THE *IN VITRO* PHARMACOLOGICAL ACTIVITIES OF *PERICAMPYLUS GLAUCUS* (LAM.) MERR. AND THEIR RELATION TO ETHNOMEDICINAL USE

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Declaration

I, Fiona Natalia Shipton, declare that this thesis is my own work. It is being submitted for the Degree of Doctor of Philosophy at the School of Pharmacy, University of Nottingham Malaysia Campus, Malaysia. It has not been submitted for a degree or examination at this or any other University.

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Date:_____

Abstract

Malaysia's rainforests have a great biodiversity, a lot of which has yet to be studied. This presents researchers with the opportunity for the discovery of new chemical structures which may be new leads in drug discovery. With problems such as antibiotic resistance and the need for improved drugs for the treatment of diseases such as cancer and neurodegenerative diseases, there is need for more research into drug discovery. The focus of the research covered in this document is on *Pericampylus glaucus*, a common climbing plant for which there is limited literature available. The aim of this study is to determine its pharmacological activities, based on what is known of its traditional use, in the hope that it can be used in the development of pharmaceutical drugs in the future.

The literature review of the traditional uses of *P. glaucus* suggests that the plant may be used in ethnomedicine for its anti-inflammatory and/or antibacterial activity as it is used to treat many conditions in which inflammation and infection is indicated. Components of *P. glaucus* extracts were investigated through a variety of phytochemical screening methods and were found to include polyphenols such as tannins and flavonoids, terpenes, sterols, alkaloids and saponins and similarities between the different extracts were observed. *P. glaucus* was found to possess antioxidant activity, with the stem chloroform extract possessing the greatest radical scavenging activity. The antibacterial assays revealed only mild antibacterial activity by *P. glaucus* extracts, making the use of the plant for treating infections seem unlikely, although this may be due to activity against specific strains of bacteria. The anti-inflammatory assays suggested a number of different mechanisms by which *P. glaucus* could exert anti-inflammatory activity, with many of the extracts and known compounds inhibiting COX-1 and COX-2 enzymes, and only the fruit hexane extract providing less than 50% inhibition of COX-1 and COX-2 at 500 µg/mL. The hexane extracts generally prevented protein denaturation to a greater extent compared to other extracts, as well as palmitic and stearic acid. The extracts showed a varying ability to prevent lysis of red blood cells, which were used as a model for lysosomes, depending on whether lysis was induced by hypotonicity or by heat. Some anticancer activity, cytotoxicity and growth promoting activity was recorded when cells were cultured with *P. glaucus* extracts. The ethanol extracts were found to exert the greatest cytotoxic activity, with the exception of the fruit ethanol extract. The root ethanol extract was the most toxic towards HK1 and MRC-5, while the stem hexane extract increased HK1 proliferation by over 200% and the root hexane extract increased MRC-5 proliferation by over 600%. Because of the growth promoting activity that was observed and the traditional use of *P. glaucus* for the treatment of wounds, the wound healing activity of the extracts was tested and found to be much less effective than the control of 10% FBS, although the greatest wound healing activity of around 20% wound coverage after 24 h was seen by the fruit extracts. Since *P. glaucus* is traditionally used for the treatment of venomous snake bites, its antivenom activity was assessed and found to have some antivenom activity, with the fruit hexane extract increasing cell proliferation by over 50% at 200 µg/mL and the alkaloid, periglaucine A, increasing proliferation by over 70% demonstrating significant antivenom activity.

The antioxidant activity is most likely attributable to the polyphenols as the total polyphenol content correlated with the results of the DPPH radical scavenging activity of the extracts. The mild antibacterial activity suggested that *P. glaucus* may be used more for its anti-inflammatory activity in traditional medicine rather than antibacterial activity. The findings of toxicity towards cancer cells supports the traditional use of *P. glaucus* for the treatment of

cancer. The results of the wound healing activity would indicate that *P. glaucus* would have a mild effect in the treatment of wounds due to its ability to promote cell proliferation and migration, however, its anti-inflammatory activity and mild antibacterial activity may be beneficial in treating wounds. It did possess antivenom activity, which supports its use for the treatment of envenomation by snakes, although the fruit extracts had stronger antivenom activity compared to the root, which is the plant part traditionally used. The mild antibacterial activity of *P. glaucus* may partly contribute to its medicinal effects, however, this study reported stronger evidence of anti-inflammatory, cytotoxic and antivenom activity which may be the properties that practitioners of traditional medicine have found valuable when selecting this plant for treatment. The pharmacological activities reported support a number of the traditional uses for the plant, *P. glaucus*, suggesting that the traditional use may have a scientific ground and could be investigated further, possibly leading to the discovery or development of new anti-inflammatory, chemotherapeutic or antivenom drugs.

Publications

1. Shipton, F., Khoo, T.-J., Wiart, C (2014). The antibacterial activity of *Pericampylus glaucus* (Lam.) Merr. Extended abstract presented at LSBE, Sapporo, Japan (pp. 67-70)

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List of Abbreviations

A

$AlCl_3$	Aluminium chloride

C

°C	Degrees celcius
ca.	Circa (approximately)
CFU	Colony forming units
CO_2	Carbon dioxide

D

dbh.	Diameter at breast height
DMSO	Dimethylsulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl

F

FBS	Foetal bovine serum
FRAP	Ferric Reducing Antioxidant Power
FRIM	Forest Research Institute Malaysia
$FeCl_3$	Ferric chloride

G

g	Gram

H

h	Hours
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HRBC	Human Red Blood Corpuscle

I

IC_{50}	Half maximal inhibitory concentration

L

LCMS	Liquid Chromatography Mass Spetrometry

M

M	Molar
MBC	Minimum bactericidal concentration
mg	Milligram
µg	Microgram
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
µL	Microlitre
mm	Millimetre
mM	Millimolar

µM	Micromolar
MRSA	Methicillin Resistant Staphylococcus Aureus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NA	Not available
NaCl	Sodium chloride
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NSAID	Non-Steroidal Anti-Inflammatory Drug

O

OD	Optical Density

P

pH	Potentiometric hydrogen ion concentration
ppm	Parts per million

R

RPMI 1640	Roswell Park Memorial Institute 1640

T

TLC	Thin Layer Chromatography
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
TSA	Tryptic soy agar
TSB	Tryptic soy broth

U

UNMC	University of Nottingham Malaysia Campus
UV/Vis	Ultraviolet/Visible

V

v/v	Volume per volume

W

w/v	Weight per volume

Z

ZOI	Zone of inhibition

Chapter 1: Introduction

1.1: A brief history

Medicine has been an important part of human culture for as far back as can be recorded, with the earliest known use of medicine being in 3300 BC, by a stone age man, who carried a preparation of *Piptoporus betulinus*, possibly to treat a parasitic worm infection (Spindler 2001).

Other examples of medicine that have early records are Ayurvedic, Mesopotamian, Greek, Chinese and ancient Egyptian medicine, which used herbalism and other forms of medicine. As humanity progressed toward the modern day, greater understanding of the nature of diseases and human physiology was gained and further medical techniques were developed, such as surgery and aseptic technique.

The discovery of penicillin was one of the most notable developments in modern medicine, which led on to the development of further antibiotics. Naturally derived medicines were the only type of medicine available, up until the mid-1800's. Chloral hydrate was the first drug discovered that did not come from nature, it was the first synthetic drug. Salicylic acid, a natural compound from the bark of *Salix alba* or the white willow tree, is an example of one of the first natural compounds to be modified for the purposes of improvement, to create acetylsalicylic acid, the first semi-synthetic drug.

With greater control of the development of drugs, many advances have been made in medicine since the 19th century, however, in many parts of the world, traditional remedies still dominate in the practice of medicine.

1.2: Traditional medicine

Traditional medicine is the knowledge of medicine that has been passed down through generations and the types of remedies used by people who rely on this type of medicine will vary between countries, counties and villages. In Asia and Africa, traditional medicine is used by up to 80% of some populations to treat a wide variety of ailments.

The knowledge that is passed down is usually done so by people who have no formal training in medicine, and the reliability and safety of traditional medicine is often questioned. Due to the questionable nature of these traditional medicines, some researchers have conducted scientific studies on the efficacy and safety of herbal remedies described by traditional healers, either proving or disproving their place in medicine.

In many cases, these traditional remedies are found to exert beneficial effects in the treatment of diseases and are sometimes found to exercise a more potent or less toxic effect in the treatment of a disease compared to modern medicine.

Hence, despite the advances made in medicine in the past few centuries and the distance that has been made between modern drugs and herbal medicine, herbal medicines are still valuable in the treatment of diseases, even in the present day.

1.3: Modern medicine

Many of the drugs that are provided to people are chemicals that are based on naturally derived compounds. Modern drugs that are used to treat diseases are typically synthetic or semi-synthetic, or are structurally identical or similar to their natural counterpart, but produced on a large scale by synthesis as opposed to extraction and isolation, which can be a more lengthy and costly process.

Research into new medicines can be guided by current knowledge, using pre-clinical tests, including structure-activity relationships, molecular docking studies or computational methods to predict activity, or isolating and identifying compounds in plant extracts that are used to treat a certain ailment in order to try and determine the compounds that are exerting the desired activity so that they can be developed further. New drugs can also be developed by high throughput screening or modifying existing compounds to alter or enhance their activity.

New drugs are continuously being developed. This process involves the discovery of a potentially useful drug and the development of this drug by further investigation. Preclinical

research is required, before clinical research can be carried out. The drug is then reviewed and, if approved, marketed (FDA 2015).

These processes have led to the development of many valuable drugs which we heavily rely on. However, a great number of diseases are still without treatment and there are many treatments which could be improved on, as such, drug research is ongoing and new drugs are being developed to cater for new and old diseases.

1.4: Motives for drug discovery

Pathogenic bacteria are one of the motivators for new drug development. Recently, the need for new, marketable antibiotics has been recognised due to the increasing incidence of antibiotic resistance in bacteria (O'Neill 2016). The ability of bacteria to adapt and tolerate antibiotic treatment has led to the need for more effective antibiotics.

Chemotherapy is another area where investigation into new drugs is quite active. Due to the large number of different cancers and the varying susceptibility of these different cancers to different drugs (Roden & George 2002; Yang et al. 2010), the discovery and development of new cytotoxic drugs for use as chemotherapeutic agents is an area of great interest to many research groups.

Due to the particularly unpleasant side effects of chemotherapy and its low success rate when used without any other form of cancer treatment (Morgan et al. 2004), research into other treatments for cancer is also very vigorous.

There are a great number of diseases for which we have no cure, such as Creutzfeldt-Jacob disease and other prion diseases, HIV, herpes virus, lupus erythematosus and asthma. Those who suffer from incurable diseases can only take drugs that can help them to manage their symptoms. With the need for improved and effective drugs, research into new drugs and methods to treat diseases has only increased.

1.5: Plants as sources of medicine

There has been some attempt to tackle the obstacles faced by researchers due to the shortcoming of modern drugs by studying plant extracts or compounds derived from plants.

The increasing antibiotic resistance and failure of modern antibiotics has raised great concern and has made research into new antibiotics a popular topic. A number of studies have found that a significant number of plants are able to kill these antibiotic resistant strains of bacteria (Revati et al. 2015; G. Li et al. 2015; Nascimento et al. 2000; Hannan et al. 2011; Nagappan et al. 2012), and that using combinations of antibiotic and plant extracts can produce a synergistic effect (Olajuyigbe & Afolayan 2012; Adwan et al. 2010), efficiently eliminating bacterial infection. New synthetic and semi-synthetic drugs are also being continually developed to keep up with antibiotic resistant strains.

Cancer treatment by chemotherapy is known to produce various negative side-effects. A number of traditional remedies and alternative medicines are used for the treatment of cancer (Cassileth 2004) but are generally shunned as "quackery" by medical professionals and media, sometimes with good reason (Fishbein 1965; Bonakdar 2002; Jarvis 1983), although some alternative medicines, when tested for their anticancer activity in the laboratory or in clinical trials, have shown promise (Sharma et al. 2001; Tröger et al. 2013; Rose et al. 2015). Some alternative medicines have become more accepted and better understood in recent years and have been proven to enhance the quality of life of patients suffering from certain illnesses (Cassileth & Chapman 1996).

Some traditional remedies claim to treat a wide variety of diseases, some of these claims can be scientifically backed (Sahin et al. 2016; Wankhede et al. 2015), while others have been disproven or lack research (Grollman & Marcus 2016). The misuse and variability in herbal medicines, as well as misidentification of herbs, can lead to herbal remedies being ineffective or harmful (Fraser & Wen 1998) and this has had a damaging effect on the reputation of the efficacy and safety of herbal medicines. Since some herbal remedies are effective in managing certain diseases (Sano et al. 1997; Baum 2008; Perry et al. 1999), the question of why these plants or their active constituents are not commonly used or prescribed begs to be answered.

1.6: Businesses in medicine

The development of medicine has been moulded not only by chemistry and nature, but by corporations and regulations.

The public depends on pharmaceutical companies to develop and produce conventional drugs, as such, the health of the public is very much dependant on the success of these companies in their endeavours. Companies can spend hundreds of millions of US dollars on a single drug, from the development stage to the approval and marketing stage, making these drugs valuable assets to the company (DiMasi et al. 2014). Naturally, businesses with similar types of products and customer base are competitive, as such, when investing a large sum of money into a product, companies want to protect their intellectual property from their competitors so that they receive an adequate return on their investment. Protection of intellectual property comes in the form of patents.

This becomes an obstacle for the success of traditional medicines on the market, because traditional knowledge and a naturally occurring substance cannot be patented according to the memorandum "2014 Procedure For Subject Matter Eligibility Analysis Of Claims Reciting Or Involving Laws Of Nature/Natural Principles, Natural Phenomena, And/Or Natural Products". This leaves the intellectual property involving natural products vulnerable to competitors, which would reduce potential profit, should the product ever be approved and marketed. Research into naturally derived compounds is also a risk for companies, as their investment may not necessarily yield a product.

Despite the current state of drug development, research into possible new drugs from plants for the treatment of diseases is ongoing and has shown the potential to provide humans with effective, new drugs. Even if naturally occurring substances are not favourable drug candidates for companies, they can provide researchers with templates to produce similar compounds which can then be developed into marketable drugs.

1.7: Objectives

Plants remain a valuable and vast resource for the discovery of new drugs and can provide researchers with novel structures and useful activities. There have been a limited number of studies published on *Pericampylus glaucus* (Lam.) Merr., as such, the present study aims to fulfill the following objectives regarding this plant:

- Identify the yields of different solvent extracts and phytochemical constituents of various parts of *P. glaucus*.

- To determine the *in vitro* pharmacological activities of the extracts, including antioxidant, antibacterial, anticancer, cytotoxic, wound healing, anti-inflammatory and antivenom activity.
- To determine the *in vitro* pharmacological activity of a number of pure compounds found in *P. glaucus*.
- To determine whether or not the traditional uses of *P. glaucus* can be correlated with the *in vitro* results and available literature.

Chapter 2: Literature review of *Pericampylus glaucus* (Lam.) Merr.

2.1: Botanical description

Pericampylus glaucus (Lam.) Merr is the accepted name of the plant which is the focus of the present study. It has the following synonyms, Menispermum glaucum Lam., *Pericampylus formosanus* Diels., *Pericampylus omeiensis* W.Y. Lien., *Pericampylus trinervatus* Yamam., *Cocculus incanus* Colebr., and *Coscinium colaniae* Gagnep. It is found in Burma, China, India, Indonesia, Laos, Malaysia, Philippines, Taiwan, Thailand and Vietnam (ThePlantList.org 2013; Tropicos.org 2016). Its common names include broad-leaved moonseed in English, areuy geureung, celuru and akar gamat in Indonesia, gasing gasing, kelempenang and taworuk in Malaysia, silong pugo, botang botang and pamago in the Philippines, salit hom kha and yan tap tao in Thailand and ch[aa]u d[ar]o and d[aa]y l[ox]i ti[eef]n in Vietnam (Wiart 2006; Ong 2001).

Pericampylus glaucus is a perennial, slender, woody climber that can grow across distances greater than 10m (eFloras.org 2016). *P. glaucus* has what resembles a tap root system on older, well established plants and a sparse, shallow root system at nodes along prostrate stems.

Young stems are herbaceous and pubescent, becoming woody and glabrous with age. Stems can be creeping or climbing, they are round or cylindrical, newer or ascending stems are green while older stems, especially older prostrate, creeping stems, are brown to dark brown. Phyllotaxy of leaves is alternate. The leaves are simple, chordate with a size up to 10 cm x 10 cm. The apex can range from being broadly rounded to obtuse and sometimes retuse or cleft. The leaf margin is entire or undulate with palmate venation. The leaves are pubescent on both sides, becoming glabrous with age. The leaf stalk ranges from 3 cm to 7 cm and is pubescent. The base of the leaves are exstipulate.

Inflorescences are determinate and arranged in cymose panicles. The inflorescence stalk is pubescent and can be 2 cm to 6 cm in length. Flowers are yellow to pale green in colour and fragrant. There are 6 wedge shaped petals of actinomorphic symmetry, 2 mm long. The male flowers have 6 stamens, 0.8 mm in length. Female flowers have 6 abortive stamens with anthers and 3 carpels, the stigma curves backward.

The endocarp is rotund around the edges and is lined with rows of spines and tubercles. The fruit is hairless and reverse egg shaped. It is a drupe and can range in colour from red to dark purple and measures approximately 6 mm x 6 mm. The seed is horseshoe shaped.

2.2: Traditional uses

The traditional uses of *P. glaucus* are summarised in **Table 2.1** below:

Table 2.1: The traditional uses of *P. glaucus*

Traditional use	**Plant part used**
Cure a swollen spleen and associated fever	Leaves, pounded and taken orally (Wiart 2006; Ong 2001).
Cure high fever	Leaves, infused and taken orally (Wiart 2006; Ong 2001).
Treat cough, laryngitis, pulmonary disease and asthma	Leaves, infused and taken orally (Wiart 2006; Ong 2001). Roots (Yan et al. 2008).
Soothe headache	Leaves, pounded and applied topically (Wiart 2006; Ong 2001).
Remedy for hair loss	Leaves, pounded and soaked before mucilage applied to the scalp (Wiart 2006; Ong 2001).
Treatment for conjunctivitis	Stem, juiced (Wiart 2006; Ong 2001).
Treatment for snakebites	Roots, fluid extract is injected at the site of the snakebite. Leaves and stems (Wiart 2006; Sam et al. 2008; Sam 2010; Ong 2001).
Narcotic properties	Root (Wiart 2006; Ong 2001).
Induce Sedation	Leaves (Yan et al. 2008).
Treat boar bites	Leaves (Yan et al. 2008).
Treat fractures	Leaves (Yan et al. 2008).
Treat boils	Roots (Yan et al. 2008).
Treatment of tumours	Stem and leaves (Zhao 2008).
Treatment of tetanus	Leaves (Yan et al. 2008; Zhao 2008).
Treat rheumatic pains	Leaves (Yan et al. 2008).
Treating itches	Leaves (Zhao 2008).

Treat eclampsia	Leaves (Zhao 2008).
Tying and basketry	Stems (Ong 2001).

When a traditional remedy is given to treat a condition and there are no scientific studies that have determined the active components of the remedy or their action, it is not easy to establish whether the remedy being given is actually treating the condition or just easing the symptoms, or both. It is also possible that the condition has not been properly diagnosed (O'Brien & Birch 2009), the treatment is ineffective and relies on the placebo effect or is harmful (Efferth & Kaina 2011). The table below summarises the causes and symptoms associated with the conditions *P. glaucus* is used to treat and the possible effect the plant could be having based on the causes and symptoms of the condition. The possible effects *P. glaucus* could be having to treat these conditions is based on the mode of action of current medicines that are used to treat those conditions and the method by which it is traditionally administered.

2.3: Possible pharmacological action of *P. glaucus*

Some of the causes mentioned may lead to the development of other symptoms which are also mentioned.

Table 2.2: Possible pharmacological activities of *P. glaucus* based on traditional use

Condition of traditional use	**Common causes/symptoms of condition***	**Current treatment* and possible effects of *P. glaucus***
Swollen spleen (splenomegaly)	**Causes:** Viral, bacterial or parasitic infections, leukemia, lymphoma, inflammatory diseases (e.g. rheumatoid arthritis), diseases affecting the liver (e.g. cirrhosis), metabolic disorders (e.g. Gaucher's disease), pressure on the veins in the liver or spleen or a clot. **Symptoms:** Pain, anaemia, fatigue,	**Current treatments:** Treatment of underlying causes or Splenectomy (surgical removal of the spleen) **Possible effects of *P. glaucus*:** Antimicrobial, antiparasitic, antiviral, anti-inflammatory, cytotoxic, analgesic, stimulant and antipyretic activity. Since the traditional treatment mentions an

	easy bleeding, fever.	associated fever, there is a greater chance that the splenomegaly *P. glaucus* is used to treat is induced by infection.
High fever	**Causes:** A variety of infections or illnesses can cause a high fever. Infection by bacteria and viruses are some of the more common causes of high fever, but causes can also include blood clots, autoimmune diseases, and diseases that cause inflammation. **Symptoms:** High fever and a number of other accompanying symptoms depending on the cause of the fever.	**Current treatments:** Antibiotics (if induced by infection) and NSAIDs. **Possible effects of *P. glaucus*:** Antimicrobial, antiviral and anti-inflammatory activity.
Cough, laryngitis, pulmonary disease	**Causes:** Infection of the respiratory tract by bacteria or viruses, hayfever, nasal drip, smoking, asthma, lung cancer, gastroesophegal reflux disease (GERD) or chronic obstructive pulmonary disease (COPD). **Symptoms:** A cough can be the symptom of a number of conditions, including laryngitis and pulmonary disease, but other symptoms that tend to accompany a cough include nasal congestion and nasal discharge, sore throat, shortness of breath and heartburn.	**Current treatments:** Expectorants, such as guaifenesin and suppressants, such as dextromethorphan and antibiotics. **Possible effects of *P. glaucus*:** Expectorant, suppressant, antihistaminic, antiviral and antibiotic activity.
Asthma	**Causes:** Exposure to airborne	**Current treatments:** Short-acting

	allergens, pollutants and irritants, respiratory infections, physical activity, exposure to cold air, certain medications and preservatives, stress and GERD. **Symptoms:** Shortness of breath, pain or tightness in the chest, coughing and wheezing.	$beta_2$-adrenoceptor agonists (SABA), anticholinergics, adrenergic agonists, corticosteroids, long-acting beta-adrenoceptor agonists (LABA), leukotriene receptor agonists and mast cell stabilisers. **Possible effects of *P. glaucus*:** *P. glaucus* may have compounds with similar activity to xanthines such as theophylline and may inhibit TGF-beta-mediated conversion of pulmonary fibroblasts into myofibroblasts or activate HDAC2. It may also have some anti-inflammatory and anti-histaminic activity.
Headache	**Causes:** Causes may include inflammation of the periosteum, meninges or associated arteries, veins and nerves, infection of the teeth or sinuses, bleeding in the brain, infection, brain cancer, trauma to the brain, inflammation and spasm of muscles covering the skull, dehydration and blood clots. **Symptoms:** Varying degrees of pain in the head and face.	**Current treatments:** NSAIDs, triptans (serotonin receptor agonist, vasoconstrictor, inhibition of pro-inflammatory neuropeptide release) and ergotamine (serotonin, dopamine, epinephrine receptor agonist, vasoconstrictor). **Possible effects of *P. glaucus*:** Anti-inflammatory or analgesic activity, agonist of serotonin/dopamine receptors, vasoconstrictor.

Hair loss	**Causes:** Heredity, hormonal changes, certain medications such as chemotherapy drugs, alopecia areata or other autoimmune disorders, infections of the scalp, trichotillomania, exposure of the scalp to radiation or harmful chemicals. **Symptoms:** Thinning of the hair, bald patches, complete loss of hair, swelling of the scalp, scaling of the scalp and broken hair.	**Current treatments:** Corticosteroids, 5α reductase inhibition (finasteride), possibly opening potassium channels and causing hyperpolarisation of cell membranes and thus increasing blood flow to follicles (minoxidil), immunotherapy, dithranol (anti-inflammatory and anti-proliferative effects). **Possible effects of *P. glaucus*:** May inhibit dihydrotestosterone production, increase blood flow, cause an immune response or sensitise skin to UV damage.
Conjunctivitis	**Causes:** infection by bacteria, viruses or parasites, chemical irritants and allergens. **Symptoms:** Ocular discharge, itching or burning of the eyes, blurred vision, increased sensitivity to light, inflammation of the sclera or inner eyelid.	**Current treatments:** Antibiotics, antihistamines, vasoconstrictors, steroids. **Possible effects of *P. glaucus*:** Antibiotic activity, anti-inflamatory activity, antihistamine activity, vasoconstriction activity.
Snakebites	**Causes:** Exposure to a snake and receiving a bite which may or may not result in envenomation and/or infection. **Symptoms:** Inflammation and pain at the site of the bite, convulsions, nausea, paralysis, difficulty	**Current treatments:** IgG antivenom, surgery, antibiotics, analgesics. **Possible effects of *P. glaucus*:** Antibiotic activity, enzyme inhibition, analgesic activity.

	breathing, blurred vision, sweating, salivating, numbness, haemorrhaging, muscle weakness and low blood pressure.	It has been noted that when the fluid extract of the roots is injected into the site of the bite, it is said to neutralise the poison by precipitating it and converting it to an inert substance.
Boar bites	**Causes:** Being bitten by a boar **Symptoms:** Pain, infection, inflammation, bleeding.	**Current treatments:** Proper wound treatment, antibiotics, antiseptics, analgesics and anti-inflammatory drugs. **Possible effects of *P. glaucus*:** Antibiotic activity, analgesic activity, anti-inflammatory activity, promotion of wound healing.
Fractures	**Causes:** Bones are fractured due to traumatic incidents but can be more likely in individuals who suffer from osteoporosis. **Symptoms:** Swelling, bruising, deformity, pain, protruding bone from skin.	**Current treatments:** Antibiotics, splinting, analgesics, anti-inflammatory drugs. **Possible effects of *P. glaucus*:** Antibiotic activity, analgesic activity, anti-inflammatory activity, promotion of wound healing.
Boils	**Causes:** *Staphylococcus* infection of the skin. **Symptoms:** Pain, swelling, inflammation, fever.	**Current treatments:** Antibiotics, draining of the boil. **Possible effects of *P. glaucus*:** Antibiotic activity, anti-inflammatory, analgesic activity.
Tumours	**Causes:** The causes of benign tumours is often unknown, however the causes of malignant tumours are vast, as outlined in **Chapter 7**.	**Current treatments:** Surgery, radiotherapy, chemotherapy, targeted therapy.

	Symptoms: Depending on where the growth is, the symptoms can vary widely, but they generally involve the growth of a mass of cells that form a lump, except in the cases of cancers such as leukaemia.	**Possible effects of *P. glaucus*:** Cytotoxic activity, interaction with cell signalling molecules.
Tetanus	**Causes:** infection of a wound by *Clostridium tetani* and subsequent release of the toxic peptides tetanospasmin (which permanently binds to neurons) and tetanoysin (which destroys tissues). **Symptoms:** Painful muscle spasms, stiffness of jaw, neck and abdominal muscles, difficulty swallowing, fever, sweating, elevated blood pressure and increased heart rate.	**Current treatments:** Antibiotics, tetanus immune globulin or antitoxin, vaccination, sedatives, beta-blockers. **Possible effects of *P. glaucus*:** Antibiotic, sedative, anticonvulsant activity.
Rheumatic pains	**Causes:** Autoimmune disorder. **Symptoms:** Pain, swelling, stiffness, loss of energy and appetite, fever.	**Current treatments:** Analgesics, antimetabolite drugs and anti-inflammatory drugs including corticosteroids. **Possible effects of *P. glaucus*:** Anti-inflammatory and analgesic activity.
Itches	**Causes:** Some of the most common causes of itching may include skin conditions such as eczema or psoriasis, allergies, parasites or insects, infections by various microbes and viruses and hormonal	**Current treatments:** Steroids, antimicrobials, calcineurin inhibitors, antihistamines, antidepressants and phototherapy. **Possible effects of *P. glaucus*:**

	changes. **Symptoms:** The symptoms will vary depending on the cause, but generally, itching can be experienced over the area of the whole body or in a localised area and may or may not include a rash or spot.	Anti-inflammatory, antimicrobial, antihistaminic, antiparasitic, anti-anxiety activity.
Eclampsia	**Causes:** Unknown, but it occurs during pregnancy the condition arises due to the placenta functioning incorrectly. **Symptoms:** Symptoms of pre-eclampsia include high blood pressure, proteinuria, swelling of face and hands, headaches, weight gain, nausea, vomiting, vision problems, problems urinating. Symptoms of eclampsia are seizures, loss of consciousness, headaches and muscle pain.	**Current treatments:** Delivery of the baby. **Possible effects of *P. glaucus*:** Hyperstimulation of the uterus, anti-convulsant activity, diuretic, analgesic activity.

* Information regarding the symptoms, causes and treatment of diseases were derived from Papadakis & McPhee 2017.

From this table, many of the conditions listed are treated with antibiotics, anti-inflammatory drugs, steroids and enzyme inhibitors. It is possible, since a single plant part can contain a very large number of compounds with varying potency, that *P. glaucus* exerts a number of activities to treat these conditions. However, traditional medicines may not always be effective in treating any ailment and their use for the treatment of those conditions is purely based on knowledge passed down through generations, by people who have limited knowledge of medicine.

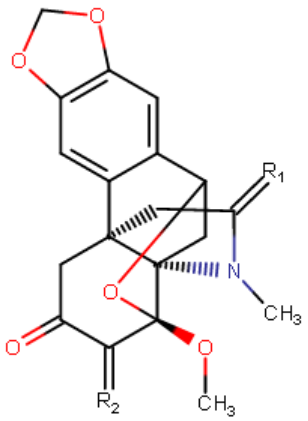
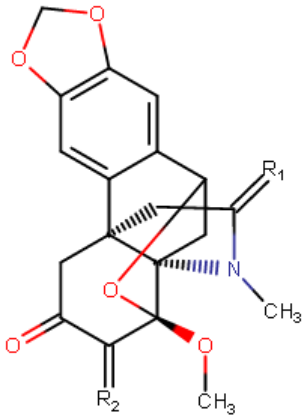
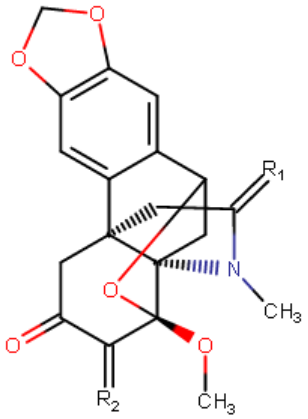
2.4: Literature on *P. glaucus*

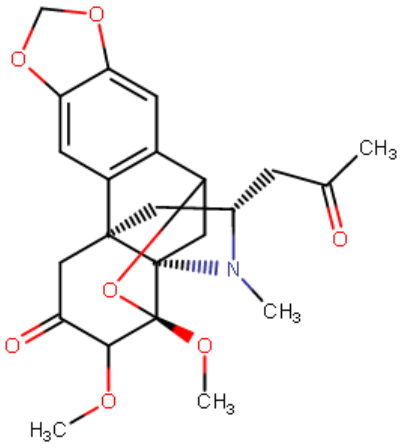
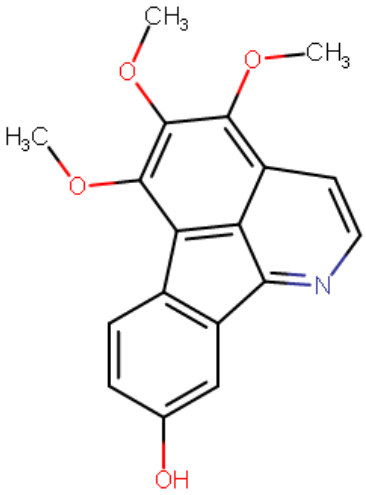
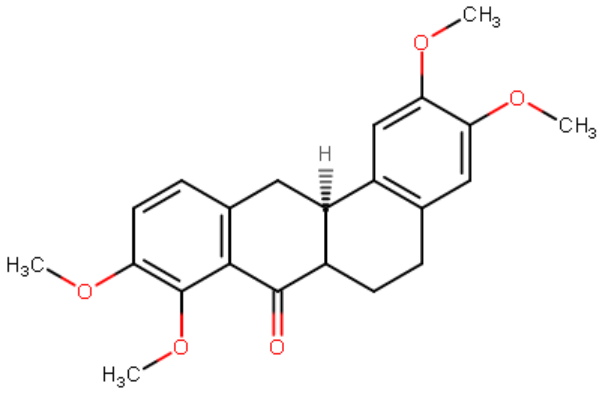
P. glaucus has been studied to some extent and has been found to possess a number of different activities. Some of the alkaloids possess anti-HIV-1 and anti-HBV activity (Yan et al. 2008). The crude extract exhibited no significant toxic effects on mice when administered orally (up to 4000 mg/kg), although some sedative effect was observed in the mice fed higher doses and aspartate transaminase and alanine transaminase levels were significantly increased, suggesting that the extract may be affecting liver function (Kifayatullah, Mustafa, et al. 2015; Giboney 2005). The ethanolic extract was found to have anti-diabetic and anti hyperlipidaemic activity by reducing fasting blood sugar, cholesterol, triglyceride, urea, LDL and creatinine levels (Kifayatullah & Sengupta 2016; Feingold et al. 1992; Sacks et al. 2014; Mitch & Walser 1986) and contained antioxidant compounds (Kifayatullah, Senguptha, et al. 2015). Triterpenes isolated from *P. glaucus* were found to have an inhibitory effect on the growth of K562 (human myelogenous leukaemia) cells (Zhao & Cui 2009) and the ethanolic extract of the branch inhibited the cell cycle of tsFT210 (mouse CDC2 mutant) cells at the G2/M phase by 25-50% at a concentration of 100 µg/mL (Zhao et al. 2005). The leaf chloroform extract strongly inhibited the growth of the parasite, *Plasmodium falciparum*, the hexane, chloroform and ethanol extracts of the stems also showed some activity against the parasite, although only the hexane extract of the stems (IC_{50} = 39.75 µg/mL) did not lyse red blood cells (Khaw, Leerach, Yap, Jaturas, Mahboob, Tan, Dungca, LosBaños, et al. 2015). It was also found to have activity against some strains of antibiotic resistant bacteria (Zhao 2008).

