our collaborator and dear friend from the University of Lagos, Nigeria (Elegbede et al 2020). Antidiabetic and anti-inflammatory effects have been seen *in vitro* and in rat models (reviewed by Chaudhary et al 2021) and it has been shown to reduce oxidative stress in a small number of human volunteers (Marnewick et al 2011, 2016; Davies et al 2019).

Sutherlandia frutescens **(cancer bush, kankerbos)**

Sutherlandia frutescens or cancer bush is another very popular medicinal plant in South Africa. Its traditional uses include (Pringle 2020):

- Diabetes
- Anti-inflammatory
- Immune booster
- Fever
- Anxiety, depression
- Cancer

Despite its common name, there is no convincing evidence in literature to support Sutherlandia's anticancer activity. The anti-diabetic effects, especially at the level of insulin resistance, have been confirmed in rat experiments that we have done with Prof Saartjie Roux (Chadwick et al 2007; MacKenzie et al 2012). We have also tested it extensively for *in vitro* antidiabetic activity and its *in vitro* anti-inflammatory effects have also been reported by our group and others.

The most significant *in vitro* antidiabetic effects of Rooibos (Pringle et al 2021) and Cancer bush (Pringle 2020) from our research are summarised in Table 1.

Table 1. The most significant *in vitro* antidiabetic effects of *Sutherlandia frutescens* (cancer bush) and *Aspalathus linearis* (Rooibos).

Activity	**Mechanism**	***S. frutescens***	***A. linearis***
Anti-inflammatory	iNOS, COX-2 expression ↓	√	
Anti-inflammatory	NFkB pathway		√
Glucose uptake increased	Insulin resistant muscle cells	√	√
Lipid accumulation decreased	Insulin resistant muscle cells	√	
Intestinal glucose uptake decreased	Intestinal epithelial cells (not digestion)	√	
Pancreatic beta cell health	Oxidative stress decreased	√	√
Pancreatic beta cell health	Proliferation increased		√
Insulin secretion	Stimulates beta cells		√
Anti-glycation	Prevents protein glycation		√

As you can see in this table, the nice thing about plant extracts as opposed to a single chemical, is that they may contain more than one compound that work at different antidiabetic targets to improve their overall effect.

Conclusion

We all know that part of a healthy diet is the inclusion of lots of fruits and vegetables, but in my opinion it could also include herbal teas and supplements. The problem with this is that herbal products are not standardised and regulated to ensure efficacy and safety. At least Rooibos has a proven safety record so combine your cup of tea with other healthy habits and who knows, it may add a few healthy years to life.

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