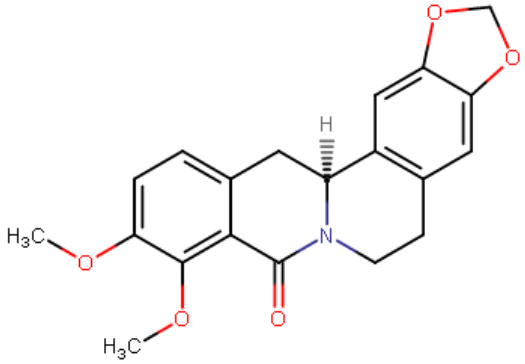
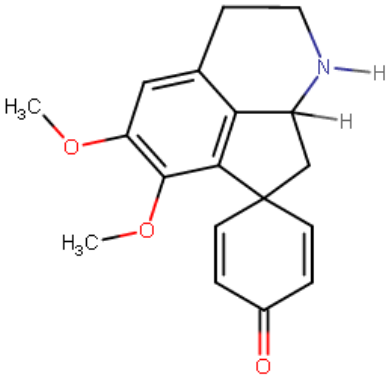
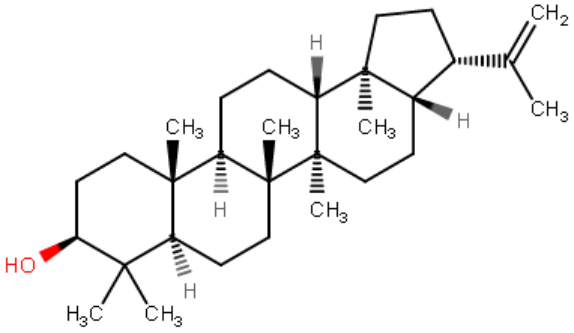
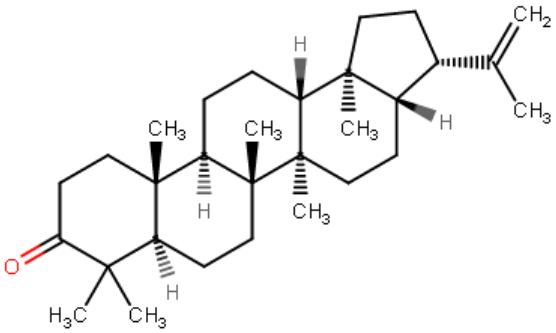
2.4.1: Known compounds and their activity

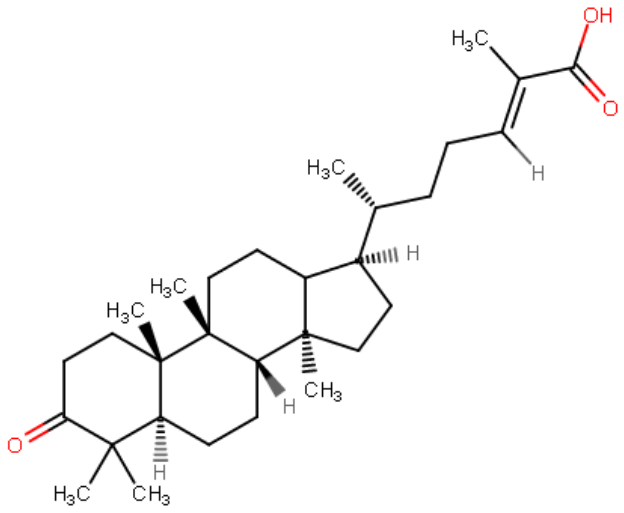
A number of studies have isolated and identified chemicals from *P. glaucus*, as summarised in **Table 2.3** below.

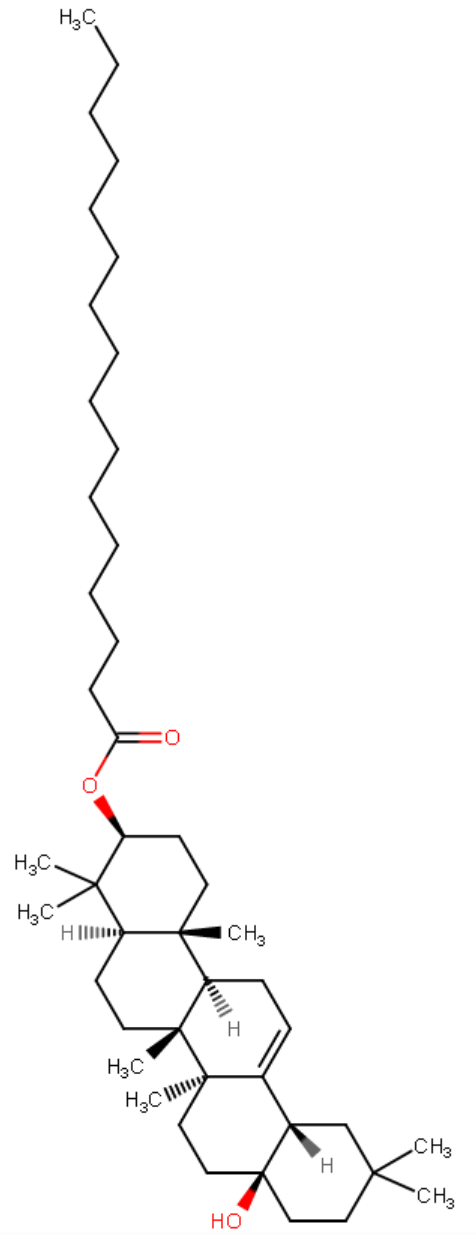
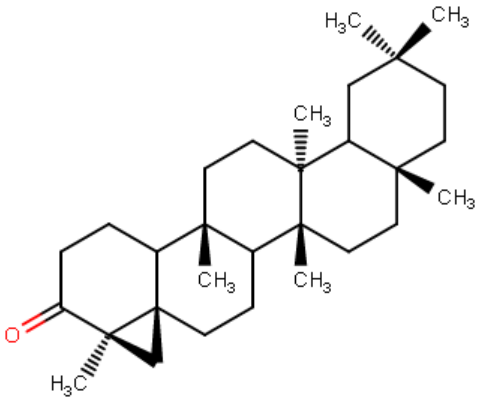
Table 2.3: Compounds isolated and identified from *P. glaucus*

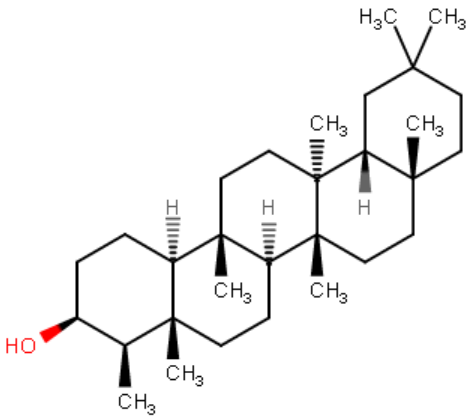
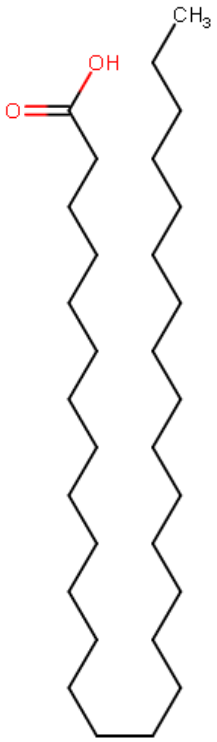
Compound structure		Compound details
	$R^1 = H_2$ $R^2 = \alpha\text{-}OCH_3$ β-H	**Name:** Periglaucine A **Class:** Alkaloid **Known activity:** Weak activity against HIV and weak to moderate activity against HBV (Yan et al. 2008).
	$R^1 = H_2$ $R^2 = \beta\text{ -}OCH_3$ α -H	**Name:** Periglaucine B **Class:** Alkaloid **Known activity:** Weak activity against HIV and weak to moderate activity against HBV (Yan et al. 2008).
	$R^1 = O$ $R^2 = \alpha\text{-}OCH_3$ β-H	**Name:** Periglaucine C **Class:** Alkaloid **Known activity:** Weak activity against HIV and weak to moderate activity against HBV (Yan et al. 2008).

	**Name:** Periglaucine D **Class:** Alkaloid **Known activity:** Weak activity against HIV and weak to moderate activity against HBV (Yan et al. 2008).
	**Name:** Norrufescine **Class:** Alkaloid **Known activity:** Good activity against HIV and weak activity against HBV (Yan et al. 2008).
	**Name:** (-)-8-Oxotetrahydropalmatine **Class:** Alkaloid **Known activity:** Good activity against HIV and HBV (Yan et al. 2008).

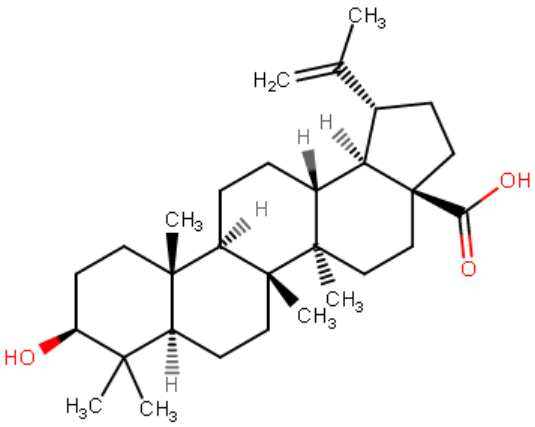
	**Name:** (-)-8-Oxocanadine / Gusanlung B **Class:** Alkaloid **Known activity:** Weak activity against HBV (Yan et al. 2008).
	**Name:** Stepharine **Class:** Alkaloid **Known activity:** Promotes neogenesis, regeneration and electrophysiological function of nerves after injury (Tomita et al. 1967; Kopylov et al. 2014)
	**Name:** Hopenol B **Class:** Triterpenoid **Known activity:** None (Zhao & Cui 2009).
	**Name:** Hopenone B / Mortenone **Class:** Triterpenoid **Known activity:** None (Zhao & Cui 2009).

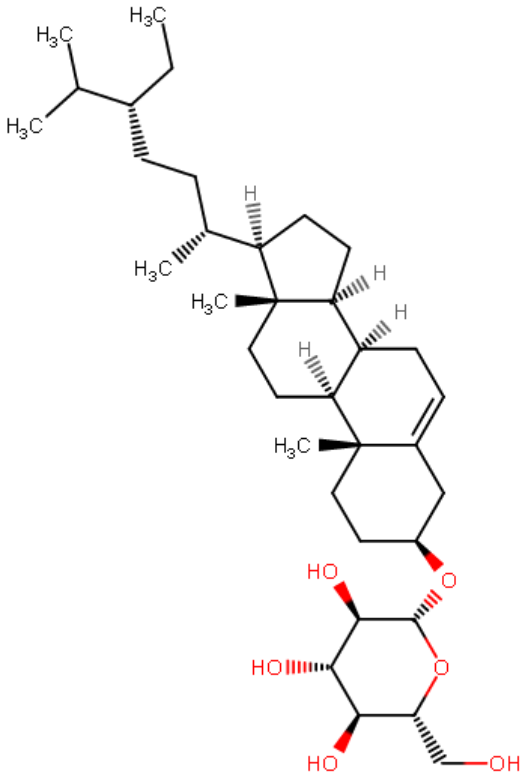
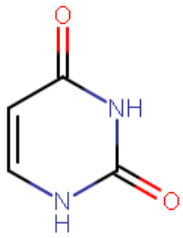
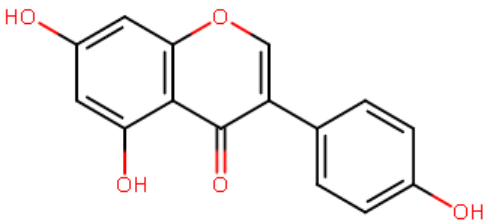
	**Name:** Mangiferonic acid / 22-hydroxyhopan-3-one **Class:** Terpene **Known activity:** Mild cytotoxicity towards B16-BL6, strong cytotoxicity towards HeLa cells, moderate cytotoxicity towards HT-29 (Zhao & Cui 2009; Ahmad et al. 2015; Li et al. 2009).

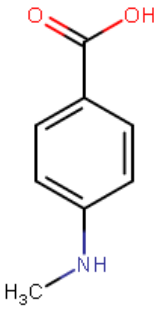
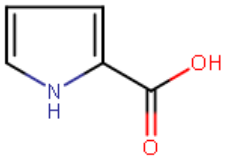
	**Name:** Erythrodiol 3-palmitate **Class:** Triterpenoid **Known activity:** None (Zhao & Cui 2009).
	**Name:** 5β,24-cyclofriedelan-3-one **Class:** Triterpenoid **Known activity:** Moderate antiplasmodial activity (Zhao & Cui 2009; Ng′ang′a et al. 2012).

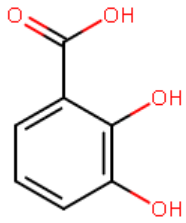
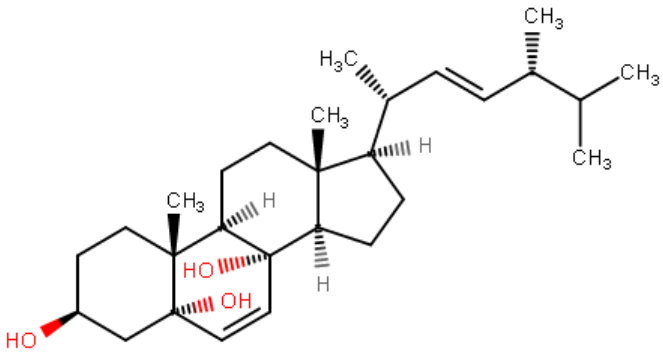
	**Name:** Epifriedelinol **Class:** Triterpene **Known activity:** Allelopathic, herbicidal (Torres-Romero et al. 2010; Trevisan et al. 2012; Liang et al. 1998).
	**Name:** Melissic acid **Class:** Fatty acid **Known activity:** None (Liang et al. 1998).

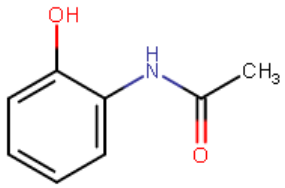
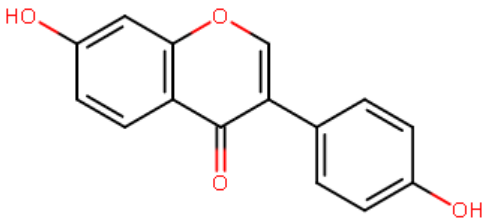
	**Name:** Palmitic acid **Class:** Fatty acid **Known activity:** Antitumour activity (Harada et al. 2002), stimulation of glucose uptake by adipocytes, promotion of tumour growth, promotion of inflammation, possible link to increased risk of cardiovascular disease (Mancini et al. 2015).
	**Name:** Stearic acid **Class:** Fatty acid **Known activity:** Cardiotoxic, cytotoxic, induces intracellular lipid accumulation, pro-inflammatory, mild antimicrobial activity (J. Choi et al. 2013; Rabkin & Lodhia 2009; Lima et al. 2002; Gupta et al. 2012; Liang et al. 1998).

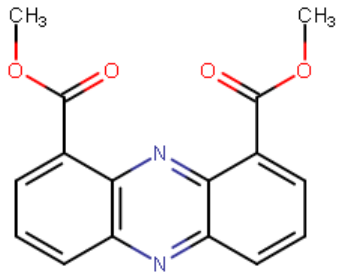
	**Name:** Betulinic acid **Class:** Triterpenoid **Known activity:** Antiviral, antplasmodial, mild anti-bacterial, anti-inflammatory, immunomodulatory and anticancer and antiangiogenic activity (Schettino et al. 2013; Xu et al. 2014; Ciurlea et al. 2010; Yi et al. 2010; Periasamy et al. 2014; Tsai et al. 2011; Yun et al. 2003; Fontanay et al. 2008; Ghaffari Moghaddam et al. 2012; Yun et al. n.d.; Surowiak et al. 2009; Zuco et al. 2002; Harris & Pierpoint 2012; Fulda 2008; Mukherjee et al. 2004; Lin et al. 2015; Hong et al. 2015; Oliveira Costa et al. 2014; De Sá et al. 2009; S. Wang et al. 2016; Liang et al. 1998).

	**Name:** Daucosterol **Class:** Sterol **Known activity:** Anticancer, immunoregulatory, antidiabetic, neuroprotective and insecticidal activity (Murillo et al. 2014; G.-Q. Wang et al. 2016; Lee et al. 2007; Asghari et al. 2015; Jiang et al. 2015; Liang et al. 1998).
	**Name:** Uracil **Class:** Pyrimidine derivative, nucleobase **Known activity:** Tumour promoting activity (Blount et al. 1997; Zhao 2008).
	**Name:** Genistein **Class:** Isoflavone **Known activity:** Interaction with various enzymes, stimulation of autophagy, stimulation of smooth muscle contraction, activation of various receptors, antioxidant activity, anti-parasitic, inflammation

	modulation, promotion of tumour growth (Choi & Kim 2013; Russo et al. 2016; Rahman Mazumder & Hongsprabhas 2016; Cho et al. 2011; Bevilacqua et al. 2011; Lepri et al. 2013; Lepri et al. 2014; Kaminska et al. 2013; Yanagisawa et al. 2012; Sakamoto et al. 2014; Kim et al. 2011; Nakamura et al. 2011; Kar et al. 2004; Luo et al. 2009; Prietsch et al. 2014; Zhao 2008).
	**Name:** 4 -(methylamino)-benzoic acid **Class:** Phenolic acid **Known activity:** None (Zhao 2008).
	**Name:** 1H-pyrrole-2-carboxylic acid / Pyrrole-2-carboxylic acid **Class:** Carboxylic acid **Known activity:** Inhibition of quorum sensing and virulence of bacteria, antifungal activity (Nguyen et al. 2015; Hassan et al. 2016;

	Zhao 2008).
	**Name:** 2,3-dihydroxybenzoic acid **Class:** Phenolic acid **Known activity:** Chelating, antimicrobial activity, cytoprotective activity, antioxidant activity (Sappey et al. 1995; Graziano et al. 1974; Parent et al. 2002; Bellaire et al. 2003; Shibumon, Benny, Sunny & Cinci 2011; Benny et al. 2010; Shibumon, Benny, Sunny & Sarala 2011; Severinsen et al. 2006; Naghibi et al. 2007; Zhao 2008).
	**Name:** Ergosterol peroxide **Class:** Sterol **Known activity:** Increase tolerance of yeast to stress, regulation of streptozotocin induced diabetes, anticancer activity (X. Li et al. 2015; Liu et al. 2013; Ang et al. 2015; Zhao 2008).

	**Name:** N-(2-Hydroxyphenyl)acetamide / 2-Acetamidophenol **Class:** Phenolic **Known activity:** Anticancer, anti-arthritic activity (Perveen et al. 2013; Hanif et al. 2014; Zhao 2008).
	**Name:** Daidzein **Class:** Isoflavone **Known activity:** Anticancer, cancer promoting, reduction of bone loss, anti-inflammatory, antibacterial, neuroprotective (Aras et al. 2015; R. C. Y. Choi et al. 2013; Yang et al. 2012; Kajta et al. 2013; Ullah et al. 2016; Han et al. 2015; Park et al. 2013; Magee et al. 2014; Lepri et al. 2014; Choi & Kim 2013; Yanagisawa et al. 2012; Tang et al. 2013; Koo et al. 2015; Tousen et al. 2011; Bae et al. 2016; Sakamoto et al. 2016; Li et al. 2014; Sakamoto et al. 2014; Zhao 2008).

	**Name:** 1,9-dicarbomethoxy-phenazine **Class:** Pyrazine **Known activity:** None (Zhao 2008).

The alkaloids (-)-8-Oxotetrahydropalmatine and (-)-8-Oxocanadine are quaternary protoberberine alkaloids. Quaternary berberine alkaloids have been found to have significant and varied activities, including antimicrobial, cytotoxic, anti-inflammatory and antimalarial activity (Grycová et al. 2007). It is possible that these two alkaloids may also possess similar such activities.

Some of the compounds identified in *P. glaucus* do not have any known activity, however, some have been well studied and found to have a variety of activities which could contribute to its medicinal properties in traditional medicine. The present study aims to further investigate the properties of the extracts of *P. glaucus* and some of its components to further elucidate the potential medicinal properties of this plant.

Chapter 3: Collection, extraction and phytochemical analysis

3.1: Introduction

The collection, identification, extraction of phytochemicals and the analysis of the resulting extracts are the first steps in conducting a study on any plant. Since there were a variety of methods to choose from which could potentially yield different extracts as well as different types of compounds, a number of these methods, are outlined in this chapter. These differences could include using solvents of different polarity, altering the extraction technique or by extracting chemicals from the plant material by taking advantage of the chemical properties of the desired compounds. The details of these processes and their importance are highlighted in this chapter.

3.1.1: Collection and Identification

The correct identification of a plant that to be collected is vitally important. Plants that look similar can have a significantly different chemical profile. Plants are sometimes misidentified, meaning the medicinal value of the herb that was collected may be different to what is expected. The resulting effect may be detrimental to health or, sometimes, an unexpected benefit (Radulovic et al. 2010; Colombo et al. 2010).

To ensure that the correct plant has been collected, the phenotypes of the plant are examined carefully and compared to a voucher specimen. A dichotomous key can also be used to deduce the plant species. Genetic testing may be carried out, which is considered a reliable method of identification for many species but has its own limitations, such as the reliability and differences between submitted genomic sequences of the same species and genetic variation within the same species (Howard et al. 2012).

3.1.2: Extraction

Before extraction, the plant material is ground into a fine powder which ruptures cells and increases the surface area of the plant material that will be exposed to the solvent and allow chemicals to diffuse out through the plant material more quickly.

The technique used for extraction of chemicals from a plant can make a large difference in the compounds extracted (Anand et al. 2005). Various techniques are adopted for the extraction of phytochemicals and the method used is largely decided based on the type of

compounds that are desired from the plant. Compounds in plants have varying solubility in solvents of different polarity at different temperatures and different pH. These are variables that can be altered to target compounds with certain properties.

3.1.2.1: Distillation and supercritical CO_2 extraction

Essential oils are typically extracted by distillation, where the plant material is boiled with water and the volatile oils are separated from the solution by water vapour and collected in the condensate. Supercritical carbon dioxide (CO_2) extraction uses liquid carbon dioxide as a solvent to extract non polar compounds, such as oils, from plant material and is adopted in the production of oils for food or cosmetics, as the solvent, CO_2, is easily and thoroughly removed from the finished product.

3.1.2.2: Solvent extraction

Solvent extraction can also be used to extract oils along with other non-polar compounds. The extraction of oils involves the maceration of plant material in a non-polar solvent such as petroleum ether or hexane, with or without heating, and subsequent filtration and evaporation of the solvent to leave the pure oil and other non-polar compounds in a non-polar extract. Solvents of increasing polarity can be used in turn to obtain chemically different solvent extracts from plant material, the resulting extracts will therefore contain chemicals that have been extracted according to polarity with the most non-polar compounds in the non-polar solvent extract and the more polar compounds in the more polar solvents used for extraction. Solvent extraction can be carried out by simple maceration, percolation, using Soxhlet apparatus or using pressurised solvent. The time of extraction and temperature applied can vary.

3.1.2.3: Ultrasonic extraction

The extraction of some chemicals can be made more efficient by the use of ultrasound or microwaves. This is an efficient method of extraction when handling small quantities of plant material. However, it has been reported that free radicals can be formed during ultrasound assisted extraction, which may result in changes in some compounds in the extract (Handa et al. 2008; Riesz & Kondo 1992).

3.1.2.4: Acid-base extraction of alkaloids

To selectively extract alkaloids from an extract, acid-base extraction can be used. Alkaloids are converted to their salts when reacted with an acid, and these salts are usually soluble in water as they are ionic. When reacted with an alkali they are converted to their free base form, which is usually insoluble in water but more soluble in some organic solvents. This generalisation of alkaloid solubility can be applied to many alkaloids, however, there are a number of exceptions, for example, atropine sulphate is more soluble in water than atropine, and pilocarpine is insoluble in organic solvents. The structure of the alkaloid will decide how basic the alkaloid is, i.e., slightly acidic, neutral, weakly basic (low pKa), strongly basic (high pKa) or amphoteric (not acidic or basic). More acidic conditions are required to convert a neutral or weakly basic alkaloid into its salt compared to a strongly basic alkaloid, which will convert to its salt in relatively low acidic solutions. Alkaloids normally exist as the salt of an acid when in a plant due to the presence of acids in the plant. The unique chemical properties of alkaloids can be exploited to facilitate their extraction.

The extraction by acid or alkali treatment can begin with either the powdered plant material or an extract of the plant material and involves the defatting and extraction of the alkaloids from the plant material either with a solvent or directly with an acidic solution, which ensures the alkaloids are in their salt form and are more soluble in water than in an organic solvent. The aqueous, acidic solution is then partitioned using a water immiscible organic solvent, such as ethyl acetate, chloroform or dichloromethane, which solubilises many of the unwanted compounds as well as neutral and weakly basic alkaloids, leaving the more strongly basic alkaloids in the acidic, aqueous solution. The aqueous acidic solution is made alkaline with the addition of an alkali, this converts the alkaloid salts into their basic form which is usually less soluble in water, causing them to precipitate.
The theoretical pH at which the base can be expected to precipitate can be calculated using the pHp (pH precipitation) equation (Bouwman-Boer et al. 2009):

Equation 3.1: pHp (pH precipitation) equation

$$pHp = pKa + log\left(\frac{S_0}{S - S_0}\right)$$

where, S_0 is the solubility of the undissociated base (moles/L) and S is the concentration of the base which has been added to the solution (in moles/L).

According to this theory, the base will start to precipitate out of solution at a pH higher than the pHp calculated by the above equation. For weakly acidic alkaloids, a similar formula can be used:

Equation 3.2: pHp equation for weakly acidic alkaloids

$$pHp = pKa + log\left(\frac{S - S_0}{S_0}\right)$$

The conversion of alkaloid salts to their free base form will depend upon the strength (pKa) of the acid used and the pKa of the free base alkaloid. The stronger the acid, the lower the pKa value and the stronger the base, the higher the pKa value. As such, the pKa values of the acidic solution and the alkaloid, along with the reaction of the alkaloid salt with the alkali as the pH is raised, will determine the conversion of the alkaloid salt into its free base form as more of the alkali is added to the acidic solution. When separating a variety of unknown alkaloids of unknown concentration and pKa from a crude extract, the use of the pHp equation may not be practically useful in precipitating an alkaloid by adjusting pH, however the principle upon which it is based is still valuable and can be practically demonstrated by the separation of alkaloids of different pKa by gradually increasing the pH and partitioning the aqueous solution with organic solvent between each raising of the pH. The resulting fraction from the partitioning with the organic solvent is the total alkaloid fraction, containing a crude mixture of most of the alkaloids from the plant material.

3.1.3: Metabolites from plants

A typical plant contains a vast number of chemicals. The compounds that a plant contains will determine its biological activity. Some of the compounds found in plants serve an essential biological function in the plant, these are primary metabolites, while others do not serve a direct role in the functioning of the plant and are called secondary metabolites.

Primary metabolites are compounds such as carbohydrates, lipids, amino acids, peptides, proteins and enzymes, these are essential in order for the plant to carry out basic functions.

Secondary metabolites serve a different purpose and consist of compounds such as phenolics, terpenoids and alkaloids.

Phenolics are compounds that consist of an aromatic ring bound to a hydroxyl group, the simplest phenolic being phenol. Polyphenols consist of a number of phenol groups. Phenolics include benzoquinones or other simple phenols and phenolic acids or aldehydes, coumarins, naphthoquinones, xanthonoids, stilbenoids, anthraquinones, flavonoids, lignans, lignins, tannins and other polyphenols. Flavonoids are water soluble and can function as pigments for attracting pollinators, filters of ultraviolet light and are involved in symbiosis, defence and numerour other cellular processes (Amalesh et al. 2011). Their pharmacological activity has been found to include anticancer, antioxidant, anti-inflammatory and antiviral activity (Liu et al. 2016; Friedman 2007; Suen et al. 2016; Koosha et al. 2016; Leyva-López et al. 2016). Tannins are typically astringent and provide defence from herbivores and are regulators of microbe populations (Teplitski et al. 2000; Kumar & Singh 1984; Moctezuma et al. 2014). They are used in the leather tanning process due to their ability to bind to proteins, but also possess anticancer, anti-inflammatory, antimicrobial and other useful activities (Haslam 1989).

Terpenoids, also called isoprenoids, include hemiterpenes (such as prenol), monoterpenes (including iridoids), sesquiterpenes, diterpenes, sesterterpenes, triterpenes (including steroids and saponins), tetraterpenes (including carotenoids and xanthophylls) and polyterpenes. Terpenes tend to be volatile and functionally useful as communicative chemicals, attracting beneficial organisms such as pollinators or predators of herbivores and for communicating with neighbouring plants (Gershenzon & Dudareva 2007). They are used by industry as fragrances and flavourings but also have significant pharmacologcal activity, such as antimicrobial and insecticidal activity (Trombetta et al. 2005; Zengin & Baysal 2014; Chaubey 2012).

Alkaloids are secondary metabolites and may come in a great variety of different structures, but all are organic, nitrogen containing bases. The number of substitutions of the hydrogen atoms that are bound to the nitrogen with an alkyl group determines whether the alkaloid is a primary ($R\text{-}NH_2$), secondary ($R_2\text{-}NH$), tertiary ($R_3\text{-}N$) or quaternary ($R_4\text{-}N$) alkaloid. Their pharmacological effects are diverse and include anti-parasitic, anti-inflammatory, stimulant,

anticancer, analgesic, cytotoxic and psychotropic activity (Wink 2012; Kakhia 2003; Kaur & Arora 2015; Souto et al. 2011; Chierrito et al. 2014).

Glycosides consist of a glycone (sugar molecule) bound to an aglycone (non-sugar molecule) via a glycosidic bond. The aglycone of a glycoside can be an alcohol, phenolic molecule, terpenoid or alkaloid (glycoalkaloid). Some glycosides may have a medicinal use, while others may be potently toxic (Mason & Foerster 1981; Gálvez et al. 2006). Their activity is dependent on the aglycone, which becomes unbound from the glycone when the glycosidic bond is hydrolysed in the digestive tract.

It is possible for the presence of some classes of compounds to be tested for using the plant material or its extract, by carrying out simple chemical reactions. Knowing what class of compounds make up the majority of the metabolites in the plant allows the mechanism of action to be speculated to a limited extent, based on the known interactions of the different classes of compounds, for example, an extract rich in tannins could be expected to have strong inhibitory activity towards enzymes due to the ability of tannins to bind proteins.

3.1.4: Thin layer chromatography for analysis

Thin layer chromatography (TLC) is the separation of compounds on a TLC sheet which consists of a stationary phase such as silica gel, spread over a flat surface such as glass or aluminium. After a compound or mixture of compounds is spotted onto a TLC sheet, it is placed into the mobile phase, which consists of a mixture of solvents, and the mobile phase is absorbed by the stationary phase and travels up the TLC sheet, carrying the sample with it. Silica binds strongly to polar compounds, but more weakly to non-polar compounds. The movement of the sample along the TLC sheet is determined not only by the affinity of the individual compounds in the sample towards the silica in the stationary phase, but also by the solvent system used. The mobile phase will affect the movement through the silica gel as compounds that are more soluble in the mobile phase will be able to move through the stationary phase more quickly. After separating a crude extract, a number of spots are observed, from which the retention factor (Rf) can be calculated based on how far the spot moved from its place of origin compared to the solvent front. If identical compounds are present in 2 different extracts, theoretically, they should have the same Rf. Compounds that are very similar may have similar, if not identical Rfs, although dissimilar compounds may

also have an identical Rf if they have similar solubility in the mobile phase and similar affinity for the stationary phase.

Other stationary phases can be used, such as alumina which, like silica, is also polar. Sephadex™ is an example of a resin that separates compounds based on their size, while C18 is an example of a non-polar resin, favouring the movement of polar compounds and retaining non-polar compounds for longer.

3.1.5: HPLC analysis of phytochemicals

High performance liquid chromatography (HPLC) can be used to determine what compounds are present in a mixture using appropriate standards and references, how many compounds there are and also what the relative polarity of the components are. HPLC can also be performed on a large scale to separate compounds for collection. Some even more advanced systems can cycle the mobile phase through the column so that the separation of peaks is increased and individual compounds can be isolated with fewer contaminants.

The mobile phase for HPLC is usually two or more organic solvents such as acetonitrile, water or methanol, while the stationary phase is a resin such as C18. C18 is a hydrophobic resin, meaning that it has a higher affinity for non-polar compounds than it does for polar compounds. When a plant extract is passed through a C18 column, non-polar compounds will pass through it much more slowly than polar compounds, so that the more polar compounds will be eluted first and the non-polar compounds will be eluted later. The mobile phase can be optimised to adjust elution time, with a more non-polar mobile phase allowing quicker elution of compounds on a non-polar resin. The mobile phase may be an unchanging mixture of solvents or may change to be increasingly non-polar over the course of the run.

The range of stationary and mobile phases that are available can allow tailored separation of compounds from a sample and identification of compounds by comparing retention times with standards. As such, this is a useful tool in generating typical spectra that can be used to standardise plant extracts and identify components of extracts (Colombo et al. 2009).

3.2: Materials and methods

3.2.1: Chemicals and reagents

Analytical and HPLC grade n-hexane, chloroform, ethanol, methanol, ethyl acetate, dichloromethane (DCM), dimethylsulphoxide (DMSO) and acetonitrile were purchased from

RCI Labscan (Thailand). Sulphuric acid and phosphoric acid were purchased from Friendemann Schmidt (Australia), ammonia, potassium iodide, gelatin, aluminium chloride, vanillin, potassium iodide and sodium chloride were purchased from RM Chemicals (India), bismuth subnitrate was purchased from Mallinkrodt (USA), acetic acid, iron chloride, and hydrochloric acid were purchased from Systerm (Malaysia).

3.2.2: Collection and identification of *Pericampylus glaucus*

Fresh *Pericampylus glaucus* was collected from the coordinates 2°56'53.4"N 101°53'51.7"E and 2°56'53.2"N 101°53'52.0"E in West Malaysia, in 2013.

A voucher specimen, consisting of leaves and stems, was identified by Mr. En Kamarudin from the Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM). The sample was positively identified as *P. glaucus* and deposited in the Kepong Herbarium of FRIM (PID 020115-02).

3.2.3: Preparation, sequential solvent extraction, ultrasonic extraction and acid-base extraction of plant material

3.2.3.1: Processing of plant material

All plant parts were dried in the shade for 2 weeks before any large plant parts were cut into 2 cm pieces. After drying, the plant parts were ground, separately, into a fine powder using a blender (Philips, China) and weighed using a weighing balance (Sartorius AG, Germany). The total dry weight of the leaves, stems, fruits and roots came to 119 g, 438 g, 1.8 g and 23.9 g respectively.

3.2.3.2: Sequential solvent extraction of plant material by maceration

The stems and leaves of *P. glaucus* were macerated for 3 days at 60 °C in hexane, chloroform and ethanol respectively and maceration in each solvent was performed 3 times with the plant material being soaked in solvent in a 1:3 ratio by volume. The solvent was evaporated from the plant material thoroughly before extraction by a new solvent commenced. The extracts obtained from each of the solvents were filtered using filter paper (Whatman International, England) and concentrated using a rotary evaporator (Buchi Labortechnik AG, Switzerland) and stored in glass scintillation vials (Kimble, USA) at -20 °C until further use. Samples were labelled PGSH, PGSC, PGSE, PGLH, PGLC, PGLE, PGRH, PGRC, PGRE, PGFH, PGFC

and PGFE, the letters representing the genus (*Pericampylus*), species (*glaucus*), plant part (stem, leaf, root or fruit) and solvent (hexane, chloroform or ethanol) respectively.

3.2.3.3: Ultrasonic extraction of plant material

Due to the small quantity of root and fruit material collected from *P. glaucus*, ultrasonic extraction was used, to get the highest yield of extract by sequential solvent extraction. The powdered root material was put into an Erlenmeyer flask and solvent was added in the ratio 1:3 (v/v). This was then placed in an ultrasonic bath for 30 min before filtering and evaporating the solvent to dryness. This ultrasonic solvent extraction was repeated 3 times with hexane, chloroform and ethanol, respectively.

3.2.3.4: Acid-base extraction of alkaloids

The plant material was collected then dried for 2 weeks before being cut into approximately 2 cm pieces. The plant material was ground into a fine powder using a laboratory blender and transferred into an Erlenmeyer flask. The plant material was defatted using hexane after which it was allowed to dry thoroughly. The plant material was macerated with methanol overnight at 50 °C, the extract was filtered and evaporated to dryness. The maceration was repeated 3 times. The dried extract was dissolved in 0.5% sulphuric acid and impurities were removed by liquid separation with ethyl acetate. The remaining aqueous solution was basified using 25% ammonia solution so that the pH of the solution reached 9.0. The basified solution was subjected to liquid separation with chloroform. The chloroform layer was then separated from the aqueous layer using a separating funnel and evaporated using a rotary evaporator to give the crude total alkaloid extract.

3.2.4: Yield of extracts

The yield of the extracts was calculated by dividing the final weight of the dried extract by the weight of the dried plant material, before extraction, which the extract was derived from and multiplying by 100 to get the yield as a percentage. The calculation is summarised in the equation below:

Equation 3.3: Calculation of extract yields

$$\text{Yield} = \frac{Mass\ of\ extract\ (g)}{Mass\ of\ plant\ material\ (g)} \times 100$$

3.2.5: Phytochemical screening of extracts

The following tests were performed to determine the presence and approximate the quantity of certain types of compounds in each extract. The presence of alkaloids, flavonoids, saponins, tannins, terpenes and steroids was determined according to the methods described by Kamalinejad*et al.* (2003) and Musa *et al.* (2009).

Extracts were also separated by thin layer chromatography (TLC) before staining. A solvent system for optimal separation of the extracts was developed and separated extracts were sprayed with different stains so that the compound class of specific spots could be identified. Hexane, chloroform and ethanol extracts were separated using hexane:ethyl acetate, 9:2, dichloromethane:methanol:hexane + NH_3, 9:1:1 and dichloromethane:methanol + acetic acid, 9:1 respectively. The alkaloid extract was separated using dichloromethane:methanol, 9:1. Samples were labelled SH, SC, SE, LH, LC, LE, RH, RC RE, FH, FC, FE and SA where the first letter represents the plant part (stem, leaf, root or fruit) and the second letter represents the solvent used for extraction (hexane, chloroform or ethanol) with the exception of the stem alkaloid extract, which was labelled SA.

3.2.5.1: Test for alkaloids

The extract was heated on a boiling water bath with 5 mL of 2 M HCl. The solution was cooled and filtered, then divided into two equal portions. To one portion a few drops of Mayer's reagent (1.358 g $HgCl_2$ in 60 mL of water added to 5 g KI in 10 mL of water and made up to 100 mL) was added, to the other portion a few drops of Wagner's reagent (2 g of Iodine and 6g KI in 100 mL of water) was added. The results of the test were graded as follows:

Negative result = No opaqueness
Weakly positive result (+) = Slight opaqueness

Positive result (++) = Definite turbidity, no flocculation
Strong positive (+++) = Heavy precipitate and flocculation

For TLC staining, Dragendorff's reagent was sprayed onto the separated extracts and allowed to develop. The development of a red/orange spot indicated the presence of an alkaloid. Spraying with an ethanolic vanillin solution (1%) and heating causes a colour change in amines and amino acids to a green, yellow, pink, red or brown colour.

3.2.5.2: Test for flavonoids

The extract was dissolved in 5 mL of DMSO. 6 drops of 36% HCl and 0.5 g of magnesium turnings were added. The development of a pink/magenta red colour within 3 min indicated a positive result.

For TLC staining, an alcoholic solution of aluminium chloride (1%) was sprayed onto the TLC sheet and dried before observing under long wave UV light. The brightening of a spot to give a light blue colour indicated the presence of a flavonoid.

3.2.5.3: Test for saponins

The extract was dissolved in 5 mL of purified water and sonicated for 15 min at 40 °C. The solution was shaken for 30 seconds then left for 45 min before observing the level of froth that remained. The results of the test were graded as follows:

Negative result = no froth
Weakly positive = less than 1cm of froth
Positive = 1-2cm of froth
Strong positive = more than 2cm of froth

3.2.5.4: Test for tannins and phenolics

10 mL of 0.9% NaCl solution was added to the extract. The solution was filtered and a solution containing 10% NaCl and 1% gelatin was added. Precipitation indicated a positive result. If a positive result was obtained, $FeCl_3$ solution was added. The development of a blue, black or green colour and precipitate indicated a positive result for phenolics.

The $FeCl_3$ solution was sprayed onto TLC sheets with separated extracts and the change of a spot into a darker, brown colour indicated the presence of a tannin.

3.2.5.5: Test for terpenoids and sterols

The extract was dissolved in 2 ml of chloroform and sonicated for 15 min. Concentrated H_2SO_4 was slowly added down the side of the test tube. The upper layer would turn a red colour and the H_2SO_4 layer would turn a yellow-green colour. A red or brown colour at the interface indicated the presence of terpenoids, while a blue or green colour at the interface indicated the presence of steroids.

For analysis of extracts separated by TLC, 40% phosphoric acid causes a colour change in sterols when sprayed onto the TLC sheet and heated on a hot plate.

3.2.6: HPLC analysis of *P. glaucus* extracts and standards

All solvents used for HPLC analysis were prepared by filtering using a 0.2 µm filter. The chloroform, ethanol and alkaloid extracts were dissolved in HPLC grade methanol to a concentration of approximately 1 mg/ml. An Agilent Technologies 1260 Infinity HPLC machine was used. A 4.6 x 150 mm Zorbax Extend C18 column with 5 micron particle size was used at a flow rate of 1 ml/min. The mobile phase used for the duration of the run was 100% H_2O and 0% acetonitrile for at time 0 with a linear gradient from 0 - 20 mins to 0% H2O and 100% acetonitrile and 0% H2O and 100% acetonitrile from 20 - 35 mins. The spectra were acquired at the wavelengths of 210 and 280 nm for all extracts and standards and at 230, 254, 274 and 293 nm for the alkaloids and periglaucine A. Hexane extracts were not analysed by HPLC to prevent damage of the HPLC column by retention of highly non-polar compounds.

3.3: Results

3.3.1: Yields from solvent extraction and acid-base extraction

The yields of the extracts shown in **Table 3.1** are a measure of the efficiency of each solvent at extracting compounds from the plant material. The highest yield was obtained from extraction with ethanol, with extraction yields of 8.58, 6.54, 5.18 and 1.04% from the leaf,

fruit, stem and root material respectively. The hexane extracts tended to give the lowest yield, with the exception of the leaf extract, where the chloroform extract yield was only slightly higher than the hexane yield by 0.39%. These results indicate that the majority of the extractable compounds in the plant material have high polarity, as the majority of the extract was gained from the ethanol extraction. The lowest yield was obtained from the acid-base extraction of the stems. The total or crude alkaloid fraction that was obtained as a result of the acid-base reaction gave a yield of 0.07%.

Table 3.1: Yield of extracts

Extract	Plant material collected (g)	Extract obtained (g)	Yield (%)
PGSH		6.59	0.83
PGSC	794.5	12.55	1.58
PGSE		41.16	5.18
PGLH		6.61	2.82
PGLC	234.4	5.70	2.43
PGLE		20.11	8.58
PGRH		0.07	0.29
PGRC	23.94	0.24	1.00
PGRE		0.25	1.04
PGFH		0.03	1.64
PGFC	1.834	0.06	3.27
PGFE		0.12	6.54
PGSA	684.2	0.47	0.07

3.3.2: Phytochemical screening of the extracts

The phytochemical screening of the extracts was performed to gain an understanding of the types of compounds present in the extracts as the class of compounds differs greatly between plants and between the parts of the same plant. **Table 3.2** and **Appendix B** display the results of the screening of the leaf and stem parts of *P. glaucus*. The roots, fruits and crude alkaloid fraction were analysed only by TLC, as shown in **Figures 3.1 - 3.8**, due to the small quantity plant material collected of the roots and fruit and resulting small quantity of extract obtained from solvent extraction and acid-base extraction of alkaloids. The ethanol extracts of the stem and leaves tested negative for steroids, whereas the other plant parts tested positive. Terpenoids were found in both ethanol extracts as well as the leaf chloroform and stem hexane extracts. No tannins, phenolics or flavonoids were detected by the methods adopted. Saponins were found only in the leaf chloroform, leaf ethanol and stem ethanol extracts, with the stem ethanol extract containing the most saponin. Alkaloids were detectable only in the chloroform extracts, with the stem testing more strongly positive for alkaloids than the leaves. The leaf ethanol and stem ethanol extracts showed the most similarity in their composition based on the phytochemical screening results.

Table 3.2: Phytochemical Analysis of Extracts

	Type of compound						
Sample	Alkaloids*	Flavonoids	Saponins	Tannins	Phenolics	Terpenoids	Steroids
PGLH	-/-	-	-	-	-	-	+
PGLC	-/+	-	+	-	-	+	+
PGLE	-/-	-	+	-	-	+	-
PGSH	-/-	-	-	-	-	+	+
PGSC	+/++	-	-	-	-	-	+
PGSE	-/-	-	++	-	-	+	-

*Two tests were carried out to test for the presence of alkaloids, the first result is from testing with Dragendorff's reagent and the second with Wagner's reagent.

3.3.3: Phytochemical analysis of extracts by TLC

The analysis by TLC allows the different compounds in the extract to be separated and visualised as spots on a TLC sheet which can then be observed under different light and stained with various stains to highlight different types of compounds. **Figure 3.1** shows the appearance of the extracts under normal light after being separated with solvent system which allowed optimal separation of all of the extracts obtained from the same solvent. A large number of compounds are observed, with the stem hexane and leaf hexane extracts showing great similarity in their chemical composition. The stem chloroform and root chloroform extracts also show similar chemical composition when observed under normal light. When observed under short wave UV light (**Figure 3.2**), some of the spots become clearer, highlighting the similarities observed under normal light, but also clearly showing the presence of simlar relatively non-polar compounds in the ethanol extracts which can also be seen under long wave UV light (**Figure 3.3**).

The staining by $AlCl_3$ shown in **Figure 3.4** caused an increase in intensity of some of the blue spots in the root hexane extract and also caused a blue spot to appear more visible in the stem hexane and stem ethanol extracts indicating the presence of flavonoids. A number of compounds were stained by $FeCl_3$ (**Figure 3.5**), particularly in the chloroform and ethanol extracts, indicating the presence of tannins. Staining by Dragndorff's reagent as shown in **Figure 3.6** revealed the presence of alkaloids in the root hexane extract and, to a lesser extent, the stem chloroform extract. Since the alkaliod content was found to be very low, the reason alkaloids were not detected in the leaf chloroform extract may be because the quantity of alkaloid present was so minute that the test was not sensitive enough to detect them, whereas in the phytochemical test which was performed directly on the extract, much more of the extract was used. The stains with phosphoric acid and vanillin (**Figures 3.7 - 3.8**) caused a colour change in many of the spots indicating the presence of sterols and amines.

The stem alkaloid extract was visualised under normal, short wave UV and long wave UV light and was stained using Dragendorff's reagent as shown in **Figure 3.9**. Although 11 distinct spots were observed unver short wave UV light, only 5 distinct spots were stained by the Dragendorff's reagent. The spots that were stained were the most visible when observed under the different light sources and likely make up the majority of the extract. The other compounds that were extracted could possible be other acidic or basic compounds with similar pH based solubility characteristics of alkaloids.

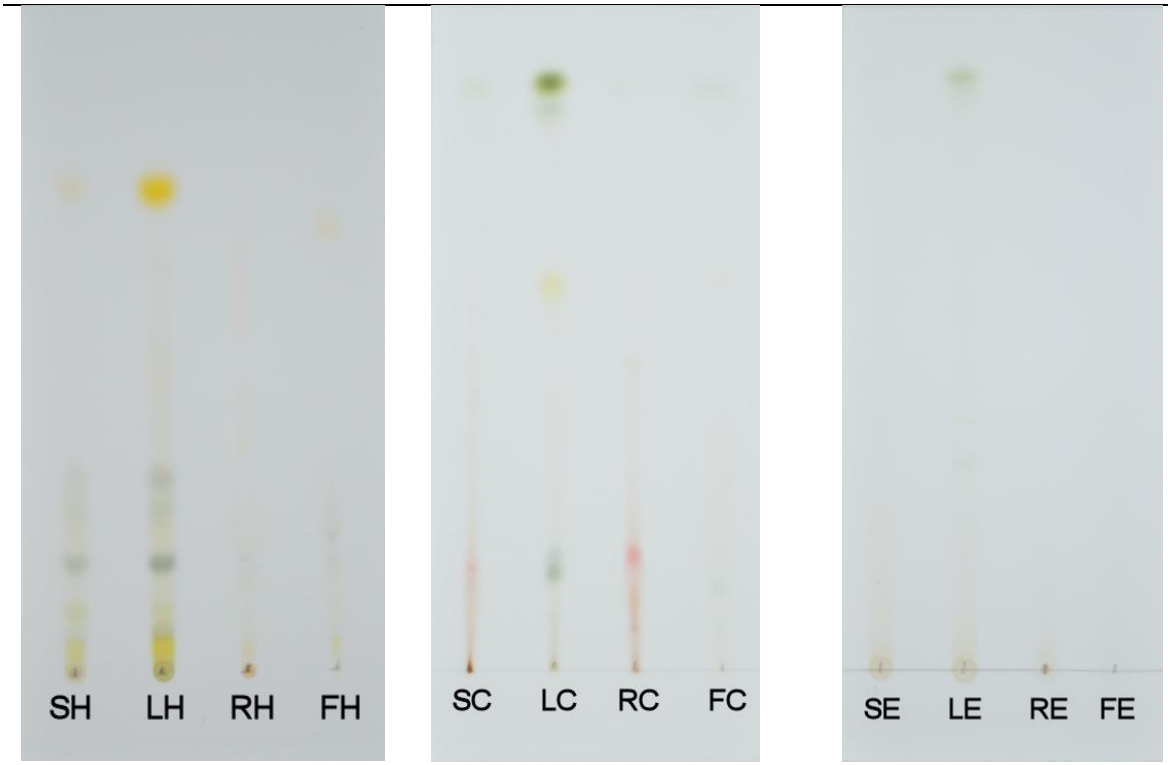


Figure 3.1: Separation of crude extracts by TLC under normal light.

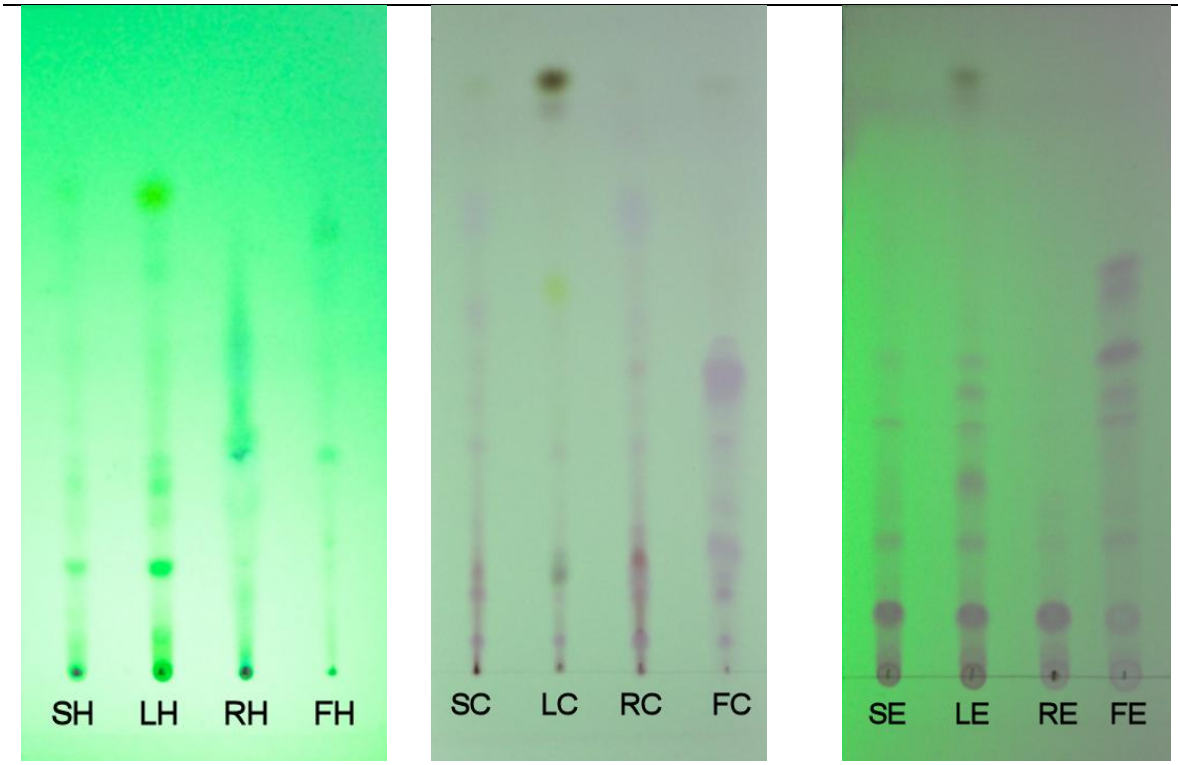


Figure 3.2: Separation of crude extracts by TLC under short wave UV light.

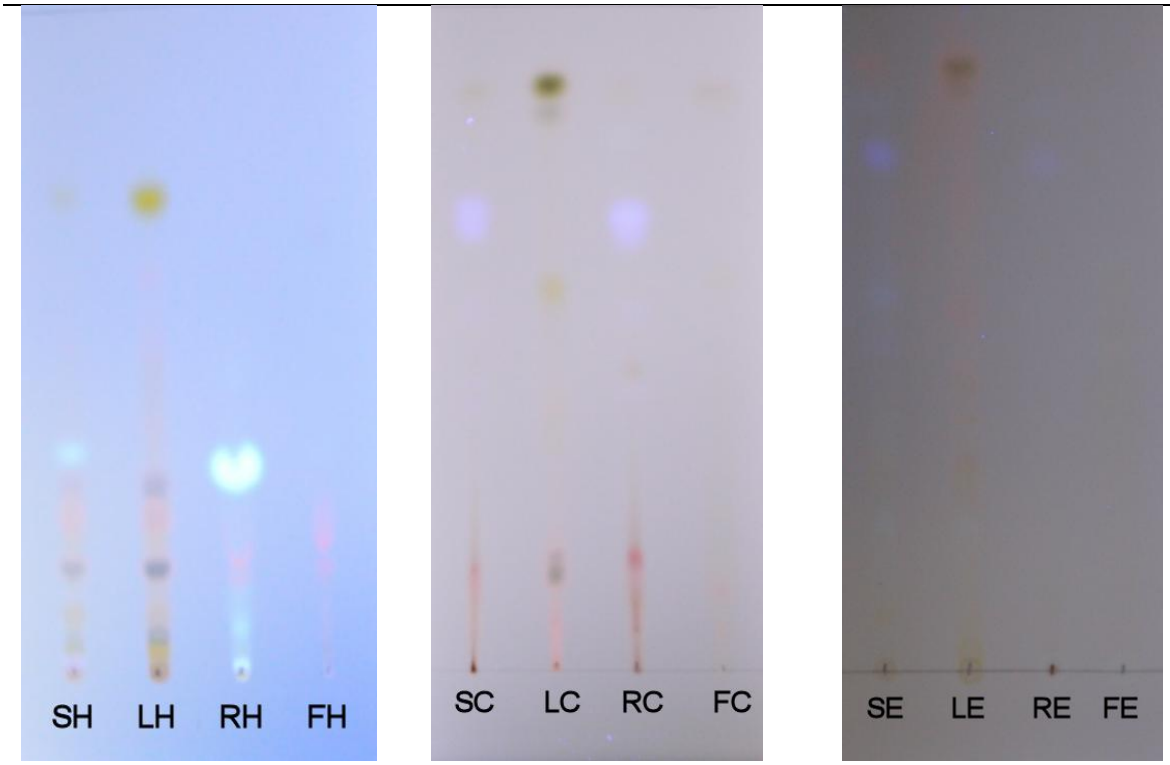


Figure 3.3: Separation of crude extracts by TLC under long wave UV light.

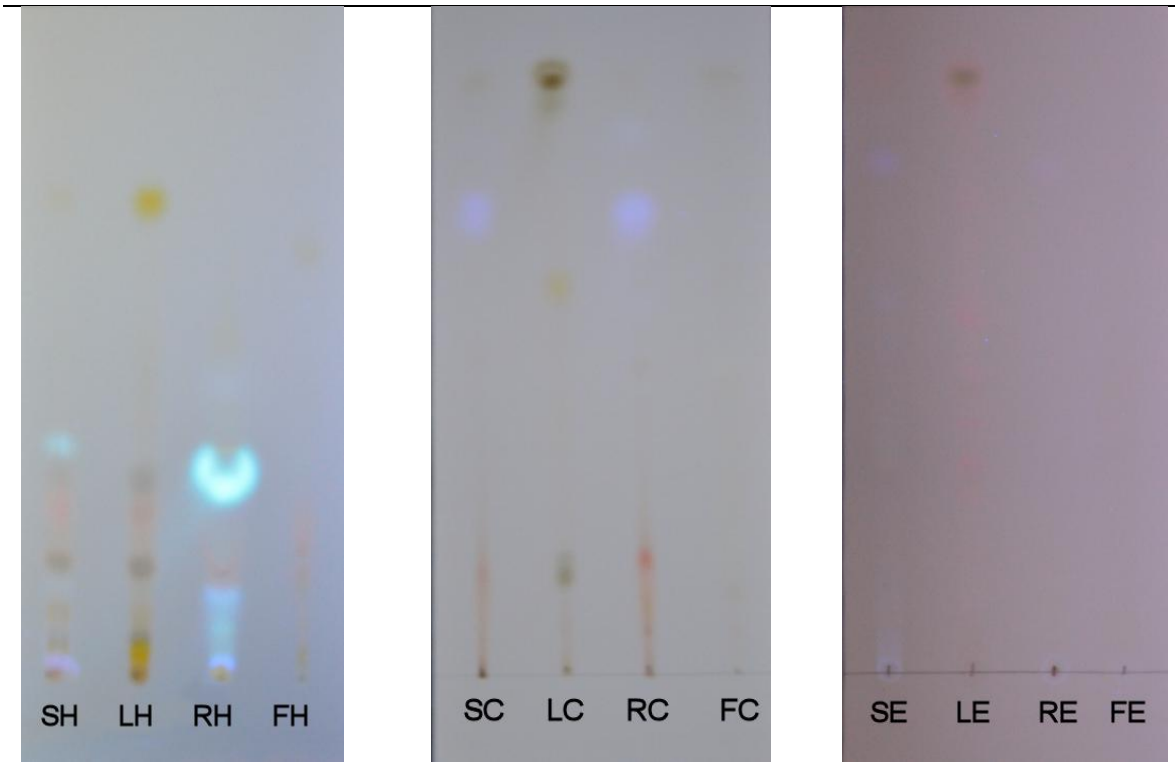


Figure 3.4: Separation of crude extracts by TLC under long wave UV light after $AlCl_3$ treatment.

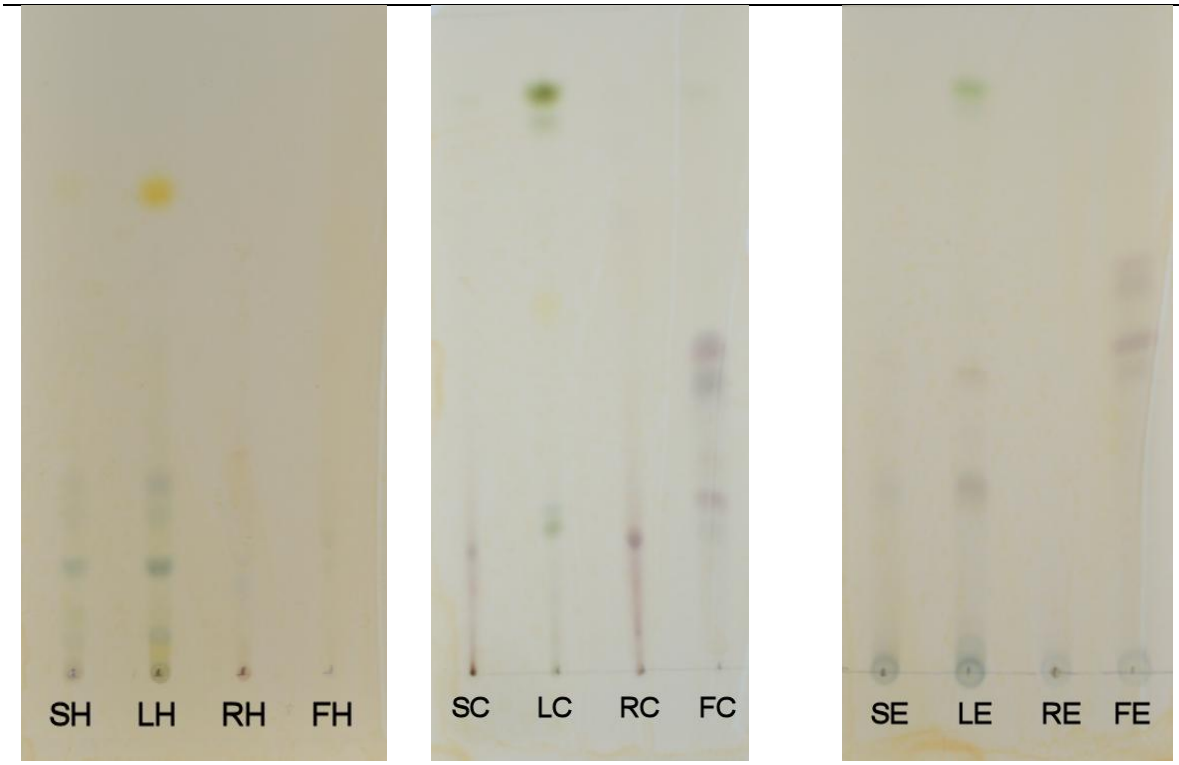


Figure 3.5: Separation of crude extracts by TLC under normal light after $FeCl_3$ staining.

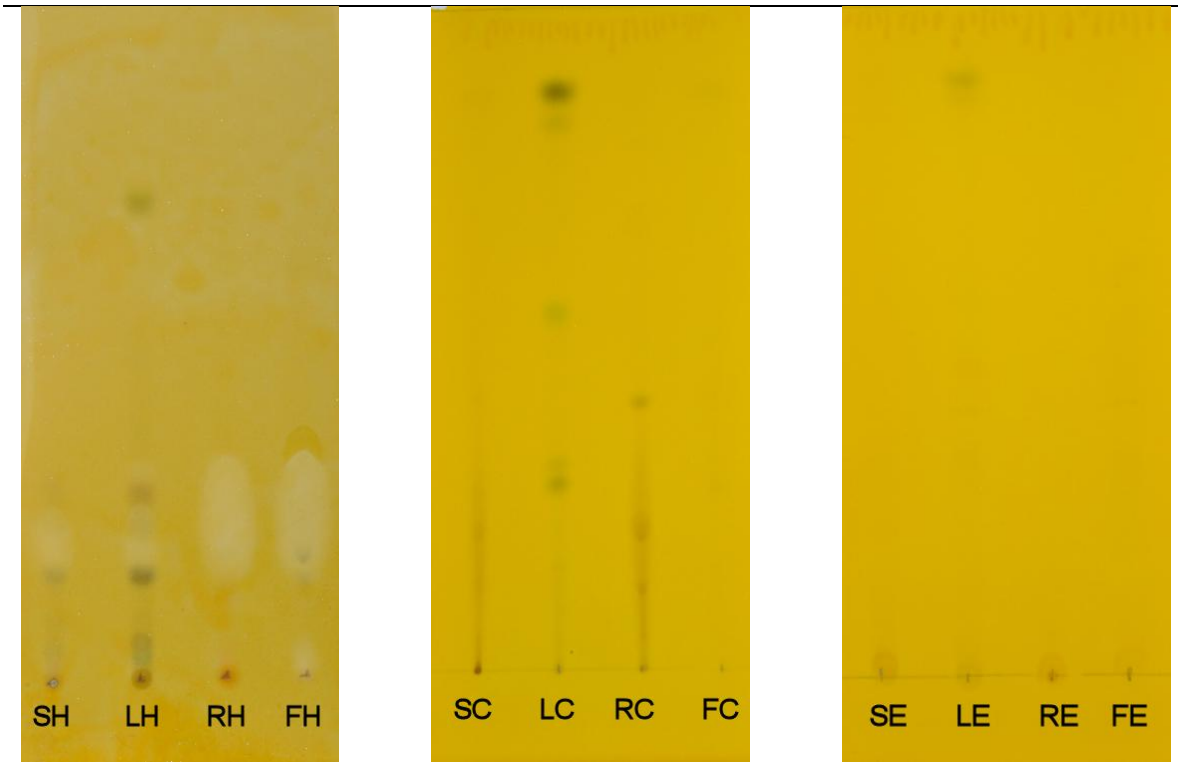


Figure 3.6: Separation of crude extracts by TLC under normal light after staining with Dragendorff's reagent.

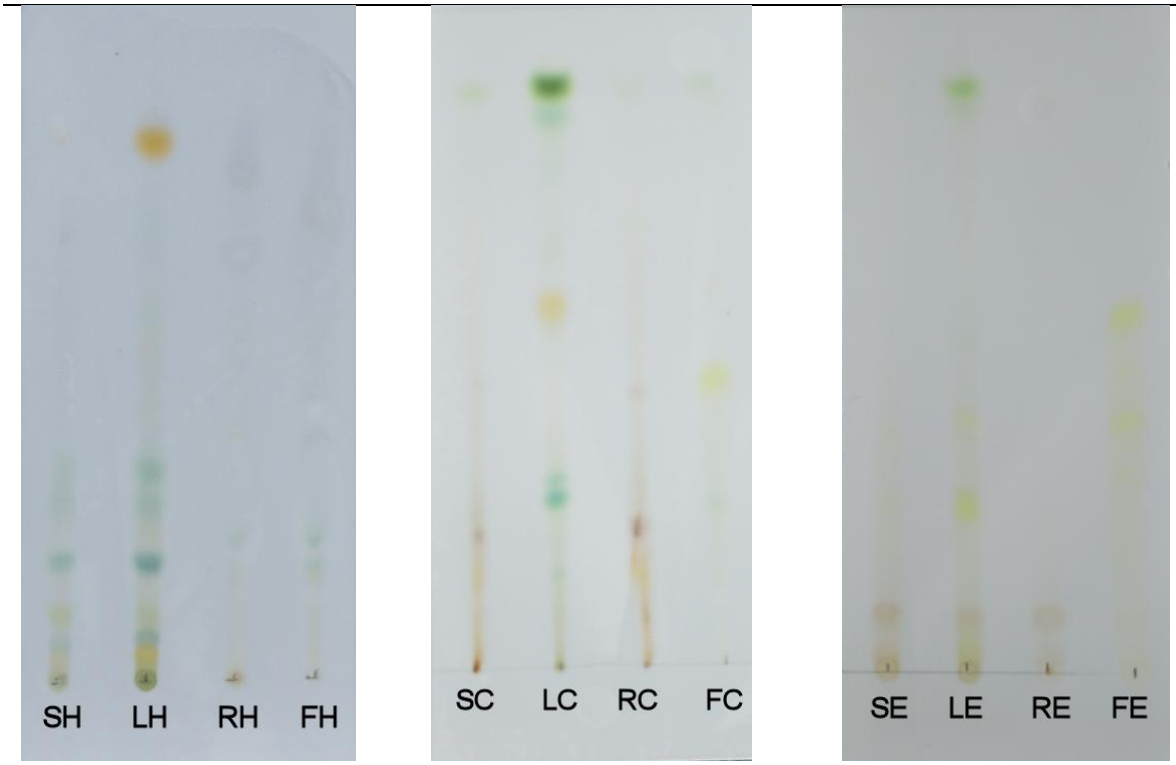


Figure 3.7: Separation of crude extracts by TLC under normal light after heating with 40% phosphoric acid.

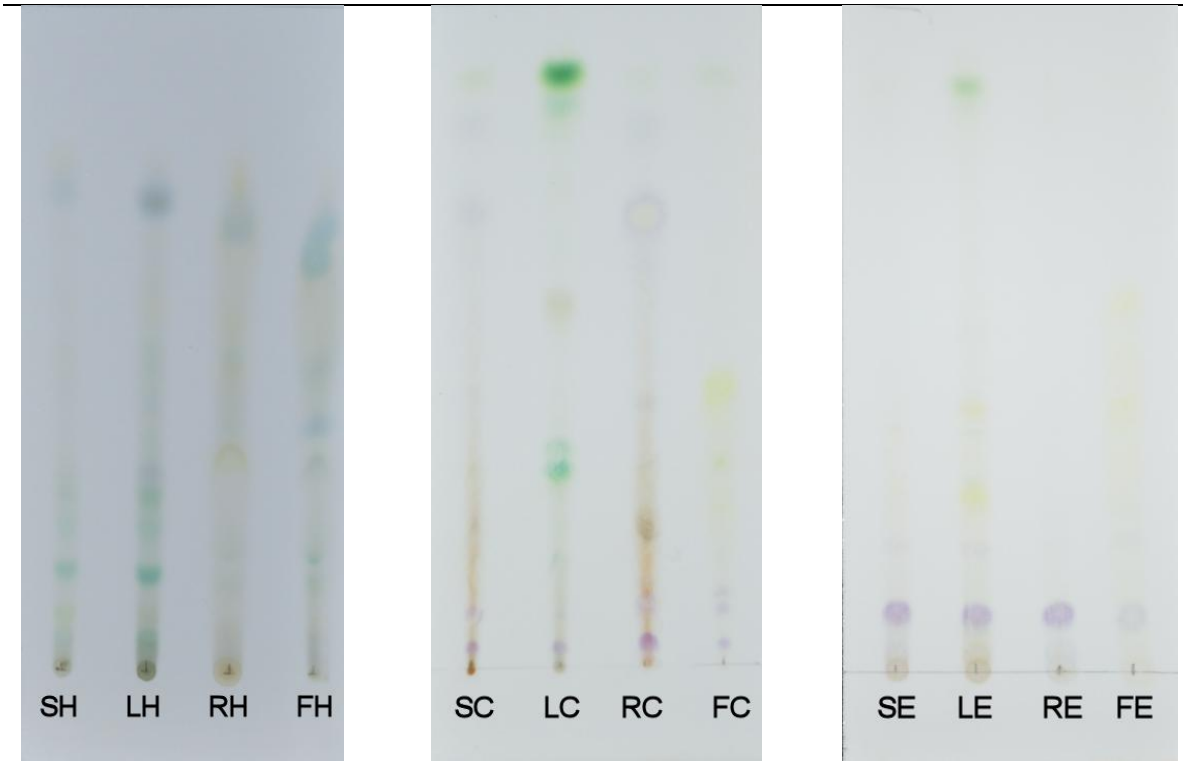


Figure 3.8: Separation of crude extracts by TLC under normal light after heating with vanillin.

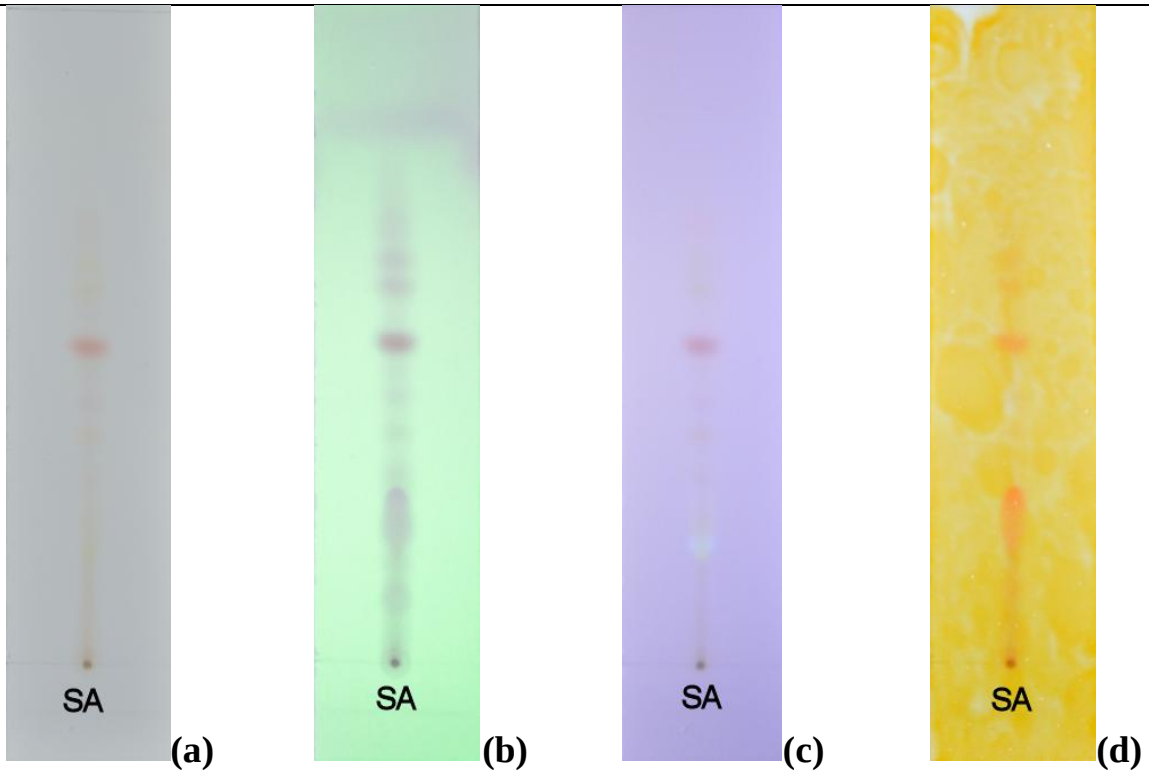


Figure 3.9: Separation of stem alkaloid extract by TLC. Separated compounds are shown (a) under normal light, (b) under short wave UV light, (c) under long wave UV light and (d) after staining with Dragendorff's reagent.

3.3.4: HPLC analysis of extracts and compounds

The HPLC analysis revealed chemical similarities between the extracts with some distinct peaks having the same retention times in numerous extracts.

When the chloroform extracts (**Figures 3.10 - 3.13**) were analysed at 210 nm, large peaks were observed between 7.083 - 7.094, 8.184 - 8.202 minutes in the stem, leaf and root extracts. The stem and leaf extracts also showed a large peak at approximately 12.27 minutes. All of the chloroform extracts demonstrated a peak at about 20 minutes which broadened and was followed by another peak at approximately 24.5 minutes. At 280 nm (**Figures 3.14 - 3.17**), fewer similarities were seen between the larger peaks, although a large peak at about 7 minutes was observed in the stem, leaf and root extracts and may relate to the same compound that showed a peak at this time at 210 nm. The same large peak just after 7 minutes was also observed in the stem, leaf and root ethanol extracts (**Figures 3.18 - 3.21**) and appeared to be the most abundant compound. The analysis of the ethanol extracts (**Figures 3.22 - 3.25**) did not show any distinct similarities.

Betulinic acid was analysed at 210 nm (**Figure 3.26**) and appeared to elute at 21.074 minutes. All of the extracts, when analysed at 210 nm, showed a peak around this time which may suggest the presence of betulinic acid in all of the extracts, with the peak being greatest in the leaf chloroform extract.

Periglaucine A was eluted at approximately 7.5 minutes as shown by **Figures 3.33 - 3.38**. The analysis of periglaucine A at the wavelengths of 230 nm and 254 nm was recommended by the manufacturer, however, analysis at 210 nm also revealed a distinct peak at the same time. The leaf chloroform did show a peak at approximately 7.5 minutes and a large number of peaks were observed around that time in the stem extract as well. Where peaks were observed at this time, there were often many other peaks making them difficult to discern.

The results of the HPLC analysis would suggest that the extracts have some distinct similarities, however it cannot be said with complete certainty that they contain betulinic acid or periglaucine A, although the elution times would suggest that it is possible some of the extracts may contain these chemicals.

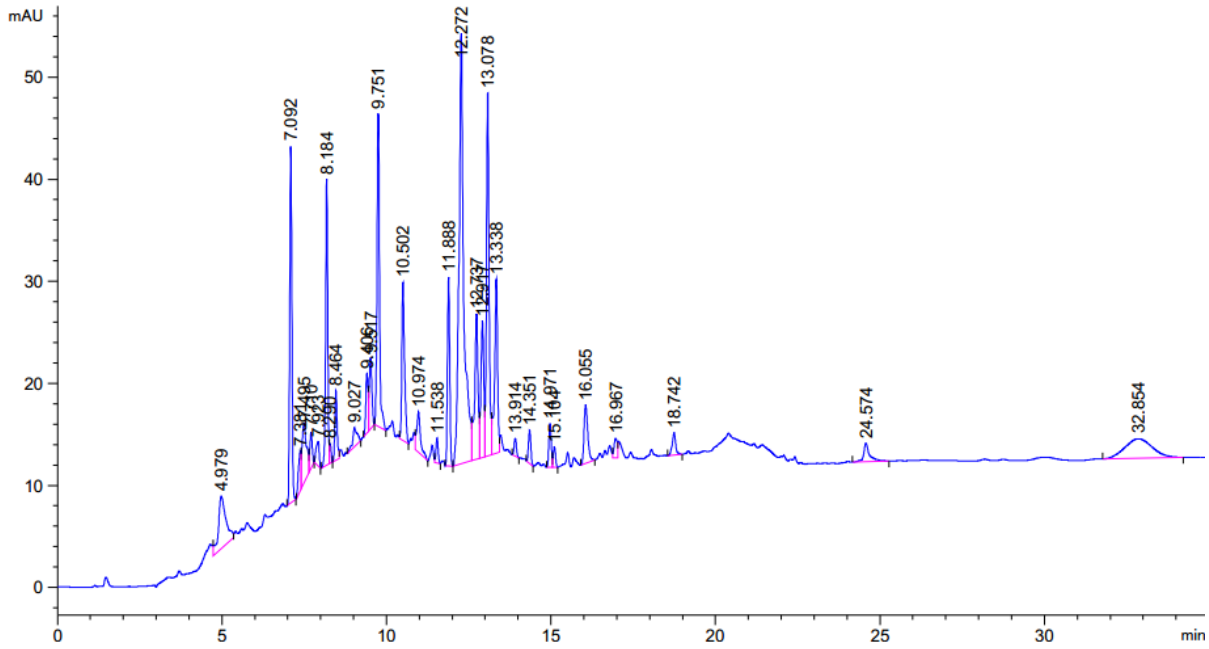


Figure 3.10: HPLC chromatogram of *P. glaucus* stem chloroform extract at 210 nm.

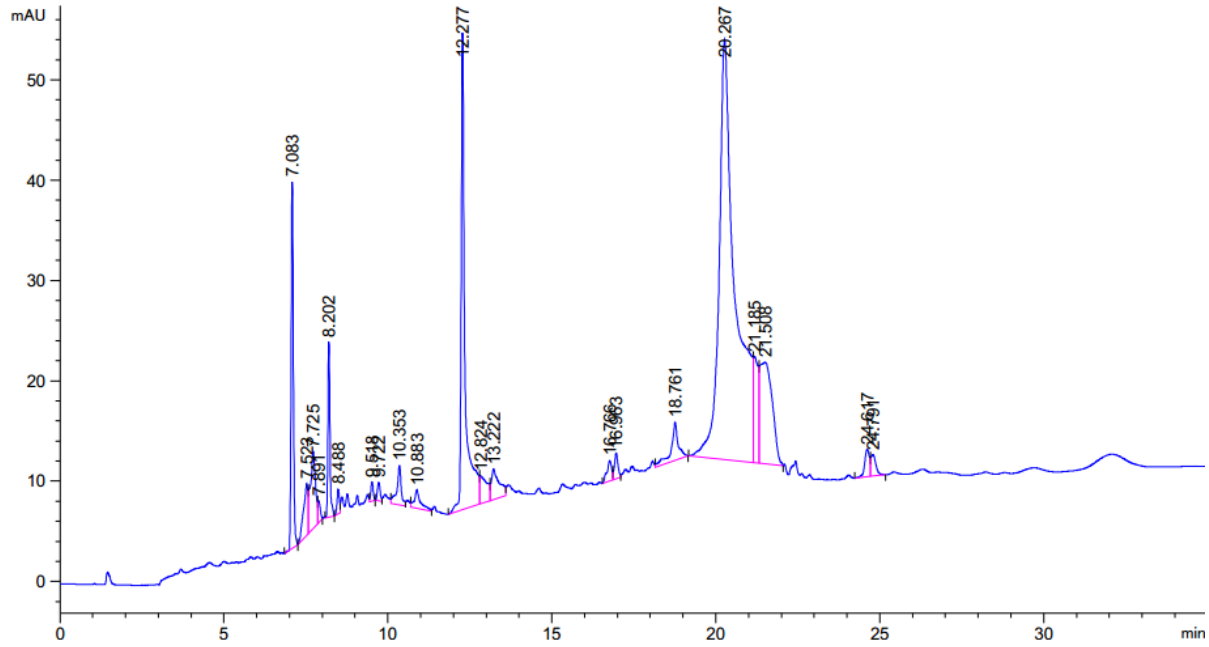


Figure 3.11: HPLC chromatogram of *P. glaucus* leaf chloroform extract at 210 nm.

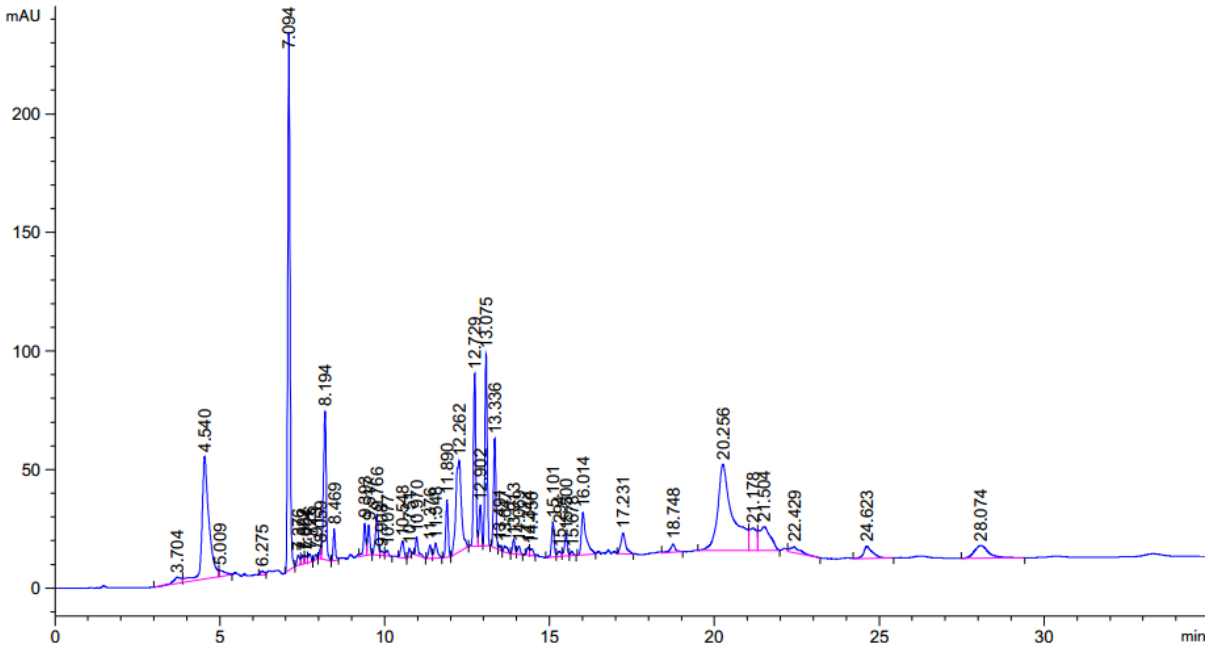


Figure 3.12: HPLC chromatogram of *P. glaucus* root chloroform extract at 210 nm.

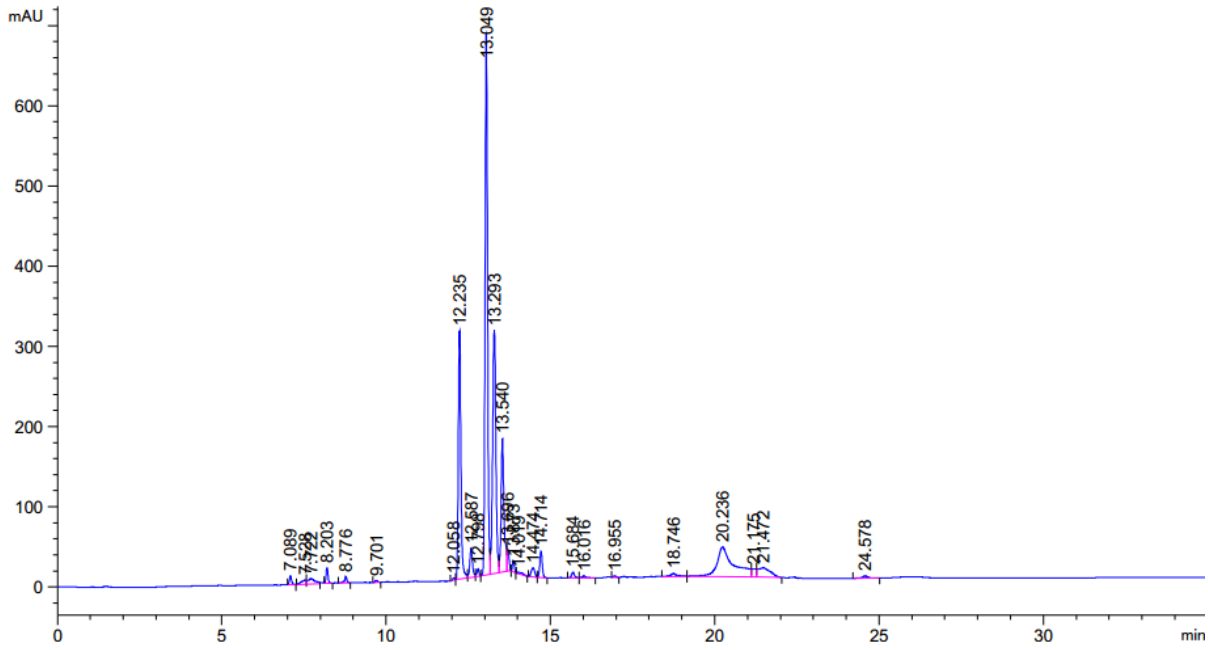


Figure 3.13: HPLC chromatogram of *P. glaucus* fruit chloroform extract at 210 nm.

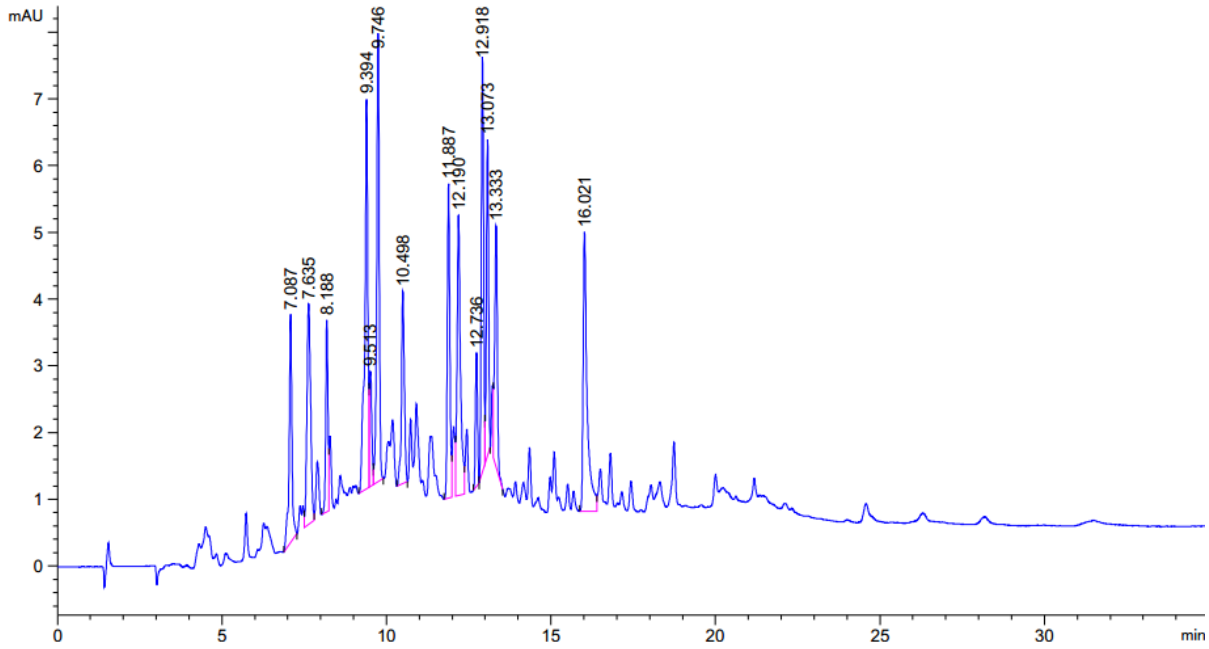


Figure 3.14: HPLC chromatogram of *P. glaucus* stem chloroform extract at 280 nm.

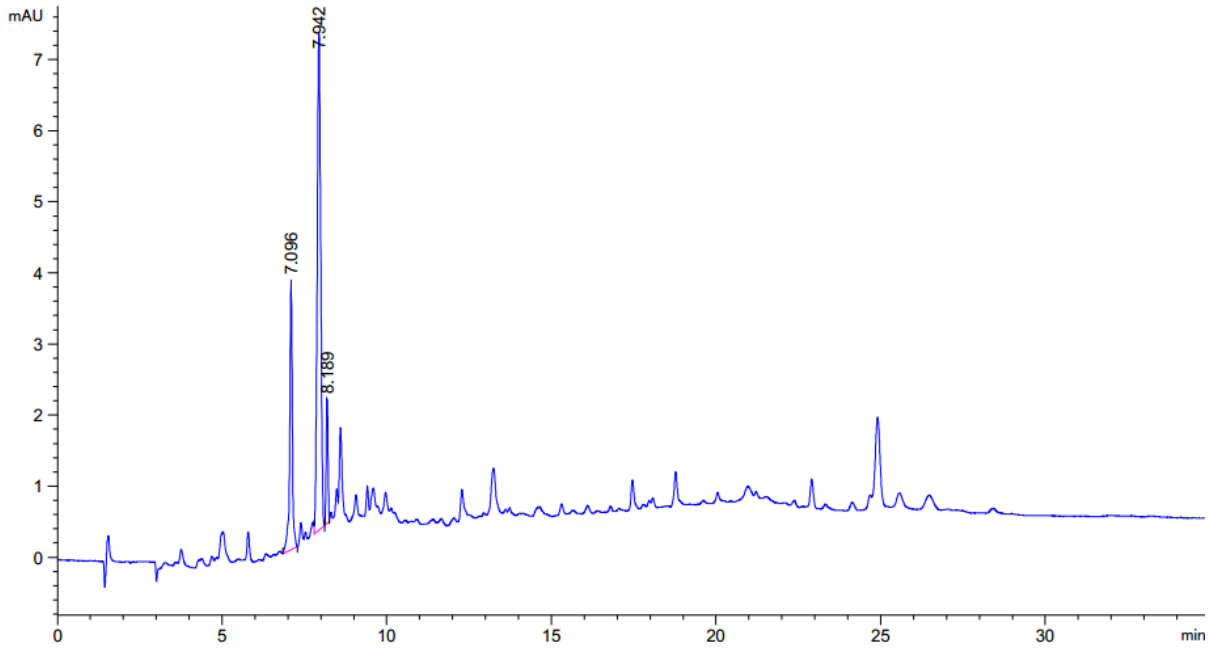


Figure 3.15: HPLC chromatogram of *P. glaucus* leaf chloroform extract at 280 nm.

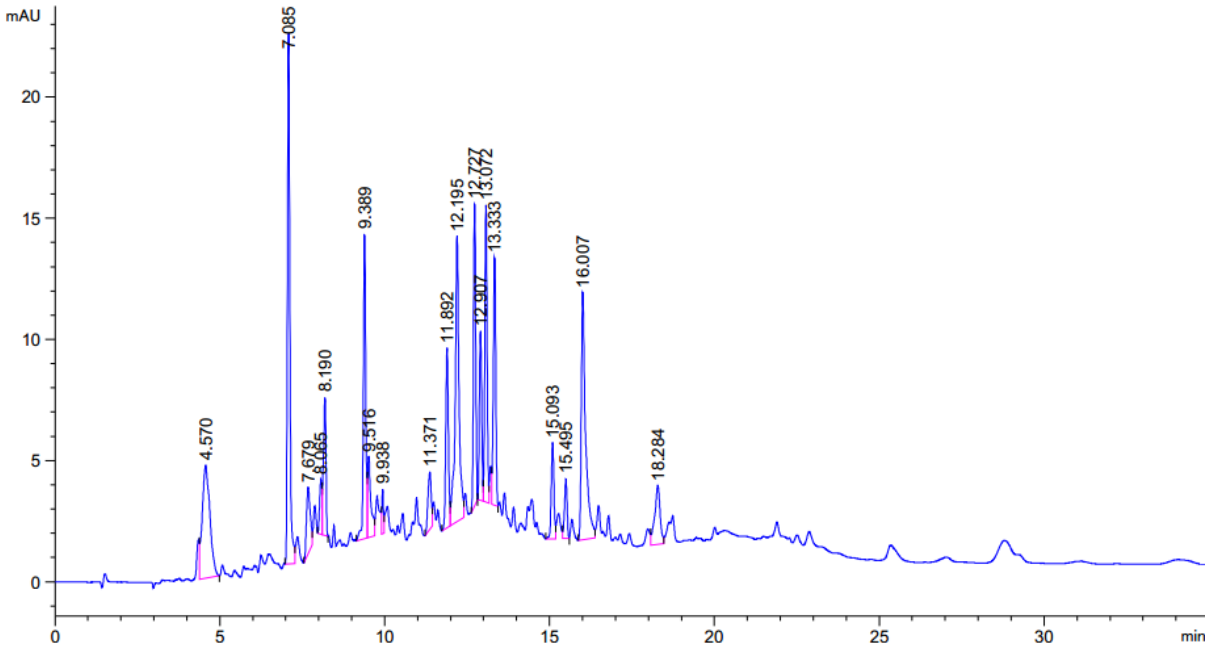


Figure 3.16: HPLC chromatogram of *P. glaucus* root chloroform extract at 280 nm.

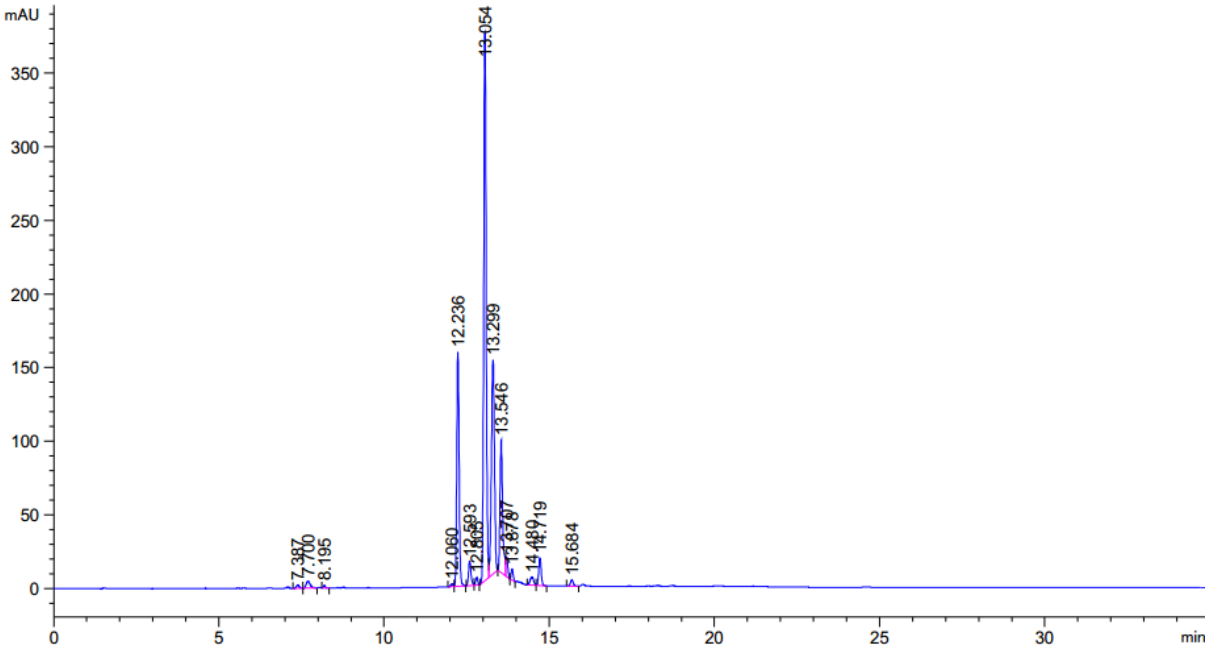


Figure 3.17: HPLC chromatogram of *P. glaucus* fruit chloroform extract at 280 nm.

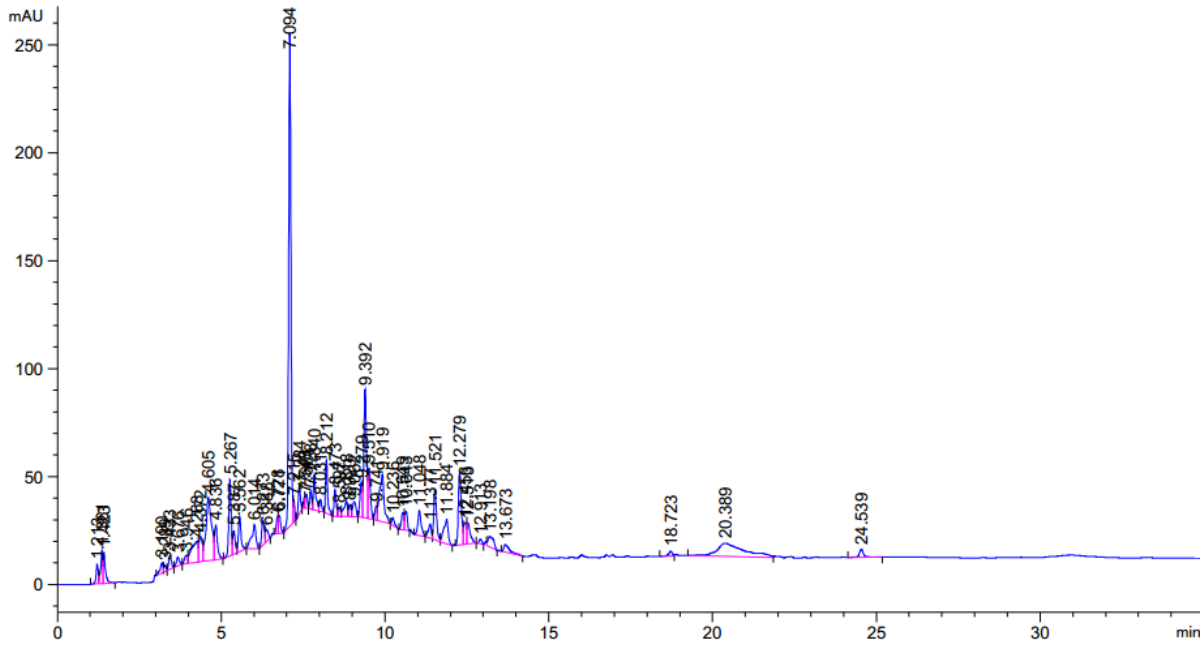


Figure 3.18: HPLC chromatogram of *P. glaucus* stem ethanol extract at 210 nm.

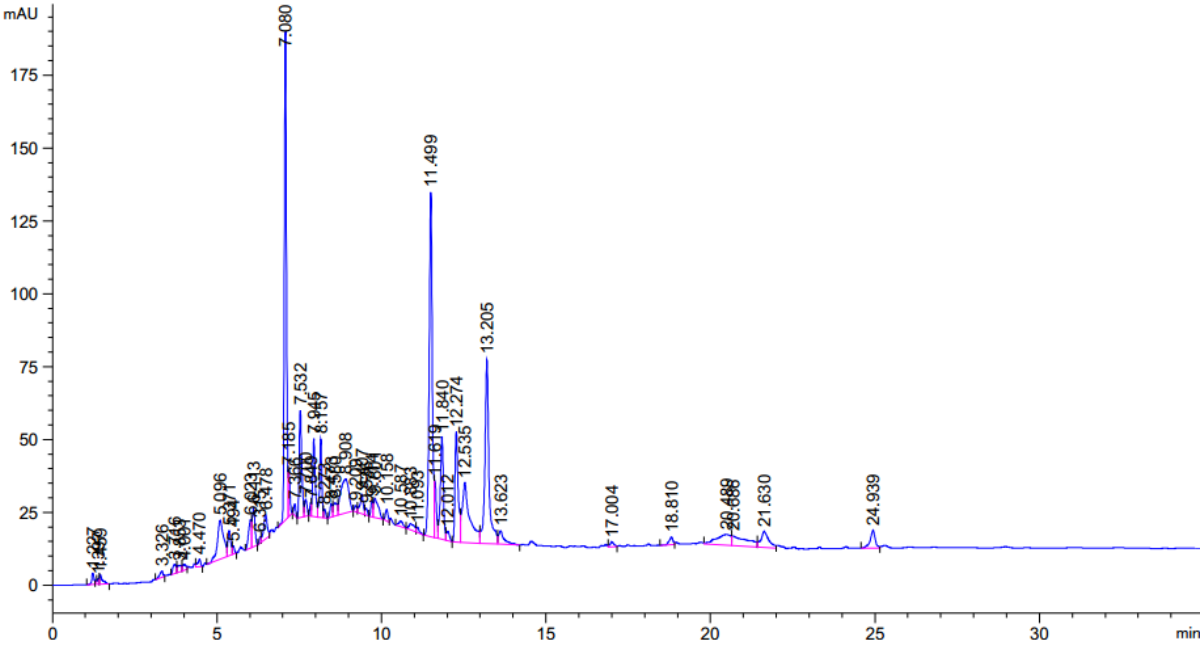


Figure 3.19: HPLC chromatogram of *P. glaucus* leaf ethanol extract at 210 nm.

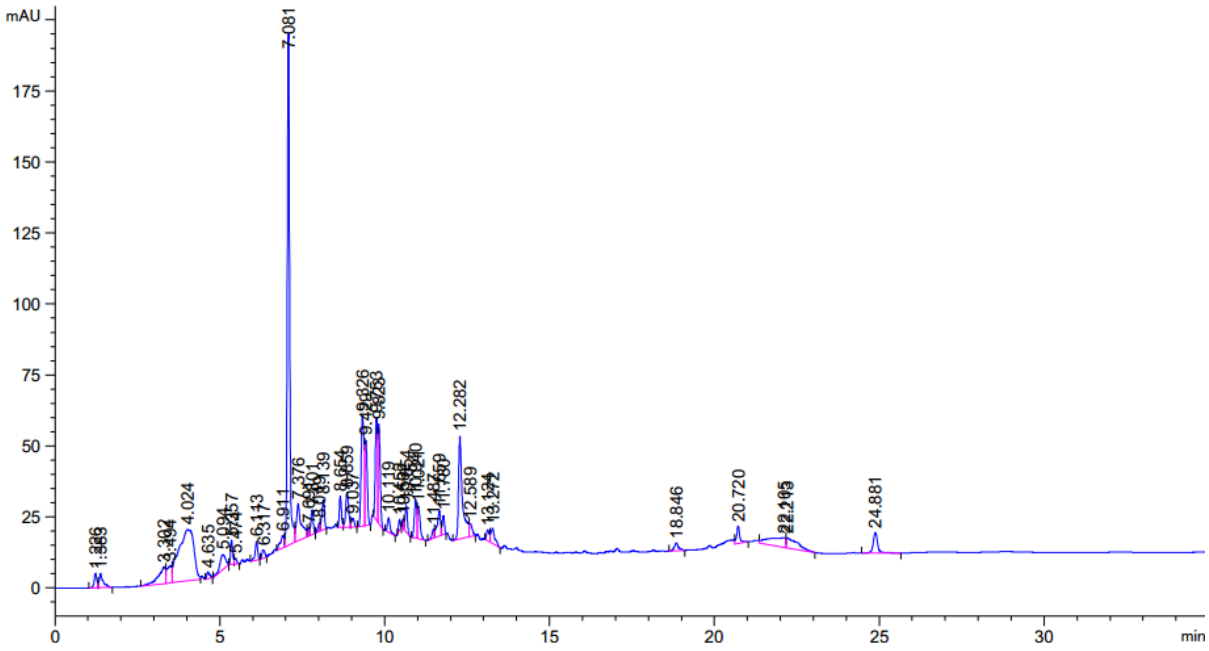


Figure 3.20: HPLC chromatogram of *P. glaucus* root ethanol extract at 210 nm.

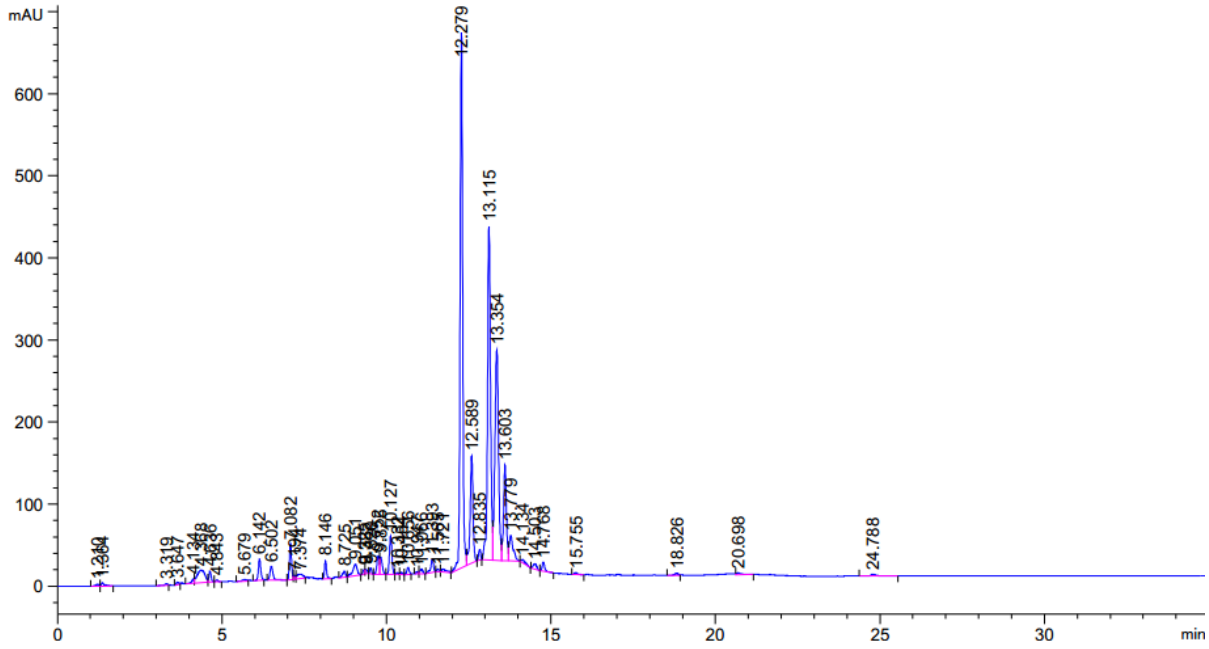


Figure 3.21: HPLC chromatogram of *P. glaucus* fruit ethanol extract at 210 nm.

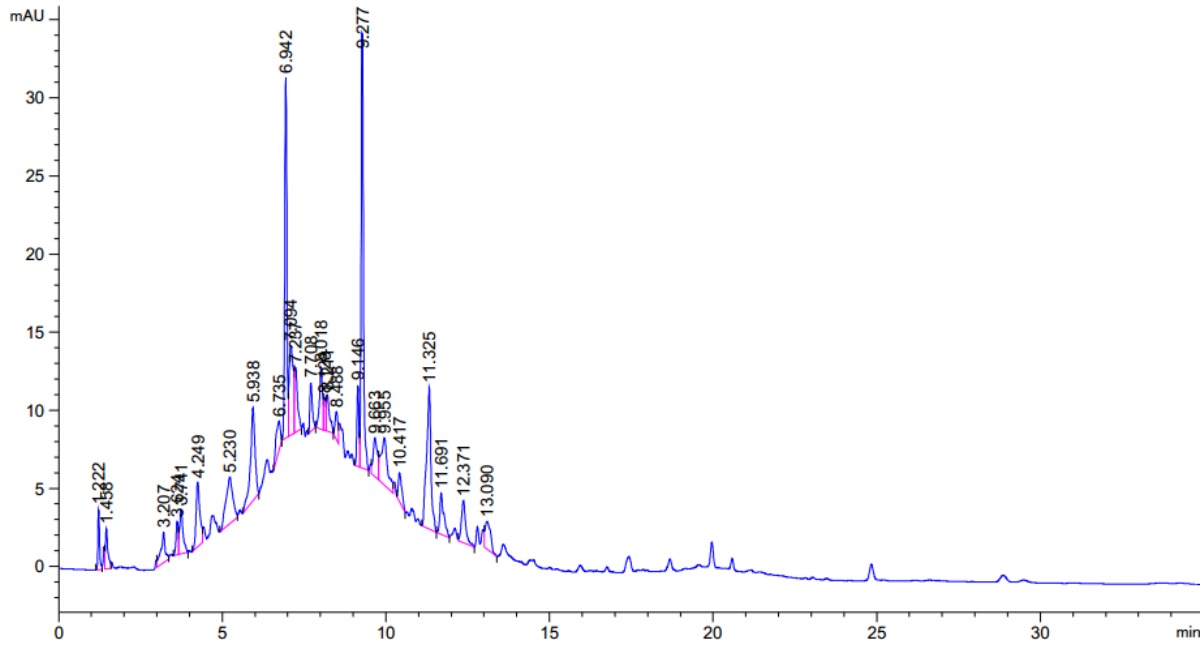


Figure 3.22: HPLC chromatogram of *P. glaucus* stem ethanol extract at 280 nm.

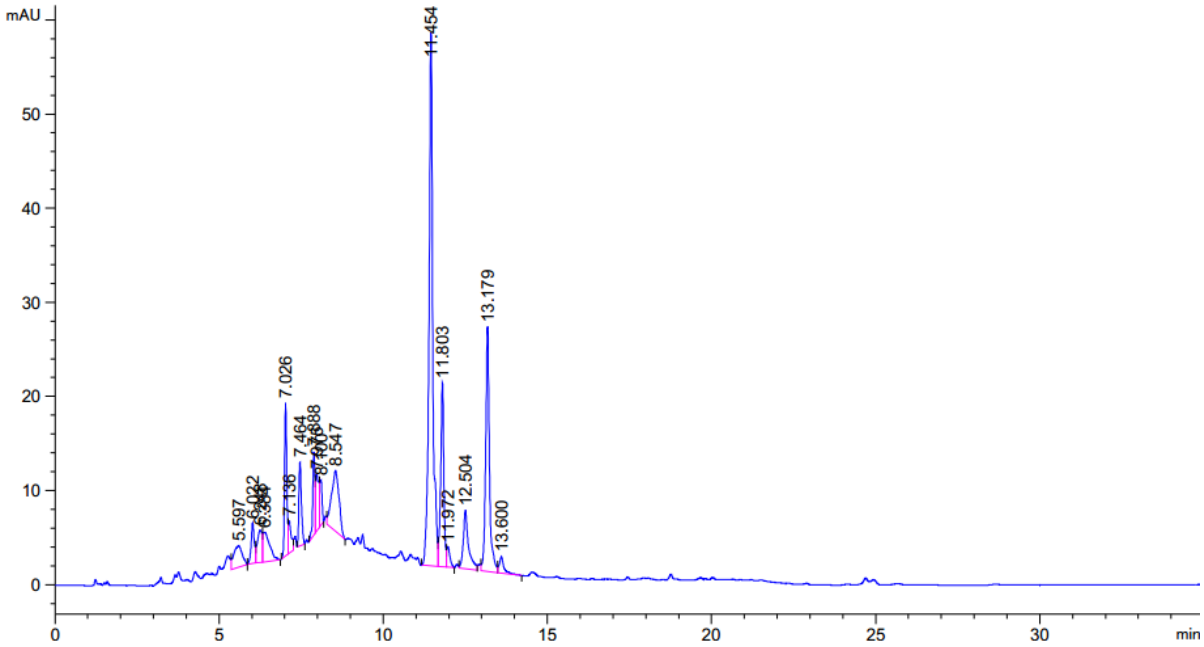


Figure 3.23: HPLC chromatogram of *P. glaucus* leaf ethanol extract at 280 nm.

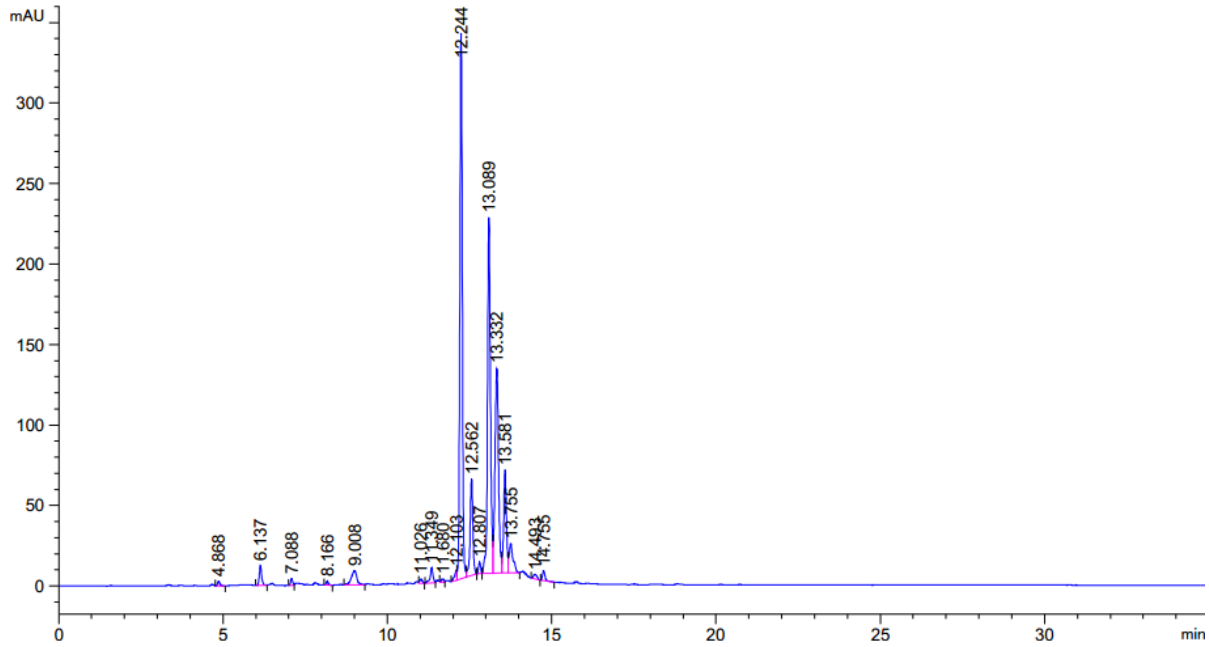


Figure 3.24: HPLC chromatogram of *P. glaucus* fruit ethanol extract at 280 nm.

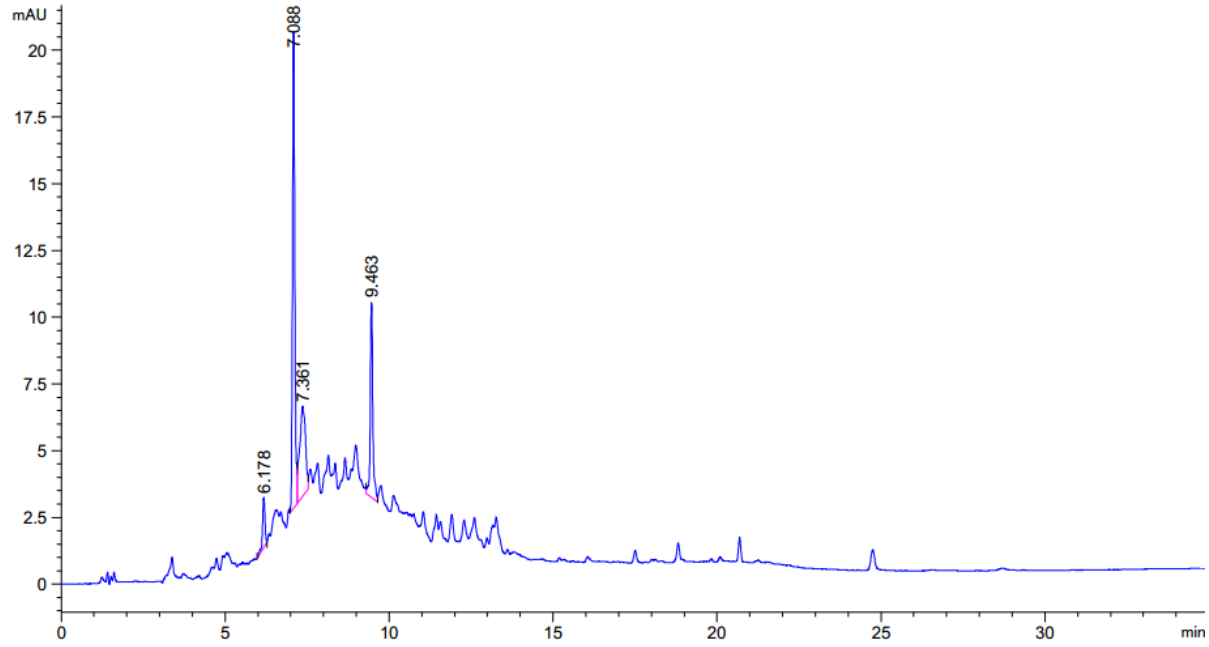


Figure 3.25: HPLC chromatogram of *P. glaucus* root ethanol extract at 280 nm.

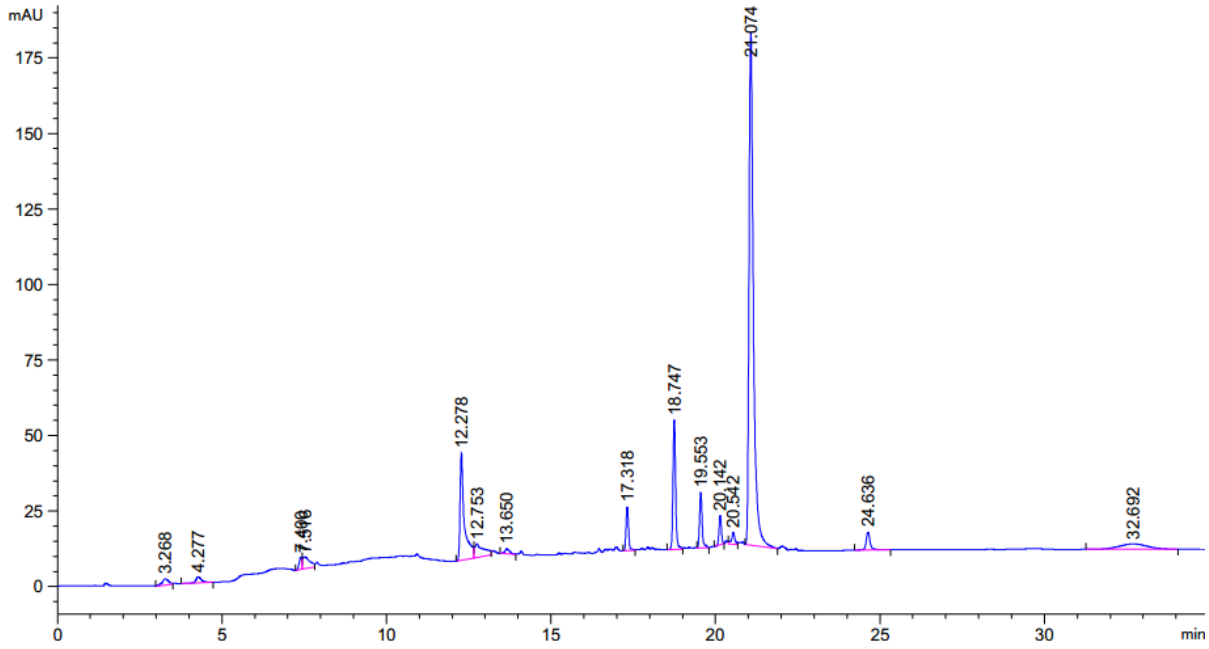


Figure 3.26: HPLC chromatogram of betulinic acid at 210 nm.

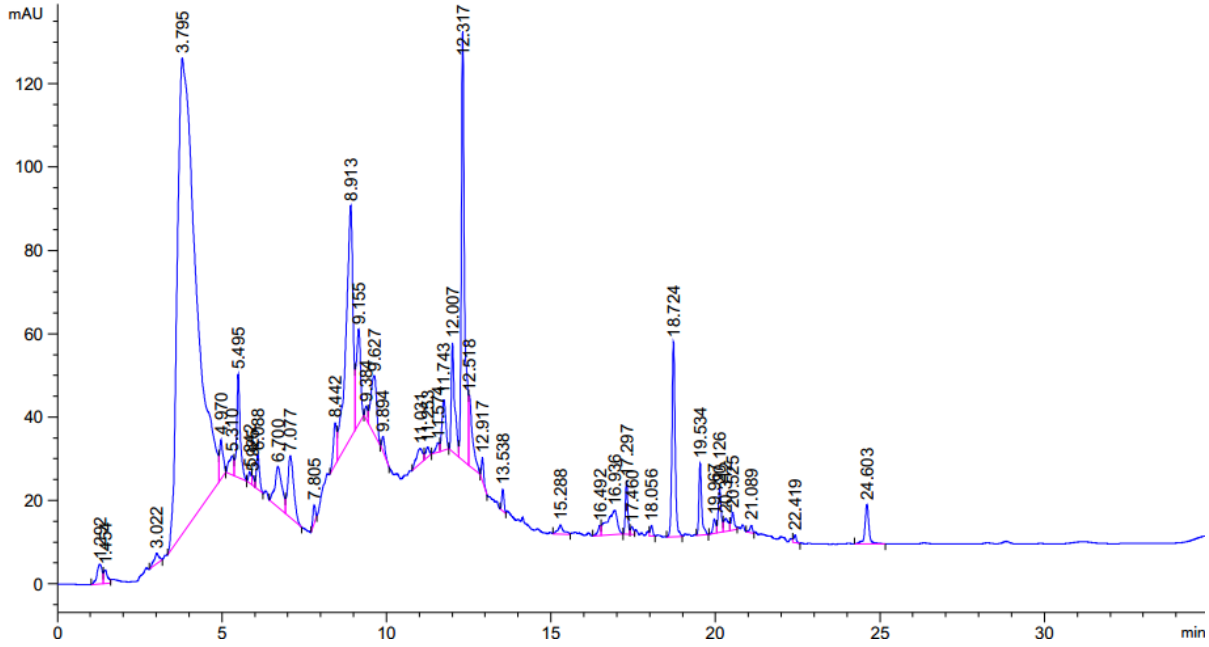


Figure 3.27: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 210 nm.

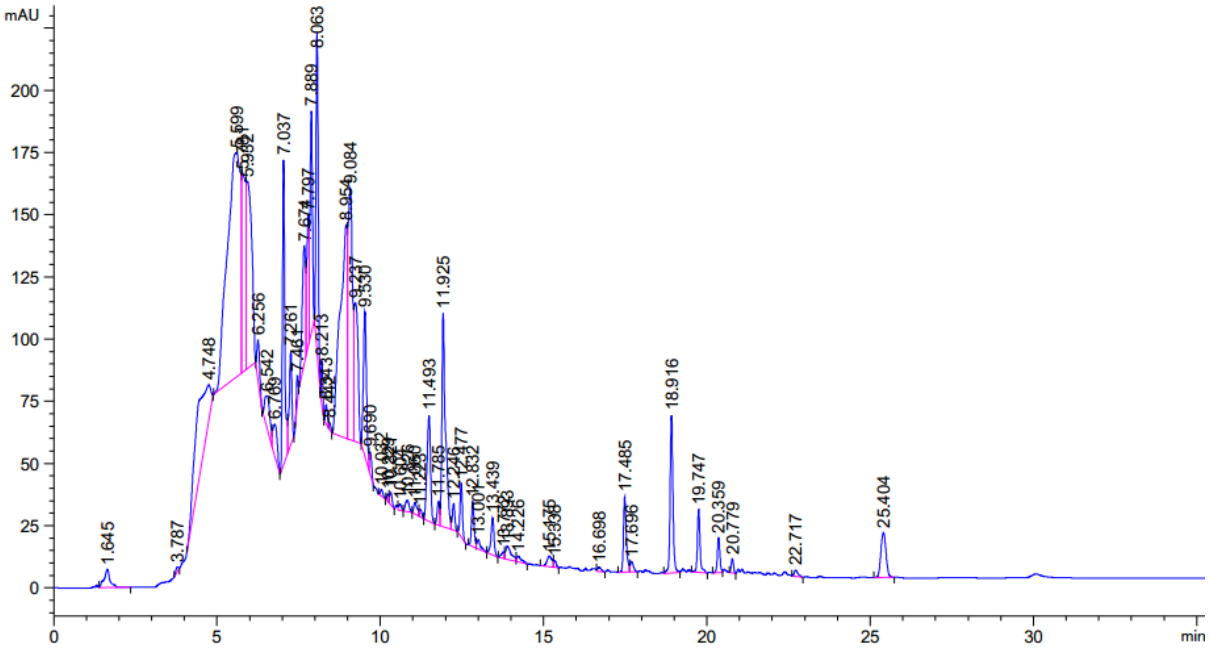


Figure 3.28: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 230 nm.

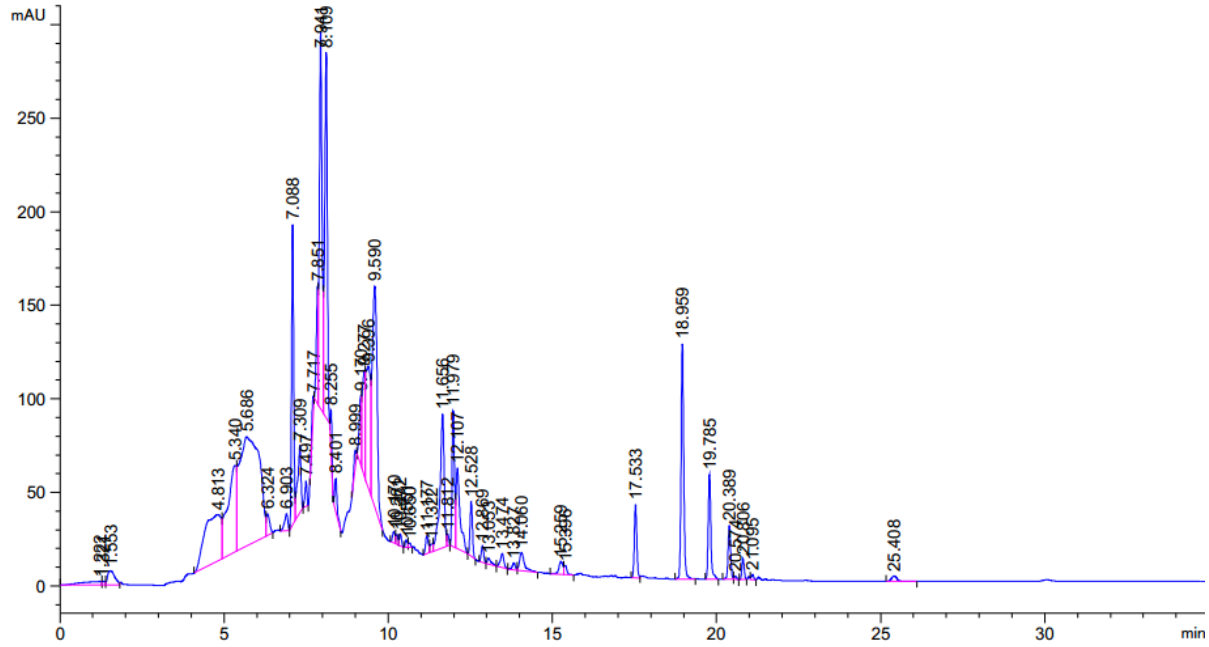


Figure 3.29: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 254 nm.

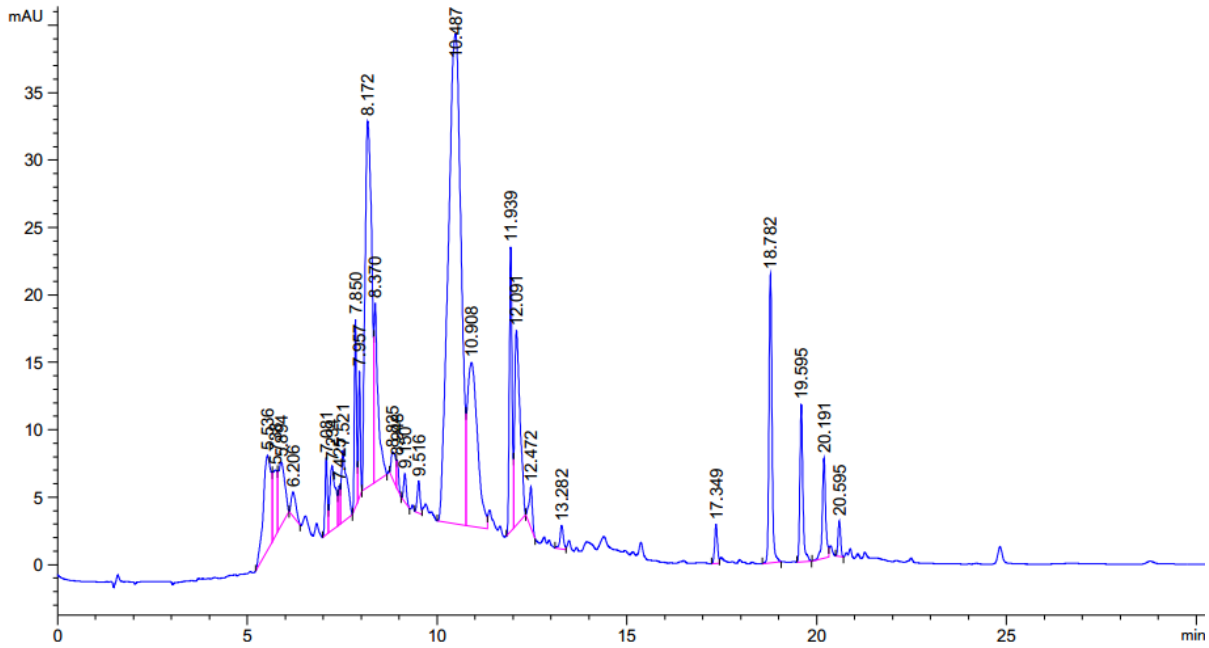


Figure 3.30: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 274 nm.

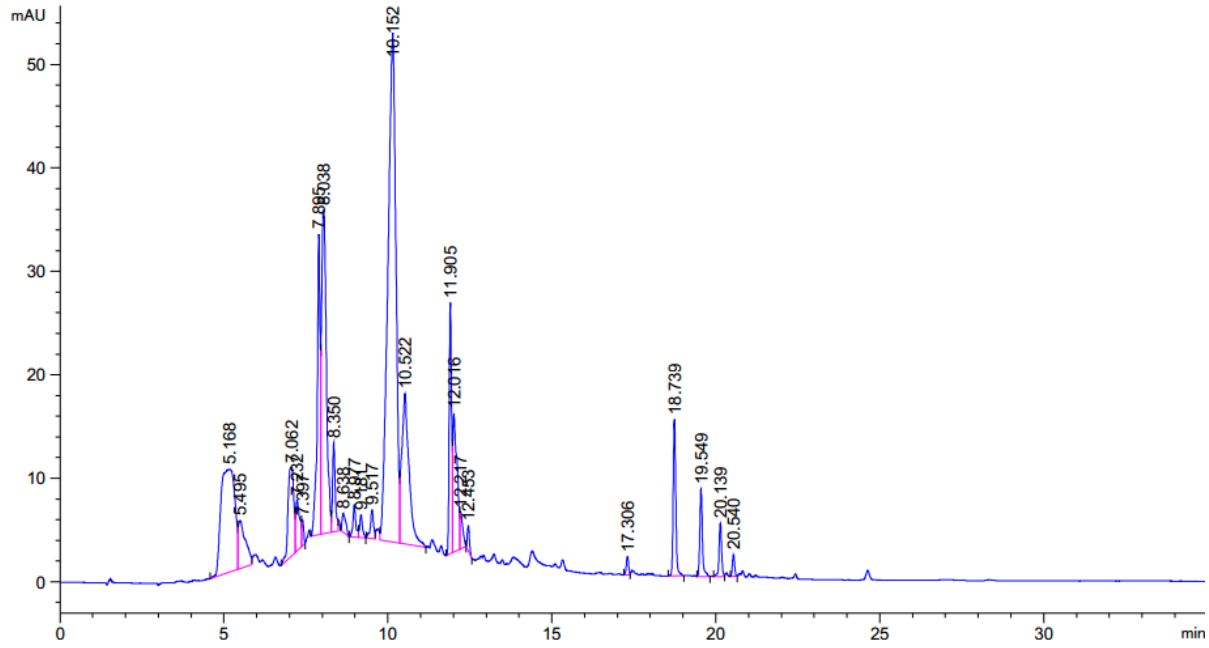


Figure 3.31: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 280 nm.

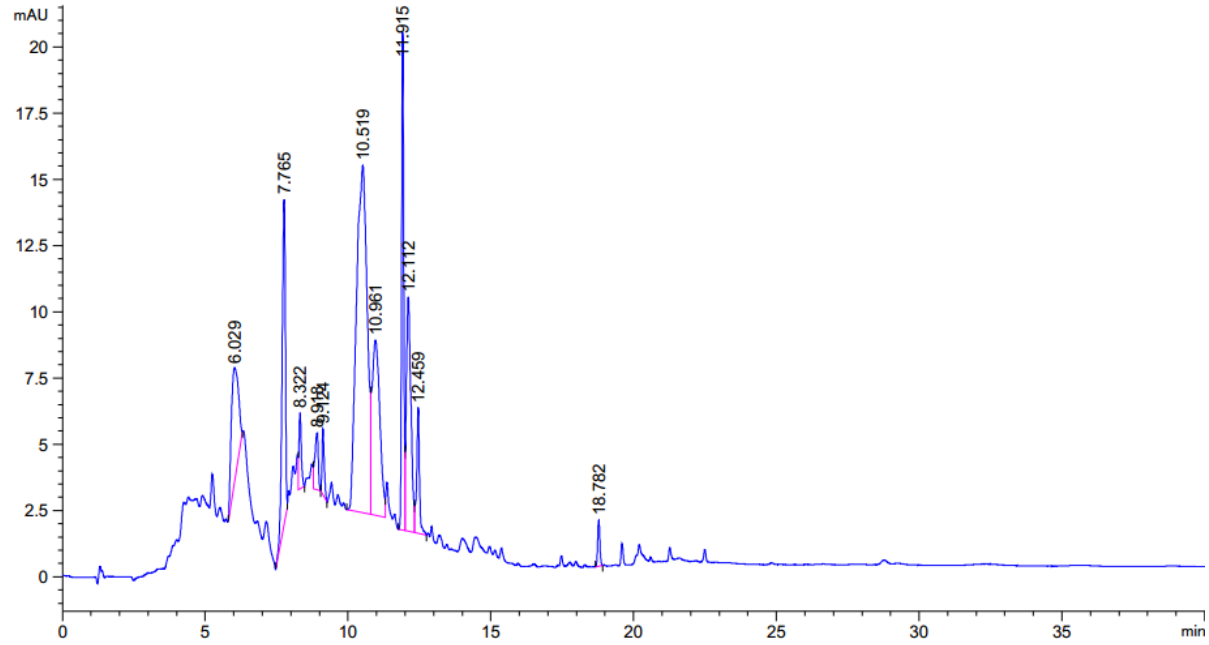


Figure 3.32: HPLC chromatogram of *P. glaucus* stem alkaloid extract at 293 nm.

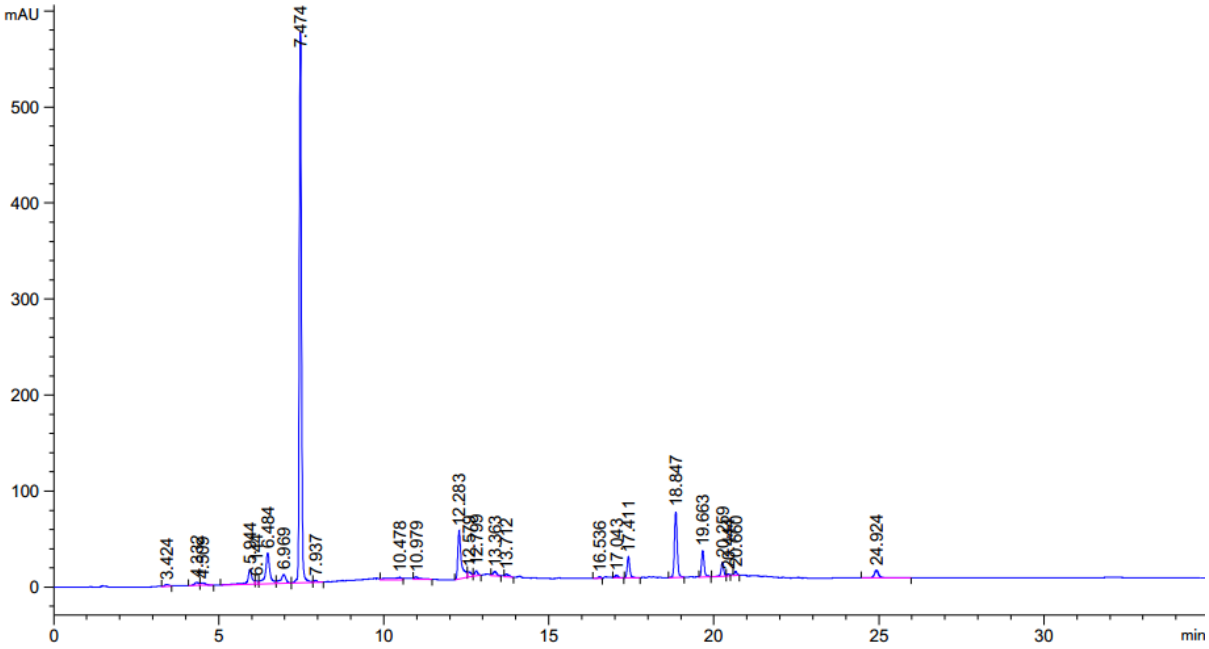


Figure 3.33: HPLC chromatogram of periglaucine A at 210 nm.

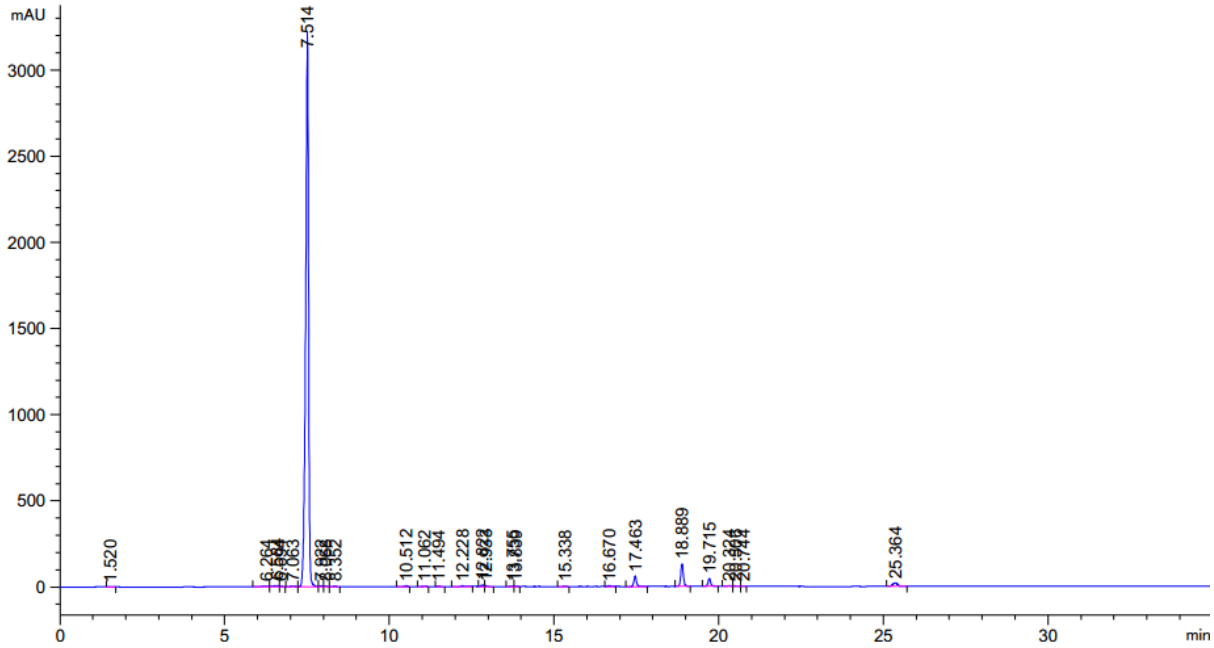


Figure 3.34: HPLC chromatogram of periglaucine A at 230 nm.

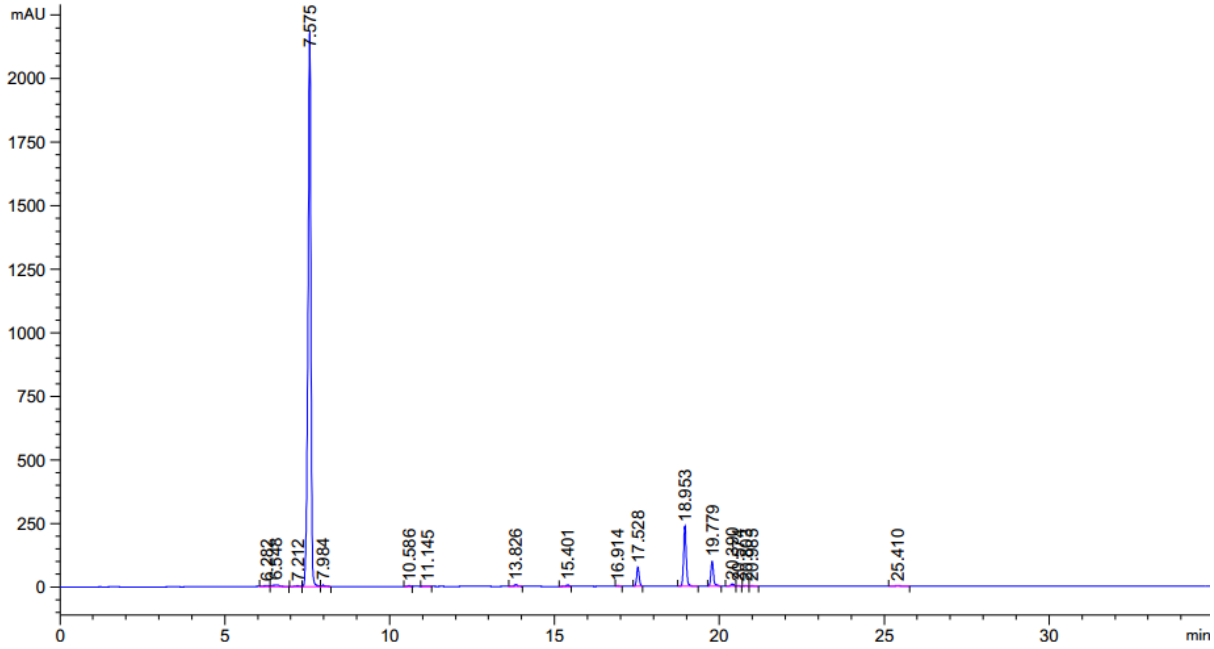


Figure 3.35: HPLC chromatogram of periglaucine A at 254 nm.

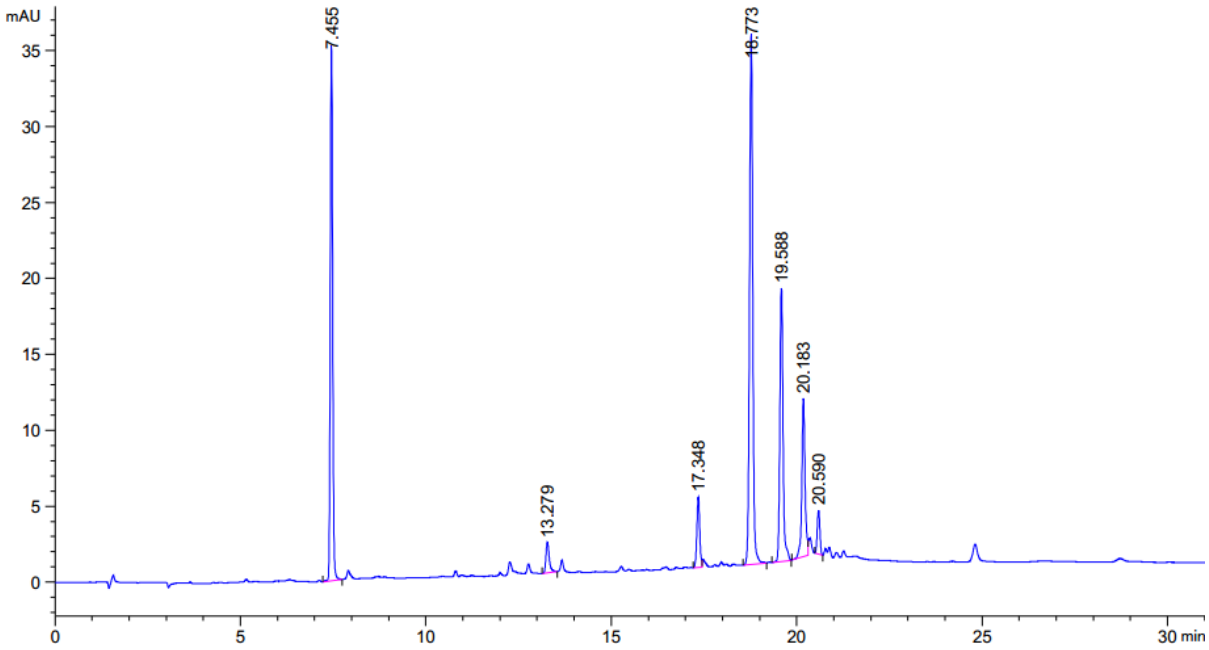


Figure 3.36: HPLC chromatogram of periglaucine A at 274 nm.

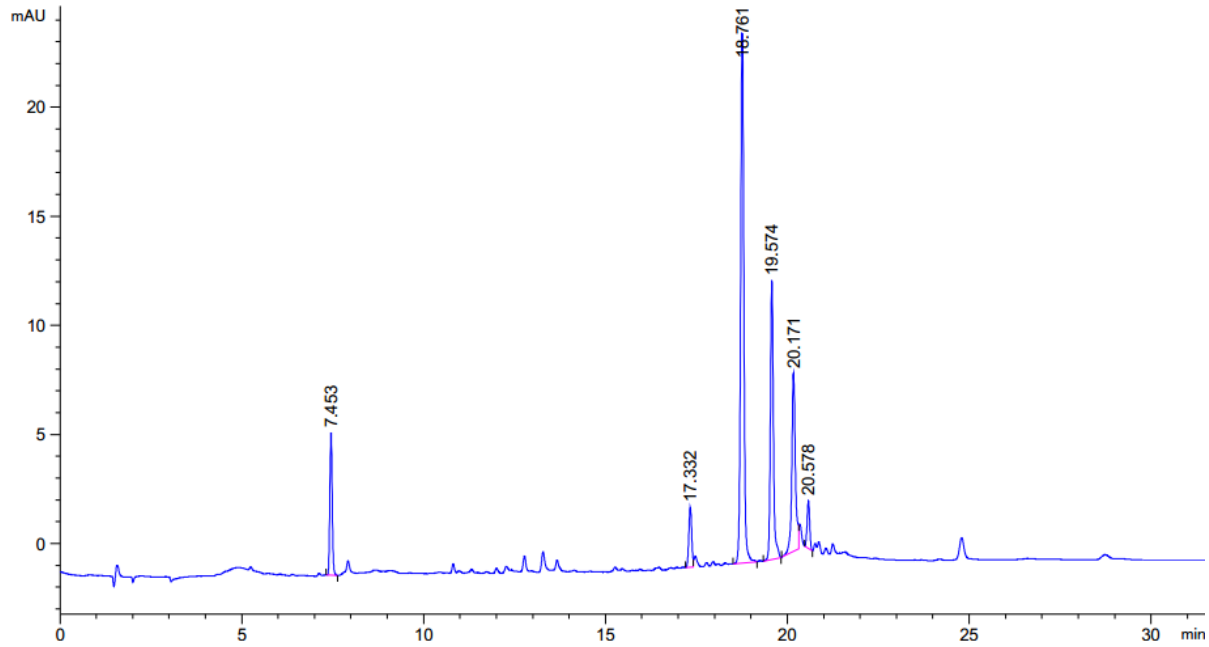


Figure 3.37: HPLC chromatogram of periglaucine A at 280 nm.

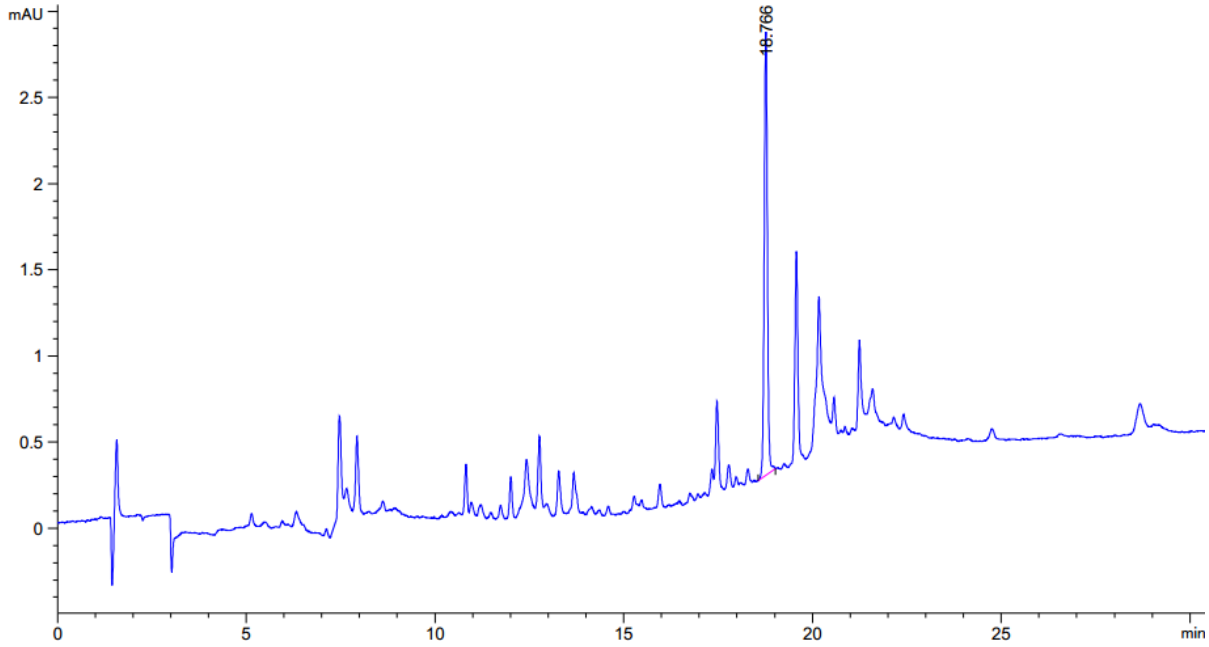


Figure 3.38: HPLC chromatogram of periglaucine A at 293 nm.

3.4: Conclusion

The ethanol extracts gave the greatest yield, as such, most of the compounds extracted were relatively polar. The phytochemical analysis revealed the presence of many types of compounds although the results of the TLC analysis and the direct analysis of the extracts gave conflicting results with regards to the flavonoid, tannin and polyphenol content as these compounds did not show up by direct analysis but were positively detected when stained after separation by TLC. This may be due to an error in the experiment, as it is unlikely that a plant would not contain any polyphenols. This error may have arisen due to an insufficient quantity of polyphenol being present in the amount of extract used to cause precipitation of the gelatin. Altering the concentration of polyphenol relative to protein, and *vice versa*, can lead to different degrees of precipitation of the protein, for example, a small quantity of polyphenol may be required to cause precipitation of a solution containing a high concentration of protein while a relatively larger ratio of polyphenol to protein is required to cause precipitation of a low concentration of protein solution (Haslam 1989). As such the use of a greater quantity of extract may have yielded different results. The HPLC analysis displayed distinct similarities and differences between the extracts with some or the large peaks being identifiable in a number of extracts, particularly in the stem, leaf and root extracts. Peaks were also found at similar elution times as betulinic acid for all the extracts. Periglaucine A may also be present in the leaf chloroform extract as a distinct peak was observed at the same elution time at 210 nm. These results allow the general phytochemical composition of the extracts and their chemical similarities to be better understood which may help further when analysing results from assays involving the extracts.

Chapter 4: The antioxidant activity of *P. glaucus*

4.1: Introduction

We are constantly being exposed to and creating free radicals, so a healthy balance between free radicals and antioxidants is required to maintain proper physiological function. Oxidative stress occurs when there is an excess of free radicals which cannot be detoxified. Oxidation can be described as the loss of an electron, while reduction is the gain of an electron. It is a well known fact that oxidative damage is capable of causing problems, such as contributing to the development of cancer, rheumatoid arthritis, neurodegenerative diseases, cardiovascular disease and aging, among others (Pham-Huy et al. 2008). Antioxidants are able to interact with oxidative compounds, neutralising them and thus protecting an organism from oxidative damage. Since the deleterious effects of oxidants and radicals are well known, as is the ability of antioxidants to quench their activity, they are thought, by some, to be beneficial to health by directly neutralising the activity of oxidants.

4.1.1: The mechanism of action

Antioxidants have been touted as being health promoting in a vast number of ways, able to neutralise free radicals and reverse the damage done by them, which has many implications in health.

In most cases, free radicals are either an atom, molecule or ion that has unpaired valence electrons, making them particularly reactive. When they react with another molecule, they target a double bond, using up one of the electrons and altering the molecule by bonding with it. This can sometimes lead to the formation of another free radical, allowing further reactions to occur. When free radicals react with proteins and DNA, this can lead to dysfunction (Riley 1994).

Antioxidants are molecules that give an electron to a free radical. Once this transfer of electrons is complete, the valence electrons are paired and the free radical loses its reactivity, no further radicals are generated from this reaction (Riley 1994).

4.1.2: Naturally produced antioxidants

A number of synthetic antioxidants exist, such as butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), metabisulphite and ethoxyquin. Some studies have found these to exhibit toxicity while others have found them to be acceptable (EFSA 2011; Williams et al. 1999; Akanji et al. 1993; Wattenberg 1974; Nair & Elmore 2003; Manson et al. 1992; Branen 1975). Instead, other antioxidants are more confidently used in foods.

Plants are a source of a wide variety of antioxidants, the phenolic and polyphenolic compounds being one of the main types of compounds responsible for this activity. Ascorbic acid, tocotrienols and tocopherols are essential vitamins with antioxidant activity that are also found in plants. Some studies have linked the intake of these antioxidants to the prevention and improvement of certain diseases, while others have disproved these claims. The benefit of antioxidants in disease management is subject to some dispute (Hajhashemi et al. 2010).
Plants are generally thought to produce polyphenols, such as tannins, as a defence against herbivores, pathogens, to provide pigmentation and regulate certain processes. When eaten by herbivores, the polyphenols in some plants are potent enough to deactivate digestive enzymes and reduce the nutritional value of the plant, making it a less appetising choice of food. Some of the polyphenols from plants are therefore not always beneficial when consumed. The consumption of tannins can also affect iron bioavailability, thus excessive consumption of tannins can lead to anaemia (Haslam 1989; Bagchi et al. 2000).

Plants are not the only source of antioxidants and humans have other means by which to manage the presence of potentially harmful free-radicals. The body is capable of producing its own antioxidants which are able to regulate the level of oxidants. Glutathione, glutathione peroxidase, catalase and superoxide dismutase (SOD) are examples of molecules produced by the body that neutralise oxidants. Albumin, transferin and ceruplasmin help to regulate the production of free radicals catalysed by metal ions (Kalyanaraman 2013). The antioxidant enzymes play a crucial role in the body. Mutation of glutathione peroxidase 4 causes Sedaghatian-type spondylometaphyseal dysplasia (Smith et al. 2014), mutations in the gene encoding catalase can lead to acatalasemia (Kishimoto et al. 1992), mutations in superoxide dismutase 1 (SOD1) are linked to amyotrophic lateral sclerosis (ALS) (Rosen et al. 1993). It is clear from these examples that the correct functioning and expression of antioxidant enzymes is essential in order for proper functioning.

4.1.3: Antioxidant supplements

With the recognition of the preservative action of antioxidants, many people have made an attempt to preserve or improve their current physiological state with the use of antioxidants in the form of supplements and cosmetics. Some of the essential vitamins required by humans have antioxidant activity and are provided as supplements and incorporated into cosmetics. Supplementation has been found to have health promoting effects in some studies, possibly helping in treating or preventing age related diseases (Wojtunik-Kulesza et al. 2016; Kumar & Singh 2015). It has been recognised that although oxidative damage contributes to the progression of a number of diseases, supplementation of antioxidants may not necessarily be beneficial in treating these diseases (Goszcz et al. 2015; Glasauer & Chandel 2014). Some researchers have found that antioxidants may increase the risk of developing certain types of cancers, possibly by protecting cancer cells from damage (Albanes et al. 1996; Omenn et al. 1996).

4.1.4: Adverse effects of antioxidants

Some studies have found that antioxidant supplements may increase the risk of developing cancer, reduce the benefits of exercise or offer no protection or relief from disease. Taking high doses of antioxidants can cause harm by acting as pro-oxidants.

In the Fenton reaction, iron(II) and hydrogen peroxide oxidise organic molecules. This process is intended to remove organic pollutants, but also generates a hydroxyl radical and a hydroxide ion. The process involves the oxidation of iron(II) to iron(III). Vitamin C (ascorbic acid), when taken in high doses, was found to recycle iron in the Fenton reaction, converting iron(III) back into iron(II), promoting the production of reactive oxygen species (Fisher & Naughton 2003).

4.1.5: Practical use of antioxidants

Antioxidants are consumed in our everyday diet and in supplement form, however they are also used for other applications. They are used as preservatives in our foods, drugs and cosmetics, they are also used in the oil/petrochemical industry to prevent the degradation of rubber and gasoline.

When taken as a supplement, the effect of antioxidants is not fully known and studies have found conflicting results. When taken as part of a normal, healthy diet, they are generally found to have a beneficial effect on health (Burton-Freeman & Sesso 2014). Their use in food preservation is undeniably beneficial as they are able to preserve the oxidisable components of a food and extend its shelf life (Zeuthen & Bøgh-Sørensen 2003).

It is also important to recognise that many polyphenols possess multiple biological activities and may be beneficial to health in ways other than by simply acting as antioxidants (Haslam 1989).

4.1.6: Methods of measuring antioxidant activity

The antioxidant activity of a compound can be measured by a variety of methods, such as the DPPH radical scavenging assay, ferric reducing ability of plasma (FRAP) assay (Benzie & Strain 1998), β-carotene bleaching assay , oxygen radical absorbance capacity (ORAC) assay, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method. The total phenolic content is determined using Folin-Ciocalteu reagent (Singleton et al. 1998).

Antioxidant assays generally work in two different ways to measure antioxidant activity, there are those that involve a hydrogen atom transfer (HAT) reaction, and those that involve a single electron transfer (ET) reaction. The Folin-Ciocalteu reagent, FRAP, ABTS and DPPH assays involve a redox reaction with an oxidant, these are ET reactions, where the reducing ability of the antioxidant is directly related to its antioxidant potential. The β-carotene bleaching and ORAC assays involve a free radical generator, a probe which can be monitored for oxidisation and an antioxidant, and their combination results in a HAT reaction where the hydrogen atom donating capacity of the antioxidant can be measured and related to its ability to break the radical chain reaction (MacDonald-Wicks et al. 2006). Although the DPPH assay involves electron transfer, DPPH may also be neutralised by a HAT reaction (Prior et al. 2005). Since both SET and HAT reactions could take place to neutralise the DPPH radical, the DPPH radical scavenging assay was adopted to measure the combined occurrence of both of these types of reaction.

The different assays produce different results when compared with each other and their biological relevance is debatable, as such, a combination of carefully chosen *in vitro* and *in*

vivo methods are often perceived as being more reliable in determining the biological relevance of an antioxidant (Frankel & Meyer 2000).

The present study adopts the DPPH radical scavenging assay and uses the Folin-Ciocalteu reagent to determine antioxidant activity and total phenolic content respectively.

4.2: Materials and methods

4.2.1: Chemicals and reagents

DPPH was purchased from Sigma Aldrich, methanol and DMSO were purchased from RCI Labscan (Thailand), Folin-Ciocalteu reagent, gallic acid and sodium carbonate were purchased from R & M Chemicals (UK).

4.2.2: DPPH radical scavenging assay

The method described by Alothman et al. (2009) was adapted to suit this experiment. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in methanol to make a 0.5 mM solution. 160 µL of the sample was added to 40 µL of DPPH solution in a 96 well plate. The colour correction wells contained 160 µL of the sample and 10 µL methanol and the blank wells contained 160 µL DMSO and 40 µL methanol. The negative control wells contained 160 µL DMSO and 40 µL DPPH solution. The plate was shaken for 2 min, incubated at 37 °C for 30 min before absorbance was read at 513 nm. Each experiment was repeated at least 3 times.

4.2.3: Total phenolic content (TPC)

The total polyphenol content was determined using an adapted form of the protocol as described by Singleton & Rossi (1965). Briefly, Folin-Ciocalteu (FC) reagent was diluted with water in a ratio of 1:10 (v/v). Samples were prepared in DMSO at a concentration of 10 mg/mL and gallic acid was prepared in various concentrations to be used as the standard. 20 µL of each sample was pipetted into separate wells of a 96 well plate. 100 µL of FC reagent was added to each well and the solution was left for 10 min. 80 µL of a 10% sodium carbonate solution was then added to each well and left for 30 min. After this incubation period, the plate was read at 743 nm. The TPC was expressed as gallic acid equivalents

(GAE) in 10 mg of extract. The concentration of polyphenols in the extracts was derived from a standard curve of gallic acid which ranged from 0.25 to 4.0 mg.

4.3: Results

4.3.1: DPPH radical scavenging assay

The results of the DPPH radical scavenging assay, as shown in **Table 4.1** and **Figures C1-5** in **Appendix C** show that the hexane extracts of *P. glaucus* had the lowest radical scavenging activities, while the chloroform extracts generally had the highest radical scavenging activities. Compared to the gallic acid standard, the radical scavenging activity of the extracts at the same concentrations was much lower. The samples with the lowest IC50s were the fruit chloroform extract (IC_{50} = 198.46 µg/mL) and the stem chloroform extract (IC_{50} = 201.07 µg/mL) as shown in **Table 4.1**.

Table 4.1: DPPH Radical Scavenging IC_{50} for *P. glaucus* extracts and gallic acid

Sample	DPPH Radical scavenging IC_{50} (µg/mL)
Stem Hexane	>800
Stem Chloroform	201.07
Stem Ethanol	985.38
Leaf Hexane	1063.92
Leaf Chloroform	492.58
Leaf Ethanol	484.71
Root Hexane	>800
Root Chloroform	519.13
Root Ethanol	>800
Fruit Hexane	>800
Fruit Chloroform	198.46
Fruit Ethanol	312.69
Gallic acid	0.92

4.3.2: Total phenolic content

The TPC was determined using the absorbance results of a gallic acid standard curve. The results of the TPC test are shown in **Table 4.2**. From the results, the chloroform extracts were found to have the greatest phenolic content of each plant part, followed by the chloroform extracts. The hexane extracts had the lowest phenolic content of all plant parts.

Table 4.2: TPC of each extract

Sample (at 10mg /ml)	Absorbance at 743nm	TPC (mg GAE/10mg extract)
Stem hexane	0.1	0.01
Stem chloroform	0.665	0.11
Stem ethanol	0.362	0.06
Leaf hexane	0.1	0.01
Leaf chloroform	0.539	0.09
Leaf ethanol	0.397	0.06
Root hexane	0.047	0.01
Root chloroform	0.37	0.06
Root ethanol	0.221	0.03
Fruit hexane	0.023	0
Fruit chloroform	1.083	0.17
Fruit ethanol	0.736	0.12

4.4: Conclusion

The results show that the chloroform extracts tended to exhibit greater antioxidant activity in the DPPH radical scavenging assay and the total phenolic content was highest in the chloroform extracts of each plant part. The total phenolic content of the extracts can be directly correlated with the DPPH radical scavenging activity. The hexane extracts could possibly contain more hydrophobic antioxidants such as carotenoids, while the ethanol extracts could contain more polar antioxidants such as ascorbic acid and the chloroform extract would contain medium polarity antioxidants. A study by Ikorska et al. 2010 found that, when comparing the antioxidant activity of flavonoids of different polarity, the most polar showed the greatest antioxidant activity. Another study found that the total phenolic content of the Makrut lime could be negatively correlated with the IC_{50} from the DPPH radical scavenging assay (Fidrianny & Johan 2015), which demonstrates that antioxidant activity may not be simply due to the quantity of phenolics present but may also be due to the potency of the components. Since the stem chloroform extract and stem ethanol extracts contained 11 times and 6 times the GAE of antioxidants, respectively, compared to the stem hexane extract, this shows that antioxidants in each of the extracts are of similar potency as the radical scavenging activity for the chloroform and ethanol extracts at the same concentration of 200 µg/mL are 5.9 and 12.9 times higher than that of the hexane extract, respectively, but at concentrations above and below 200 µg/mL, these ratios change greatly, as such, the ability of the extracts to scavenge free radicals relative to each other changes depending on the concentration and relative potency cannot be used to accurately determine how much more effective an extract is at scavenging radicals compared to other extracts, although the chloroform extract exhibited stronger radical scavenging activity not only due to the higher phenolic content but was also found to have stronger radical scavenging activity after comparing the ratio of phenols to radical scavenging activity at different concentrations (**Appendix C**). The type of antioxidant test may also produce different antioxidant activities as the different techniques measure antioxidant activity based on different mechanisms (Ioannou et al. 2015). In order to get a clearer picture of the antioxidant activity of a sample, ideally, a number of different assays that work using different principles should be adopted.

Chapter 5: The antibacterial activity of *P. glaucus*

5.1: Introduction

Bacteria are single cell organisms that are usually only a few micrometers long and can come in a variety of shapes, such as oval, elongated or partly flattened cocci which may form bunches or chains, rod shaped bacilli or coccobacilli which may form pairs or chains and spiral shaped bacteria which may appear as curved rods or spirals with many loops.

Bacteria can be classed as Gram negative of Gram positive and are differentiated by Gram staining. Gram negative bacteria generally have a thin peptidoglycan cell wall surrounded by an outer membrane and when stained, they are unable to retain the crystal violet stain during the de-staining process as it is easily washed from their thin cell walls. Gram positive bacteria have a thicker cell wall which is retains the crystal violet stain during the Gram staining procedure (Holt et al. 1994). A more accurate way of identifying bacteria, especially when distinguishing strains of bacteria, is through genetic testing such as PCR (Barghouthi 2011).
Bacteria live in soil, fresh and marine waters, on plants and inside animals as well as in many extreme environments which are inhospitable to most other organisms. They play an important role in the decomposition of organic matter and are beneficial to some hosts and essential to others. Bacteria in the soil can provide plants with nutrients and protect them from harmful pathogens (Hayat et al. 2010; van der Heijden et al. 2015). Bacteria found inside the human gut can break down indigestible components in the diet, making the products of their decomposition absorbable by the host (Flint et al. 2012), they can also suppress the growth of harmful bacteria and produce vitamins, hence the use of some bacteria in human foods became popular (Tamang et al. 2016).

5.1.1: Pathogenic bacteria

Although many bacteria provide organisms and ecosystems with indispensible benefits, some can be harmful to other organisms. Bacteria that form a parasitic relationship with a host organism are regarded as pathogenic bacteria.

Pathogenic bacteria cause infections and are responsible for a number of diseases that cause millions of deaths worldwide, every year. Some of the diseases bacteria are responsible for include pneumonia, which can be caused by *Psuedomonas* spp. and *Streptococcus* spp.,

tetanus, caused by *Clostridium tetani*, food poisoning, which can be caused by a large number of different bacteria including *Salmonella* spp., certain strains of *Escherichia coli* and *Campylobacter jejuni*, and *Staphylococcus* spp. and *Streptococcus* spp. are the causes of many skin infections. Bacterial infections may lead on to other diseases, for example, *Helicobacter pylori* infection in the stomach has been linked to the development of cancer (Backert et al. 2016).

Different bacteria have a tendency to affect particular parts of the body, they may also be present without causing any adverse symptoms (Belizário & Napolitano 2015).

5.1.2: Bacterial induced cytotoxicity

There are a number of ways bacteria can cause harm to their host. Damage can be caused by growth and replication of bacteria inside a host cell, causing it to rupture or by physical damage as bacteria enter and exit a host cell, they can also attach to a host cell, sequestering nutrients from that cell and excreting toxic byproducts (Janeway et al. 2001). Many bacteria produce toxins such as endotoxins, exotoxins and siderophores. Endotoxins are lipopolysaccharides that provide structural support to the outer membrane of bacteria, but are released when the bacterial cell dies and can produce an immune response in the host organism. Exotoxins are produced by bacteria as part of their normal metabolism, but these toxins are released when a bacterial cell lyses and can cause damage to the surrounding tissues. Some toxins, such as hyaluronidase, are secreted to ease the penetration of bacteria through the tissues of the host. Siderophores are iron chelators, used by bacteria to bind iron from the host so that it may be used for metabolism by the bacteria (Girish et al. 2009; Mossialos & Amoutzias 2009; Ramachandran 2014). These harmful effects can cause the discomfort experienced with a bacterial infection, but are sometimes serious enough to cause significant tissue damage or death.

5.1.3: Antibiotics

Historically, plants and fungi were used to treat bacterial infections and, since the 1600's, the use of mould for treating infections was recommended and explored. In the 1900's, Sir Alexander Fleming discovered penicillin from *Penicillium notatum* and this allowed the effective treatment of bacterial infections (Wainwright 2012). Later, streptomycin, chloramphenicol and tetracycline were discovered (Torok et al. 2009).

Antibiotics are compounds that are able to either halt the growth of bacteria (bacteriostatic) by interfering with protein production, DNA replication or metabolism, or kill bacteria (bactericidal) by interfering with the synthesis of the bacterial cell wall or its components, some act against a wide range of bacteria (broad spetrum), while others only work against a few (narrow spectrum) (Torok et al. 2009). If they can retain their activity after passing through the digestive tract, they can be given orally, otherwise they are administered intravenously, intramuscularly or topically. There are different classes of antibiotics that work in different ways to tackle a bacterial infection (Neu & Gootz 1996).

The β-lactam antibiotics include penicillins, cephalosporins, monobactams and carbaphenems. All of these antibiotics contain a β-lactam ring and act by inhibiting cell wall synthesis. Other antibiotics, such as the sufonamides and quinolones, interfere with the activity of enzymes produced by bacteria. Interference of protein synthesis by tetracyclins and disruption of the cell membrane by polymyxins are also effective ways to control a bacterial infection. Disruption of any of these bacterial functions can halt growth or cause death of the bacteria, however, bacteria are adaptable and some have developed ways of resisting the effects of antibiotics (Neu & Gootz 1996).

5.1.4: Antimicrobial resistance (AMR)

Antibiotics do not work against all bacteria and are usually effective against a certain group of bacteria. Some bacteria are naturally resistant to certain types of antibiotic, however, resistance can also be acquired (Torok et al. 2009).

Antibiotic resistance is acquired by bacteria after they are exposed to an antibiotic agent. Although the antibiotic may be able to kill all of the bacteria at a certain dose and over a certain period of time, if any of the bacteria are able to survive exposure to these chemicals, there is a chance that they may find a way to adapt and thus increase their chances of survival. It is thought that the overuse and misuse of antibiotics by people is one of the major causes of antibiotic resistance (Vanden Eng et al. 2003), however, considering the majority of antibiotics that are manufactured are given to livestock to treat sickness, to prevent sickness and to promote growth, there is reason to believe that agriculture may be more to blame for the development of antibiotic resistant strains than the general public's consumption of antibiotics (Morris et al. 2016).

Bacteria can achieve resistance to an antibiotic by altering the permeability of their cell walls and by using drug (efflux) pumps, altering the structures that antibiotics target or obtain resistance genes via bacterial conjugation or scavenging of DNA (Davies 1994; Munita et al. 2015; Collu & Cascella 2013). They may also produce enzymes that break down the antibiotic, one example of this is the resistance of bacteria to β-lactam antibiotics by synthesising β-lactamase, which targets the β-lactam ring, rendering the antibiotic inactive. This particular mechanism of resistance can be counteracted with the use of β-lactamase inhibitors (Maiti et al. 1998).

Antibiotic resistant bacteria are difficult to manage and in cases of antibiotic resistance, antibiotics can facilitate infection by the drug resistant bacteria by allowing opportunistic bacteria to cause infection without regulation by other microbes (Verdugo et al. 2016). Methicillin Resistant *Staphylococcus aureus* was one of the first bacteria to present a widespread problem throughout communities and hospitals (Kempe 1956; Klimek et al. 1976). It is responsible for a large number of deaths worldwide (Ventola 2015) and although new antibiotics have been developed that are effective against MRSA and there are other treatments and therapies that can treat infections by resistant bacteria, these have yet to be developed into a marketable and widely available drug (Dou et al. 2016).

5.1.5: Development of new antibiotics

Antibiotic development has made progress with new marketable antibiotics being made by synthesis, however, drug companies have been struggling to keep up with the new stains of resistant bacteria that have emerged (Dubravko Jelić & Antolović 2016). This is because researchers have worked less on creating new classes of antibiotics and more on the synthesis of new generations of antibiotics, which has led to the development of new drugs that have the same mechanism of action but may be effective against different strains of bacteria (Zuchora-Walske 2014). The fact that the production of medicines is up to businesses makes the development of new drugs more complicated than if it were simply a matter of producing an effective drug to treat a disease. Antibiotics are less profitable than many other drugs, people take them only for a short time, and only when they have an infection or are at high risk of infection, i.e. after a surgical procedure. The development of new generations of antibiotics tends to increase the cost of production due to the more complex process in synthesising them, which means that the drug will be more expensive to purchase and less

attractive to customers. The regulations involved in their approval are also complicated and as a result, very few new antibiotics have gained approval in recent years (Sukkar 2013).
Recently, a group of researchers has made progress with the discovery of lugdunin, a new class of antibiotic which was derived from human nasal *Staphylococcus lugdunensis* (Zipperer et al. 2016). This particular study has given researchers a possible new source from which they can attempt to discover new antibiotics.

5.1.6: Toxicity of antibiotics

Although antibiotics can be lifesaving, they can also do damage to our cells. Antibiotics, as discussed previously, target specific bacterial functions, so are considered generally harmless to mammals. However, the mitochondria within our cells are also speculated as being ancient bacteria that have formed a symbiotic relationship with us. This hypothesis has been strengthened by findings that antibiotics which cause the production of reactive oxygen species (ROS) not only disrupt bacterial function, but also mitochondrial function. This disruption of mitochondrial function can lead to DNA damage, damage to lipids and proteins and other signs of oxidative damage (Kalghatgi et al. 2013). Some antibiotics such as gentamicin are known to have ototoxic activity, causing hearing loss and dizziness (Sheehy 1961). Gentamicin is also particularly nephrotoxic (Bowman et al. 1977). Antibiotics can produce adverse reactions such as a rash, diarrhoea, nausea, lead to superinfection or cause an allergic reaction (Cunha 2001). Taking antibiotics also kills the flora required for normal, healthy functioning . Some people who go on a course of antibiotics will experience bloating due to the lack of certain microbes and overgrowth of others which were more resistant to the antibiotic (Dethlefsen & Relman 2011).

5.1.7: Antimicrobial compounds from plants

With the approach of the "post antibiotic era", a large number of researchers have focused their efforts towards developing new antibiotics, including plant extracts and natural compounds, against various types of bacteria and antibiotic resistant bacteria. Plants, having their own need for defence against pathogens, produce a great variety of compounds that possess antimicrobial activity.

Garlic (*Allium sativum*) has been used to treat a number of diseases historically and is still recognised as a medicinal plant. Many studies have worked on garlic and its components,

investigating their antibacterial activity, and many have found that extracts of garlic do indeed posses significant antibacterial activity (Cutler et al. 2009) and is even effective against antibiotic resistant bacteria (Tsao et al. 2007; Hannan et al. 2011; G. Li et al. 2015; Shaheen et al. 2015). One of the components of garlic, allicin, is reported as being one of the components responsible for this activity, possessing antibacterial activity (Arzanlou 2016; Ranjbar-Omid et al. 2015; Borlinghaus et al. 2014). This particular compound also has been shown to enhance the activity of standard antibiotics when used in combination with them (Cai et al. 2007).

The flower buds of *Syzygium aromaticum*, better known as cloves, are noted for their analgesic and antimicrobial activity. Studies have found that extracts of cloves have antibacterial activity, even against antibiotic resistant strains of bacteria (Akdemir Evrendilek 2015; Revati et al. 2015; Mirpour et al. 2015). Since eugenol can make up approximately 68% or more of the essential oil of cloves (Akdemir Evrendilek 2015), it considered the main component that is responsible for the observed antibacterial activity. This has been demonstrated by studies which have found that eugenol does have antibacterial activity and is active against antibiotic resistant bacteria (Dash et al. 2016).

A great number of plants that are not widely available have been traditionally used for the treatment of bacterial infections and scientific studies have supported these claims (Catteau et al. 2015; Bussmann et al. 2011). Plants have been proven to be a source of antibiotics that are effective against a variety of strains of bacteria, including antibiotic resistant strains. When used in combination with other antibiotics they can be particularly effective (Diarra et al. 2013; G. Li et al. 2015), as such, research into naturally derived compounds as a source of antibiotics is invaluable considering the current situation of antibiotics.

5.1.8: Natural products as prescription antibiotics

Antibiotics are vital medicines in the healthcare industry and are invaluable for treating infections and preventing infections after surgery. With the increase in antibiotic resistance, there is an increased demand for new antibiotics that are effective against these new, resistant strains, however, the production of new antibiotics has its limits. The main obstacle in producing antibiotics does not appear to be that they are difficult to find, but more that they are not favourable investments as mentioned in section 1.6: Businesses in medicine. Because

of these hurdles, the possibility of directly using natural products as antibiotics is unlikely and would require significant changes in the practice of drug development, manufacture and sales.

5.1.9: Techniques for testing antibacterial activity

Antibacterial activity can be tested using a variety of *in vitro* and *in vivo* methods. In the current study, antibacterial activity will be tested *in vitro*, using the disc diffusion and broth dilution methods. These tests involve exposing the cultured bacteria to different concentrations of sample and determining the antibacterial activity based on inhibition of growth.

The disc diffusion test is done using agar as the medium for bacteria to grow on. This requires that the sample is able to diffuse through the agar and inhibit the growth of bacteria in the areas it has diffused into. Since the test measures the zone of inhibition around the disc containing the sample, during analysis, it assumes that the rate of diffusion of each sample is equal. This test also favours the diffusion of polar substances through agar, as the agar is a water based jelly and is hydrophilic. Considering the fact that many non-polar compounds exert significant toxicity towards bacteria (Hammer et al. 1999), the disc diffusion may not be able to accurately demonstrate the efficacy of non-polar compounds against bacteria due to their inability to efficiently diffuse through the agar medium. This is a commonly adopted method for testing the antibacterial activity of antibiotics and a standardised version of this is the Kirby-Bauer antibiotic test (Bauer et al. 1966).

The broth dilution test involves dissolving the sample in broth that is subsequently inoculated with bacteria (Fleming 1929) and the lowest concentration that inhibits all bacterial growth is termed the Minimum Inhibitory Concentration (MIC). All sample concentrations that produce no growth can be streaked on nutrient agar to determine whether growth was only inhibited or if the bacteria were killed at a certain concentration. The lowest concentration that causes death of the bacteria is called the Minimum Bactericidal Concentration (MBC). Since this test also uses a water based medium, hydrophobic compounds are poorly soluble and their antibacterial activity may not be fully demonstrated using this method, unless an emulsion is made and bacterial growth is monitored using a colourimetric indicator (Weseler et al. 2005).

In the present study, the zone of inhibition, MIC and MBC are determined using the tests mentioned, taking into consideration the limitations of these tests. The disc diffusion test allows large numbers of samples to be quickly screened for antimicrobial activity, while the MIC and MBC provide more detailed information about the activity of the samples, such as the concentration at which they inhibit or kill bacteria. These tests are not only simple and affordable, but adopted as a popular choice for determining antibacterial activity by many labs worldwide, allowing the results from these tests to be compared and related more easily so that they may be put into context.

5.2: Materials and methods

5.2.1: Chemicals and reagents

Tryptic soy broth, Mueller-Hinton broth and Mueller-Hinton agar were purchased from Difco Laboratories (USA), glycerol was purchased from R & M Chemicals (UK), chloramphenicol was purchased from Sigma Aldrich (USA), DMSO was purchased from RCI Labscan (Thailand).

5.2.2: Bacterial strains

Six ATCC strains were used, three Gram positive bacteria, *Staphylococcus aureus* (11634), *Bacillus cereus* (1178) and *Kocuria rhizophila* (9341), and three Gram negative bacteria, *Pseudomonas aeruginosa* (10145), *Citrobacter freundii* (8090) and *Escherichia coli* (8739). A number of clinical isolates were also tested, these included *E. coli*, *P. aeruginosa*, *P. mirabilis*, *S. aureus*, Methicillin Resistant *S. aureus*, *K. pneumonia* and MDR (Multi Drug Resistant) *P. aeruginosa*.

5.2.3: Maintenance of bacteria

Bacteria were streaked on tryptic soy agar plates and grown in tryptic soy broth. For long term storage, bacteria were grown in broth to an OD of 0.4, then 1 mL of this solution was taken and mixed with a 40% glycerol solution to make a final concentration of 20% glycerol. This glycerol stock of bacteria was kept frozen at -80 °C until further use.

5.2.4: Disc diffusion test

For this assay, the extract was dissolved in a solvent and applied to sterile paper discs, 6 mm in diameter, made from No. 1 filter paper. Each disc contained 1 mg of extract and was allowed to dry overnight before being used. The bacteria were grown overnight in Mueller-Hinton broth. The optical density of the bacteria was adjusted to 0.5 before being streaked onto plates of Mueller Hinton agar using a sterile cotton swab. The paper discs containing the extracts were applied to the surface of the plates. Negative controls were a paper disc with no extract and the positive controls were discs containing chloramphenicol. Results were shown excluding the diameter of the 6 mm disc.

5.2.5: Broth dilution test

This test was used to find the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extracts.

Bacteria were freshly grown in Mueller Hinton broth (MHB) and diluted to an optical density (OD) of 0.4 using MHB. Extracts were dissolved in 100% DMSO, before diluting with MHB to make a final concentration of 2 mg/mL in 20% DMSO. 100 µL of MHB was added to each well in a 96 well plate and serial dilutions of the extract samples were made by adding 100 µL of the sample to the first well, mixing the contents of the well, then taking 100 µL of this mixture and adding it to the second well and repeating the process until 8 different concentrations were made, ranging from 1000 µg/mL to 7.8 µg/mL. Wells were also prepared with varying concentrations of DMSO, ranging from 16% to 4% DMSO to test the sensitivity of the bacteria to DMSO. Wells with only MHB were used as the negative control and chloramphenicol was used as the positive control. 10 µL of the bacteria culture was pipetted into each well except for the sterile control well containing MHB only. The plates were covered and incubated for 24 h before checking for bacterial growth. The lowest concentration of sample where no bacterial growth was visible was recorded as the MIC. All wells showing no bacterial growth were streaked onto a Mueller Hinton agar (MHA) plate and incubated overnight. The lowest concentration of a sample that showed no bacterial growth on the plate after 24 h was used recorded as the MBC.

5.3: Results

5.3.1: Disc diffusion test

The results of the disc diffusion tests are shown in **Tables 5.1 - 5.3** and **Appendix C**. At a concentration of 100 µg/disc, only the stem hexane, fruit hexane and fruit chloroform exhibited antibacterial activity and this was seen against only one of the bacteria, *S. aureus*. When the concentration was increased to 1 mg/disc, no significant increase in the zone of inhibition (ZOI) of the stem hexane extract was seen against *S. aureus*, and in some cases the ZOI was smaller at this higher concentration. This would suggest that the concentration is not a limiting factor of the antibacterial activity of the stem hexane extract and may instead be the rate of diffusion of the active components through the agar.

The stem hexane extract also showed activity against *B. cereus* and a clinical isolate of *S. aureus* at a concentration of 1 mg/disc. The stem chloroform extract showed activity against only MRSA and the zone of inhibition measured was only 0.5±0.29 mm. The stem ethanol extract was mildly active against *B. cereus*, and moderately active against *K. rhizophilla* and *C. freundii*, with 4 mm ZOIs. The leaf hexane extract showed activity against *S. aureus* and *B. cereus* with zones of inhibition of 5 mm and 4.5±0.29 mm respectively, but the chloroform extract of the leaves was found to be inactive against all strains of bacteria tested. The leaf ethanol extract was active against 5 of the bacteria and was most effective against *K. rhizophilla* with a ZOI of 8±1.15 mm. It showed moderate activity against *C. freundii* and the clinical isolate of *S. aureus* with ZOIs of 4 mm for each bacteria. None of the ZOIs produced by the extracts were comparable to the ZOIs produced by chloramphenicol, with the exception of the leaf ethanol extract against the ATTC strain of *P. aeruginosa*, which showed mild antibacterial activity with a zone of inhibition of 2 mm, compared to no ZOI produced by chloramphenicol against the same bacteria.

The chloroform extracts appeared to be the least active against bacteria, producing no ZOIs, except in the case of the fruit chloroform extract, which produced a ZOI of 4±0.58 mm against *S. aureus* at 100 µg/disc.

Table 5.1: Zones of inhibition of ATTC strains from the Disc diffusion test with100 µg/disc.

	Bacteria					
Sample	*S. aureus*	*B. cereus*	*K. rhizophilla*	*P. aeruginosa*	*C. freundii*	*E. coli*
Stem hexane	**4.5±0**	0	0	0	0	0
Stem chloroform	0	0	0	0	0	0
Stem ethanol	0	0	0	0	0	0
Leaf hexane	0	0	0	0	0	0
Leaf chloroform	0	0	0	0	0	0
Leaf ethanol	0	0	0	0	0	0
Root hexane	0	0	0	0	0	0
Root chloroform	0	0	0	0	0	0
Root ethanol	0	0	0	0	0	0
Fruit hexane	**4.5±0.29**	0	0	0	0	0
Fruit chloroform	**4±0.58**	0	0	0	0	0
Fruit ethanol	0	0	0	0	0	0
Stem alkaloid	0	0	0	0	0	0

Table 5.2: Zones of inhibition of ATTC strains from the disc diffusion test at 1 mg/disc.

	Bacteria					
Sample	*S. aureus*	*B. cereus*	*K. rhizophilla*	*P. aeruginosa*	*C. freundii*	*E. coli*
Stem hexane	**4±0.58**	**1.5±0.58**	0	0	0	0
Stem chloroform	0	0	0	0	0	0
Stem ethanol	0	**0.5±0**	**4±0**	0	**4±0**	0
Leaf hexane	**5±0**	**4.5±0.29**	0	0	0	0
Leaf chloroform	0	0	0	0	0	0
Leaf ethanol	**3±0**	0	**8±1.15**	**2±0**	**4±0**	0
Chloramphenicol (50 µg/disc)	**20±0.58**	**14±0**	**30±0**	0	**21.5±0**	**24±0.29**

Table 5.3: Zones of inhibition of clinical isolates from the disc diffusion test at 1 mg/disc.

	Bacteria					
Sample	*E. coli*	*P. aeruginosa*	*P. mirabilis*	*S. aureus*	*Methicillin Resistant S. aureus*	*K. pneumonia*
Stem hexane	0	0	0	**9±0**	0	0
Stem chloroform	0	0	0	0	**0.5±0.29**	0
Stem ethanol	0	0	0	0	0	0
Leaf hexane	0	0	0	0	0	0
Leaf chloroform	0	0	0	0	0	0
Leaf ethanol	0	0	0	**4±0**	0	0
Chloramphenicol (50 µg/disc)	**21±0.29**	0	**10±0**	**23±0.58**	**22±0.58**	**9±0**

5.3.2: Broth dilution test

The results of the broth dilution test produced the minimum inhibitory concentrations (MICs) as shown in **Tables 5.4 and 5.5**, and minimum bactericidal concentrations (MBCs) as shown in **Tables 5.6 and 5.7**, of each of the extracts. When tested against the ATCC strains (**Table 5.4**), the stem hexane, stem chloroform, leaf chloroform, leaf ethanol, fruit chloroform, fruit ethanol and stem alkaloid extracts produced inhibitory activity against some of the bacteria. The strongest activity was seen by the stem hexane and chloroform extracts against *K. rhizophilla* and the fruit chloroform and ethanol extracts against *B. cereus* with MICs of 250, 125, 62.5 and 250 µg/mL respectively. These values were significantly higher than the MICs obtained for chloramphenicol, for which MICs were less than 3.9 µg/mL for all bacteria except *P. aeruginosa*. When the stem and leaf extracts were tested against the clinical isolates (**Table 5.5**), the chloroform and ethanol extracts showed some activity against a number of the bacteria, although the MICs obtained were no lower than 1000 µg/mL for any extract.

The stem hexane extract was the only sample for which an MBC was found, giving an MBC of 1000 µg/mL against *B. cereus*. From these results, some of the extracts appear to have antibacterial activity, although the activity is mild in comparison to chloramphenicol and mostly inhibitory activity was observed at the concentrations tested. A crude extract can be expected to have milder activity than a pure antibiotic, since it is a mixture of a large number of compounds.

Table 5.4: MICs of ATCC strains from the broth dilution test.

	Bacteria					
Sample	*S. aureus*	*B. cereus*	*K. rhizophilla*	*P. aeruginosa*	*C. freundii*	*E. coli*
Stem hexane	>1000	**1000**	**250**	>1000	>1000	>1000
Stem chloroform	**500**	**500**	**125**	>1000	>1000	**1000**
Stem ethanol	>1000	>1000	>1000	>1000	>1000	>1000
Leaf hexane	>1000	>1000	>1000	>1000	>1000	>1000
Leaf chloroform	>1000	>1000	>1000	>1000	>1000	**1000**
Leaf ethanol	>1000	>1000	**1000**	>1000	>1000	>1000
Root hexane	>250	>250	>250	>250	>250	>250
Root chloroform	>250	>250	>250	>250	>250	>250
Root ethanol	>500	>500	>500	>500	>500	>500
Fruit hexane	>125	>125	>125	>125	>125	>125
Fruit chloroform	>125	**62.5**	>125	>125	>125	>125
Fruit ethanol	>500	**250**	>500	>500	>500	>500
Stem alkaloid	>1000	**1000**	**1000**	>1000	>1000	**1000**
Chloramphenicol	**<3.9**	**<3.9**	**<3.9**	**62.5**	**<3.9**	**<3.9**
DMSO	>16%	**10%**	**8%**	**10%**	**10%**	**16%**

The values in bold are for samples where an MIC was obtained.

Table 5.5: MICs of clinical isolates from the broth dilution test.

	Bacteria						
Sample	*E. coli*	*P. aeruginosa*	*Multi Drug Resistant P. aeruginosa*	*P. mirabilis*	*S. aureus*	*Methicillin Resistant S. aureus*	*K. pneumonia*
Stem hexane	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Stem chloroform	>1000	**1000**	>1000	**1000**	**1000**	>1000	>1000
Stem ethanol	>1000	**1000**	>1000	>1000	>1000	>1000	>1000
Leaf hexane	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Leaf chloroform	**1000**	**1000**	>1000	**1000**	**1000**	>1000	>1000
Leaf ethanol	>1000	**1000**	>1000	>1000	>1000	>1000	>1000
Root hexane	-	-	-	-	-	-	-
Root chloroform	-	-	-	-	-	-	-
Root ethanol	-	-	-	-	-	-	-
Fruit hexane	-	-	-	-	-	-	-
Fruit chloroform	-	-	-	-	-	-	-
Fruit ethanol	-	-	-	-	-	-	-
Stem alkaloid	-	-	-	-	-	-	-
Chloramphenicol	**<3.9**	**250**	>1000	**62.5**	**<3.9**	**250**	**31.25**
DMSO	**16%**	**10%**	**10%**	**10%**	**16%**	**>16%**	**16%**

Table 5.6: MBCs of ATCC strains from the broth dilution test.

	Bacteria					
Sample	*S. aureus*	*B. cereus*	*K. rhizophilla*	*P. aeruginosa*	*C. freundii*	*E. coli*
Stem hexane	>1000	**1000**	>1000	>1000	>1000	>1000
Stem chloroform	>1000	>1000	>1000	>1000	>1000	>1000
Stem ethanol	>1000	>1000	>1000	>1000	>1000	>1000
Leaf hexane	>1000	>1000	>1000	>1000	>1000	>1000
Leaf chloroform	>1000	>1000	>1000	>1000	>1000	>1000
Leaf ethanol	>1000	>1000	>1000	>1000	>1000	>1000
Root hexane	>1000	>1000	>1000	>1000	>1000	>1000
Root chloroform	>1000	>1000	>1000	>1000	>1000	>1000
Root ethanol	>1000	>1000	>1000	>1000	>1000	>1000
Fruit hexane	>1000	>1000	>1000	>1000	>1000	>1000
Fruit chloroform	>1000	>1000	>1000	>1000	>1000	>1000
Fruit ethanol	>1000	>1000	>1000	>1000	>1000	>1000
Stem alkaloid	>1000	>1000	>1000	>1000	>1000	>1000
Chloramphenicol	**500**	**125**	**<3.9**	**500**	**500**	**250**
DMSO	>16%	**10%**	**8%**	**10%**	**10%**	>16%

Table 5.7: MBCs of clinical isolates from the broth dilution test.

	Bacteria						
Sample	*E. coli*	*P. aeruginosa*	*Multi Drug Resistant P. aeruginosa*	*P. mirabilis*	*S. aureus*	*Methicillin Resistant S. aureus*	*K. pneumonia*
Stem hexane	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Stem chloroform	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Stem ethanol	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Leaf hexane	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Leaf chloroform	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Leaf ethanol	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Root hexane	-	-	-	-	-	-	-
Root chloroform	-	-	-	-	-	-	-
Root ethanol	-	-	-	-	-	-	-
Fruit hexane	-	-	-	-	-	-	-
Fruit chloroform	-	-	-	-	-	-	-
Fruit ethanol	-	-	-	-	-	-	-
Stem alkaloid	-	-	-	-	-	-	-
Chloramphenicol	>500	>500	>500	>500	>500	>500	>500
DMSO	>16%	>16%	**10%**	>16%	>16%	>16%	>16%

5.4: Conclusion

The extracts were found to have mild antibacterial activity with the most notable activity being shown by the leaf ethanol extract against *K. rhizophilla* and the stem hexane extract against the clinical isolate of *S. aureus* in the disc diffusion test. The fruit chloroform extract gave the lowest MIC out of all the extracts and this was against *K. rhizophilla*, however, only the stem hexane extract yielded an MBC of 1000 µg/mL against *B. cereus*, which was not significant when compared to chloramphenicol. The results of the disc diffusion test and broth dilution test were not correlatable, with only the stem hexane extracts activity against *B. cereus* and the leaf ethanol extracts activity against *K. rhizophilla* allowing any relationship between the extracts and their activity against the bacteria in these two assays to be made. This may be due to the fact that the samples were prepared differently for the disc diffusion and broth dilution tests. Both tests used water based mediums, with the disc diffusion relying on the diffusion of compounds through agar and the broth dilution relying on the solubility of the compounds in the liquid medium which may have been enhanced by the DMSO which was used in this particular assay. The different properties of the compounds in the extracts may have affected the activity of the compounds in these assays. Although some studies have found the results of these tests to be correlatable (Mayrhofer et al. 2008; Hoelzer et al. 2011), other tests involving plant extracts have also found that the results of broth dilution and agar diffusion tests can contradict each other (Chomnawang et al. 2005; Hammer et al. 1999; Klančnik et al. 2010).

None of the extracts exhibited significant inhibitory or bactericidal activity against *P. aeruginosa*, however, a colour change was observed on the agar plates of the disc diffusion test. The stem ethanol, fruit chloroform and fruit ethanol extracts caused a change in colour of the pigments produced by the bacteria, which were observed as a purple/red ring around the discs which contrasted with the green/yellow colour that was observed normally. The change in colour of pyocyanin from *P. aeruginosa* is likely due to a change in pH of the agar as the extracts diffused through the medium. It has been demonstrated previously that pyocyanin turns more red in colour when exposed to acidic conditions (Conroy 1959). The pH of the environment must be lower than 4.9 in order to produce a shift to red colouration, however a colour change can also be brought about due to oxidation or reduction (Friedheim & Michaelis 1931). The findings of the present study indicate a significant change in the pH of the agar medium or oxidation of the pyocyanin. This demonstrates that extract did diffuse

a significant distance through the gel but had no antibacterial activity in these areas. Even though a shift in pH to below 4.9 has been found to reduce growth in another study (Tsuji et al. 1982) the effects that the lowered pH may have had on the growth of *P. aeruginosa* were not significant enough to visibly or measurably reduce growth. If the colour change was due to oxidation, the extent of oxidative action may not have been strong enough to overcome the ability of *P. aeruginosa* to adapt to conditions of oxidative stress and affect its growth significantly (Sabra et al. 2002). Since the extracts were found to have antioxidant activity, it is less likely that oxidation is occurring and more likely that the pH is being altered as the sample diffuses across the media.

Generally, the extracts tended to show the greatest activity towards Gram positive bacteria, with the exception of some of the leaf and stem ethanol and chloroform extracts exhibiting antibacterial activity towards *C. freundii*, *P. aeruginosa*, *E. coli* and *P. mirabilis*. The greater antibacterial activity towards Gram positive bacteria indicates that the compounds in the active extracts may be disrupting the cell wall of the bacteria but are less able to penetrate the membrane found in Gram negative bacteria.

The solvent used to dissolve the extracts, DMSO, was found to exhibit bacteriostatic and bactericidal activity towards some of the strains used for this experiment and, although it was used at a concentration which was shown not to affect bacterial growth, it may have affected bacterial growth when combined with the extracts since it is known to act synergistically with antibiotics (Kamiya et al. 1966).

The disc diffusion test is accepted as a useful qualitative test for initial screening of samples for antimicrobial activity, however due to the discrepancies between the results of this test and the broth dilution test combined with the fact that it does not favour the testing of hydrophobic compounds, it cannot be used alone. The broth dilution test, although its results may not correlate with the disc diffusion test, is still a useful *in vitro* test for determining the antibacterial activity of extracts and may be more accurate than the disc diffusion test (Klančnik et al. 2010), however, the use of a colorimetric test and the preparation of plant extracts as an emulsion may prove more accurate in determining the antibacterial potential of a sample which contains compounds of varying solubility in water (Zhang et al. 2008).

Chapter 6: The anti-inflammatory activity of *P. glaucus*

6.1: Introduction

Inflammation is triggered as a response to damage, such as mechanical or chemical damage. Chemicals that induce inflammation could include irritants or toxins from plants, insects or animals, it could also include toxins released by microorganisms such as fungi or bacteria. Inflammation is the body's response to damage that works to promote healing, but causes unpleasant side effects such as pain and swelling.

6.1.2: Inflammation in disease

Despite the beneficial purpose of inflammation in the healing process, inflammation is a contributing factor in some chronic diseases.

Rheumatoid arthritis, asthma, multiple sclerosis, Alzheimer's disease (AD), Parkinson's disease, inflammatory bowel disease, cardiovascular disease can cancer are examples of diseases which involve chronic inflammation. The deregulation or prolonged activation of inflammation causes damage to cells and the cause, site and mechanism of inflammation determines the disease (Laveti et al. 2013).

6.1.2.1: Inflammation as a contributor to the development of cancer

Damage by a carcinogen triggers the healing process which can lead to further stimulation of the cells if it occurs in the presence of irritants. Irritants can cause the recruitment of inflammatory cells, increase ROS production and impair DNA repair. This can follow with the unusually persistent expression of pro-inflammatory cytokines, leading to chronic inflammation which induces cell death, leaving only the cells that have been sufficiently altered to escape programmed cell death, essentially selecting for cells that have become deregulated. This type of chronic inflammation can lead to the development of cancer (Coussens & Werb 2002).

6.1.2.2: Inflammation in Alzheimer's disease

In the case of Alzheimer's disease, the inflammation that is associated with the disease is driven mainly by myeloid cells (microglia) upon binding of amyloid-β (Aβ) to receptors and

activation of the immune system, although it is still uncertain whether inflammation is a contributing cause or a product of the disease (Wyss-Coray & Rogers 2012). Although inflammation is a normal part of the healing process and could be considered a product of the disease, this does not necessarily mean that anti-inflammatory drugs are the best choice of drug to treat chronic inflammatory diseases such as AD. Some clinical studies have found that administration of NSAIDs is not effective in treating Alzheimer's disease (Thal et al. 2005; Aisen et al. 2003) although they may be useful in protecting against the early stages of AD (Côté et al. 2012).

6.1.2.2: The role of inflammation in the progression of atherosclerosis

Atherosclerosis is an inflammatory disease, which involves lipid peroxidation, and haemostasis. Atherosclerosis is the thickening of the artery walls due to the recruitment of white blood cells in response to chronic inflammation, leading to a build up of dead and living cells and cholesterol and triglycerides. Macrophages, foam cells and increased proliferation of smooth muscle cells are also found in atherosclerotic plaques and their composition in a plaque can help to indicate the stability of the lesion. Ruptures can lead to the formation of blood clots which can cause thrombosis, narrowing of the lumen of a blood vessel due to the build up of plaque and can eventually lead to ischemia (Wang et al. 2015).

Infection by certain viruses has been linked to atherosclerosis, such as Marek's disease herpesvirus in chickens (Fabricant & Fabricant 1999) and cytomegalovirus which infects humans and monkeys (Nikitskaya et al. 2016). Diabetes, smoking and vitamin B_6 deficiency can increase the risk of developing atherosclerosis and is also more likely to occur in the elderly and males (Sofi et al. 2016). Certain diets may also increase the risk of atherosclerosis, with the intake of oxidised fats promoting atherosclerosis (Nogueira et al. 2016).

6.1.2.3: Fatty acids and inflammation

A fatty acid is a hydrocarbon (aliphatic) chain with a carboxylic acid group at one end. They can be saturated, where there are no carbon-carbon double bonds, monounsaturated, where there is one carbon-carbon double bond, polyunsaturated, where there are two or more carbon-carbon double bonds or branched chain, where the hydrocarbon chain of the fatty acid has branches. The number of carbon atoms in a fatty acid chain can vary with short chain fatty acids having less than 6 carbons, medium chain fatty acids having 6-12 carbons, long

chain fatty acids having 12 to 22 carbons and very long chain fatty acids having more than 22 carbons. The position and number of unsaturated carbon-carbon bonds will determine the shape of the fatty acid as double bonds will cause the chain to bend.

Fatty acids can be metabolised to yield ATP, so are used as a source of energy. Animals produce fatty acids from carbohydrates and these free fatty acids will usually be combined with glycerol to form a triglyceride. Triglycerides are a part of phospholipids, making up the hydrophobic tail. Phospholipids make up the membranes of all cells and organelles. When triglycerides are broken down in animals, free fatty acids will be released into the bloodstream and bind to serum albumin (Campbell et al. 1964). Cells can then take up these fatty acids and break them down into CO_2 and water, releasing energy to synthesise ATP (J. M. Berg et al. 2002). Fatty acids are also the precursor to signalling molecules, acting as ligands for transcription factors (Jump et al. 2006). Because of these numerous functions, they play an important role in the normal biological processes in an animal (Tvrzicka et al. 2011).

An elevated free fatty acid concentration promotes inflammation and induces oxidative stress by increasing reactive oxygen species production which degrades lipids (lipid peroxidation). Saturated fatty acids can activate the apoptotic pathway via intrinsic pathways including organellar dysfunctions, which include destabilisation of the lysosomal membrane and stress to the endoplasmic reticulum (Munoz & Costa 2013; Arrigo et al. 2015). They can also act on extrinsic pathways, activating apoptosis through death receptors and Tumour Necrosis Factor Related Apoptosis Inducing Ligand-Receptors (TRAIL-R). The death of cells will trigger an inflammatory response which helps to promote defence and tissue repair (Savary et al. 2012). However, the inflammatory response to cell death can also cause further tissue damage and can promote the progression of certain diseases.

The level of free fatty acids in a cell is regulated by their delivery and catabolism. When this homeostasis is not correctly regulated, it can lead to the excess fatty acids being stored as triglycerides, particularly in adipocytes. When too much of these lipids accumulates in non-adipose tissues, such as the heart, pancreas or liver, this can lead to lipotoxicity. Lipotoxicity occurs when the fat storage capacity of non-adipose tissues is exceeded causing cellular dysfunction and cell death (lipoapoptosis). Lipotoxicity occurs as a part of a number of

diseases where metabolism is dysregulated, such as obesity, type-2 diabetes, dyslipidemia and hypertension (Savary et al. 2012).

Inflammation is implicated in a number of diseases which affect a large number of people in the developed and developing countries. Many of these diseases involve the chronic inflammation of certain tissues for which the cause may or may not be known. Anti-inflammatory drugs could possibly help to manage some of these diseases and there are a number of enzymes that are expressed specifically in inflamed tissues, as such, these are the targets of anti-inflammatory drugs.

6.1.3: Cyclooxygenases 1 and 2 (COX-1 and COX-2)

The COX enzymes (cyclooxygenases 1 and 2), also known as PTGS (prostaglandin-endoperoxide synthase/prostaglandin G/H synthase/prostaglandin H2 synthase 1 and 2) are the main enzymes involved in the conversion of arachidonic acid to eicosanoids such as prostaglandins and leukotrienes. Prostaglandins are a group of lipid compounds that are able to exert a variety of different effects in animals, similar to the effect of hormones and their levels are increased by COX enzymes as an inflammatory response or during growth. Derivatives of prostaglandins are prostacyclins which are involved in the inhibition of platelet activation and vasodilation and thromboxanes which are involved in clot formation. The activities of prostaglandins and thromboxanes regulate the body's response to vascular damage (Ricciotti & FitzGerald 2011).

COX-1 is found in sites of the body where there is inflammation. It is also found in the stomach where it reduces stomach acid secretion, reduces pepsin in the stomach and promotes the production of the mucosal lining of the stomach (Ricciotti & FitzGerald 2011).
COX-2 is found in tissues during inflammation, where prostaglandins are upregulated. It has also been found to be upregulated in the tissues of many cancers. Mutations in the gene that encodes this enzyme can lead to an increased risk of gastric cancer. It reduces the pain threshold and also reduces the production of prostaglandins and prostacyclins that are involved in the protection of the stomach, so upregulation of this enzyme may cause gastric ulcers. COX-2 also increases vasodilation and decreases platelet aggregation (Ricciotti & FitzGerald 2011; Neitzel 2010).

When a cell undergoes damage, there is an increase in intracellular calcium which activates phospholipase A2 (PLA-2). PLA-2 is an enzyme that releases arachidonic acid by hydrolysing the sn-2 acyl bond of phospholipids. When arachidonic acid is released from membrane phospholipids by the action of PLA2, the COX enzymes convert this arachidonic acid into prostaglandins. The prostaglandins then go on to exert a variety of effects in the tissues (Neitzel 2010).

6.1.4: Enzyme inhibitors

Enzymes may be inhibited in a number of different ways, some of which are reversible and some which are irreversible. In order to catalyse a reaction, an enzyme will have an active site which will fit a substrate and modify the substrate. In the case of the COX enzymes, the substrate is arachidonic acid which, after binding to the active site of the enzyme, is converted to prostaglandins and thromboxanes.

An inhibitor may act by binding to the active site of the enzyme, preventing the substrate from binding. Since the inhibitor is not the true substrate of the enzyme, it will not be converted into a product. The binding of the inhibitor may be temporary, in which case inhibition would be competitive, or it may be permanent. Aspirin is an example of a COX inhibitor, which forms a covalent bond with a serine residue in the active site of the enzyme, permanently deactivating it (Tóth et al. 2013).

6.1.5: Inhibitors of the COX enzymes

Since the COX enzymes are responsible for producing prostaglandins which lead to some of the unpleasant side effects of inflammation, such as increased pain and swelling, they are often the target for new non-steroidal anti-inflammatory drugs (NSAIDs).

NSAIDs are a class of drugs that have analgesic, antipyretic and anti-inflammatory effects. They inhibit COX-1 and COX-2 which prevents the upregulation of prostaglandins and thromboxanes. Since COX-2 is responsible for the reduction in pain threshold and increase in vasodilation, the inhibition of this enzyme provides much relief from the symptoms of inflammation. Inhibition of COX-1 however, can lead to gastrointestinal bleeding due to an increase in stomach acid, lowered protection of the stomach lining due to lowered mucus lining production and inhibition of platelet aggregation.

NSAIDs are used to treat a wide variety of conditions, such as arthritis, headache, pain and inflammation from tissue injury, dysmenorrhoea and migraines. Due to the numerous effects that inhibition of the COX enzymes can cause, use of NSAIDs is contraindicated for people with certain conditions, such as peptic ulcers and cardiovascular disease (Weisman et al. 1985).

Since COX-2 produces prostaglandins that are responsible for the unfavourable symptoms of inflammation and inhibition of COX-1 can cause problems, particularly with the gastrointestinal tract, there is a greater inclination towards developing a selective COX-2 inhibitor. Although selective COX-2 inhibitors would appear to be better than a non-selective COX inhibitor, inhibitors of COX-2 have been shown to increase the risk of a vascular event (Howard & Delafontaine 2004). Despite the potential adverse effects, the COX-2 inhibitors have proven useful in treating inflammation and diseases related to inflammation. COX-2 inhibitors also carry a lower risk of gastrointestinal bleeding compared to non-selective inhibitors although the risk of myocardial infarction is greater (Meek et al. 2010).

6.1.6: Medical importance

Since COX enzymes are upregulated in cancerous tissues, research has been conducted on the use of COX inhibitors in the treatment of cancer, with some success. Inflammation, particularly chronic inflammation, is not only an uncomfortable part of disease, but can lead to further disease. Chronic inflammation can lead to damage of the surrounding tissues which must be repaired. The increase in turnover of cells in the damaged area leads to a greater possibility of genetic mutation and thus cancer (Moss & Blaser 2005). In the case of chronic arthritis, not only is there a large turnover of cells but the possibility of calcification and an increase in pain (Loeser 2010).

Inhibitors of COX are an important class of drugs that provide relief from inflammation and its associated symptoms. The side effects of COX inhibitors are a problem for certain groups of people and for those who take them for prolonged periods of time, as such, there is room for the discovery and development of new anti-inflammatory drugs which could have reduced side effects and still provide the beneficial effects of NSAIDs.

6.1.7: Protein stability and disease

Proteins are an essential part of living organisms and perform a vast number of different functions, some of which have yet to be fully explored. Proteins can be damaged chemically or by radiation, this damage will cause the protein to denature and form aggregates. The mutation of genes that encode certain proteins can lead to the production of faulty proteins which can in turn lead to disease. Protein denaturation and DNA damage are also often implicated in inflammation.

One significant example of the importance of a correctly functioning protein can be demonstrated by the p53 protein, which is responsible for tumour suppression by activating DNA repair, arresting the cell cycle and initiating apoptosis. A mutation in the gene that expresses this protein leads to the production of the p53 protein which does not function correctly and can cause the disorder Li-Fraumeni syndrome, which greatly increases the affected individual's chances of developing cancer (Rivlin et al. 2011).

Protein aggregation is also indicated in a number of neurodegenerative diseases, prion diseases, amyloidosis and rheumatiod arthritis (Meredith 2005; Berlett & Stadtman 1997).
Protein damage can also be caused by UV damage and heat. Spending time in the sun can lead to high exposure to UV rays and cause damage, not only to DNA, but to proteins as well (Pattison & Davies 2006). When a burn is received, since proteins in the body are generally heat sensitive, they can become denatured by high temperatures.

6.1.8: Protein stabilisers

In order to stabilise proteins that have been isolated, certain chemicals can be added to prevent the protein from denaturing, such chemicals include glycerol, mannitol, maltose, sucrose and DMSO. These chemicals are not chaperones such as the ones that the body creates for the purpose of correct protein folding, they are chemicals that are able to bind to the hydrophobic sites of proteins and hydrogen bond with water simultaneously which increases protein solubility, prevents aggregation and also denaturation by heat (Papp & Csermely 2006).

A number of anti-inflammatory drugs are able to inhibit the denaturation of proteins in a dose dependant manner when proteins are exposed to heat. These drugs are able to protect against

conditions where protein denaturation plays an important role, such as cataracts and arthritis. Much research regarding anti-inflammatory drugs focuses around NSAIDs that are able to inhibit the COX enzymes which lead to the production of prostaglandins that bring about the symptoms associated with inflammation. However, it has been found that although some NSAIDs work by inhibiting the COX enzymes, some also posses the additional ability to prevent protein denaturation (Lee & Stupans 2002), furthermore, bindarit has been found to work not only by regulating the NFκB pathway, but possibly by protein stabilisation (V et al. 1992; Mora et al. 2012). Therefore, some chemicals that are able to inhibit heat induced protein denaturation may have the potential to be used as anti-inflammatory agents.

6.1.9: Lysosomes in inflammation

Lysosomes are organelles which are found in most animal cells and contain enzymes that are capable of breaking down many other molecules within a cell. Their normal function is to clear debris, organelles, bacteria, viruses or other structures within a cell by autophagy. The enzymes of lysosomes are contained by the lysosomal membrane, protecting the contents of the rest of the cell from exposure. The pH within the lysosome is acidic as the functioning of the enzymes is optimal at this pH, it also prevents damage to the cell in the event of leakage of lysosomal contents into the cytosol, as the function of these enzymes will be impaired in the more alkaline conditions.

Lysosomal enzymes play an important role in inflammatory diseases. During inflammation, lysosomes will release their enzymes causing damage to the cell and triggering inflammation of the surrounding tissues. Anti-inflammatory drugs, such as indomethacin and hydrocortisone, have been found to stabilise the membranes of lysosomes, preventing further damage and inflammation (Ignarro 1972).

In this chapter, the ability of the samples to inhibit COX-1 and COX-2 was explored using a COX fluorescent inhibitor screening assay kit which measures the ability of the samples to inhibit the COX enzymes directly. The ability of the extracts to prevent heat induced protein denaturation was also explored, using ovine albumin as the model protein. HRBCs were used as a model for lysosomes, and the ability of the samples to prevent rupture of the membranes of RBCs under stressors including heat and hypotonicity was tested.

6.2: Materials and methods

6.2.1: Chemicals and reagents

DMSO was purchased from RCI Labscan (Thailand), diclofenac sodium was purchased from Sigma Aldrich (USA), PBS was purchased from Thermofisher, fresh hen eggs were purchased from a local supermarket, tri-sodium citrate dehydrate and NaCl were purchased from R & M Chemicals (UK), COX Fluorescent Inhibitor Screening Assay Kit was purchased from Cayman Chemical (USA), periglaucine A was purchased from ChemFaces (China), palmitic acid, stearic acid, melissic acid and betulinic acid were purchased from Guokang Biotechnology (China).

6.2.2: Inhibition/enhancement of protein denaturation

For this test, samples were prepared by dissolving in DMSO to give a stock solution of 10 mg/mL. Serial dilutions of the sample were made so that the final concentration of the samples in the reaction mixture ranged from 200-12.5 µg/mL. Diclofenac sodium was used as the positive control, PBS and DMSO were used as the negative control. The reaction mixture consisted of 0.1 mL fresh hen's egg albumin, 2.35 mL of PBS and 0.05 mL of the sample. The reaction mixture of each sample was added to the wells of a 96-well plate. The plate was then incubated for 5 min at 70 °C in water bath. The plate was carefully cooled from the underside by running tap water over it, before being dried and a reading at 660 nm taken. Experiments were repeated in triplicate of triplicates (n=9). The % of inhibition of protein denaturation was calculated using the following formula:

Equation 6.1: % inhibition of protein denaturation

$$\%\ Inhibition = \frac{Control\ Absorbance - Sample\ Absorbance}{Control\ absorbance} \text{ x } 100$$

6.2.3: HRBC membrane stabilization assay

6.2.3.1: Collection and preparation of blood

Ethical approval for the collection of blood from a human participant was granted by the Science and Engineering Research Ethics Committee at the University of Nottingham

Malaysia Campus, the application identification number was "FS090714", please see **Appendix E**. Blood was collected from a healthy human volunteer who had not taken any anti-inflammatory drugs for at least 2 weeks prior to the time of the experiment. 5 mL of blood was collected and tri-sodium citrate dehydrate (3.8%) was added to the blood in the ratio of 9:1 (blood : citrate) to prevent coagulation. The blood was centrifuged at 1,300 g for 3 min and the supernatant was discarded. The RBCs were washed 3 times in PBS before being reconstituted in PBS to make a 10% (v/v) suspension.

6.2.3.2: Heat induced lysis of HRBCs

Samples were dissolved in PBS. The reaction mixture consisted of 100 µL of the sample, 880 µL of PBS and 20 µL of the 10% RBC suspension in test tubes. Diclofenac sodium was used as the internal standard and PBS was used for the control. Serial dilutions of the samples were made to give final concentrations ranging from 200-6.25 µg/mL in the final reaction mixture. The reaction mixture was incubated at 54 °C for 30 min, after which the test tubes were cooled under running water and centrifuged at 1,300 g for 3 min. Experiments were run in triplicate of triplicates (n=9).The absorbance of the supernatant was measured at 540 nm.

The following formula was used to determine enhancement or inhibition of haemolysis:

Equation 6.2: % inhibition or enhancement of haemolysis

$$\%\ Inhibition = \frac{Control\ Absorbance - Sample\ Absorbance}{Control\ absorbance} \text{ x } 100$$

6.2.3.3: Hypotonicity induced lysis of HRBCs

Samples were dissolved in PBS to make a stock solution of 2 mg/mL. The reaction mixture consisted of 100 µL of the sample, 880 µL of hyposaline (0.25% NaCl, w/v) and 20 µL of the 10% RBC suspension in test tubes. Diclofenac sodium was used as the internal standard and hyposaline was used for the control. Serial dilutions of the samples were made to give final concentrations ranging from 200-6.25 µg/mL in the final reaction mixture. The reaction mixture was incubated at room temperature for 10 min, after which the test tubes were

centrifuged at 1,300 g for 3 min. The absorbance of the supernatant was measured at 540 nm. Experiments were run in triplicate of triplicates (n=9).

The following formula was used to determine the percent of protection from haemolysis assuming 100% haemolysis in the control reaction mixture:

Equation 6.3: % Protection from haemolysis

$$\%\ Protection = 100 - \frac{Sample\ Absorbance}{Control\ absorbance} \text{x } 100$$

6.2.4: COX-1 and COX 2 inhibition assays

The COX Fluorescent Inhibitor Screening Assay Kit, from Cayman Chemicals, was used to determine the enzyme inhibitory activity of the extracts. Instructions were followed as described in the kit booklet. Briefly, 150 µL of assay buffer was added to the Initial activity (IA) and sample wells, 160 µL of assay buffer was added to the Background activity (BA) wells. 10 µL of heme was added to each well and 10 µL of enzyme was added to the IA and sample wells. 10 µL of DMSO was added to the IA and BA wells and 10 µL of the sample was added to the sample wells. The plate was incubated at room temperature (RT) for 5 min. 10 µL of ADHP was added to each well and 10 µL of arachidonic acid was quickly added to each well to initiate the reaction. The plate was incubated at RT for precisely 2 min before the plate was read at an excitation of 530-540 nm and emission of 585-595 nm. Experiments were run in triplicates (n=3).

The percent of inhibition was calculated using the following formula:

Equation 6.4: % inhibition of COX-1/COX-2

$$\%\ Inhibition = \frac{Initial\ Activity - Sample\ Activity}{Initial\ Activity} \text{x } 100$$

6.3: Results

6.3.1: Inhibition/enhancement of protein denaturation

The hexane extracts showed the greatest ability to prevent protein denaturation induced by heat (**Figures 6.1 - 6.4**), almost completely inhibiting protein denaturation at higher concentrations with the exception of the stem hexane extract which inhibited denaturation by up to 42.63%. The leaf chloroform extracts of the stems, leaves and fruits also showed good protective activity with the fruit chloroform extract showing almost identical inhibitory activity at the highest concentration tested. Enhancement of protein denaturation was seen by the root chloroform extract, although this effect was very mild. The stem alkaloid extract inhibited protein denaturation at the two lowest concentrations (**Figure 6.5**), however, this declined between 25 µg/mL and 100 µg/mL and enhanced protein denaturation at 200 µg/mL. The fatty acids (**Figure 6.7**), palmitic and stearic acid, also demonstrated strong inhibition of protein denaturation, followed by melissic acid, suggesting a link between the ability of the fatty acids to prevent heat induced protein denaturation and the length of the fatty acid chain. Betulinic was also able to prevent protein denaturation by approximately 50% at 200 µg/mL. The ability of the fatty The ability of diclofenac to inhibit protein denaturation was mild (**Figure 6.6**) in comparison to most of the extracts that exhibited dose dependant inhibition of protein denaturation.

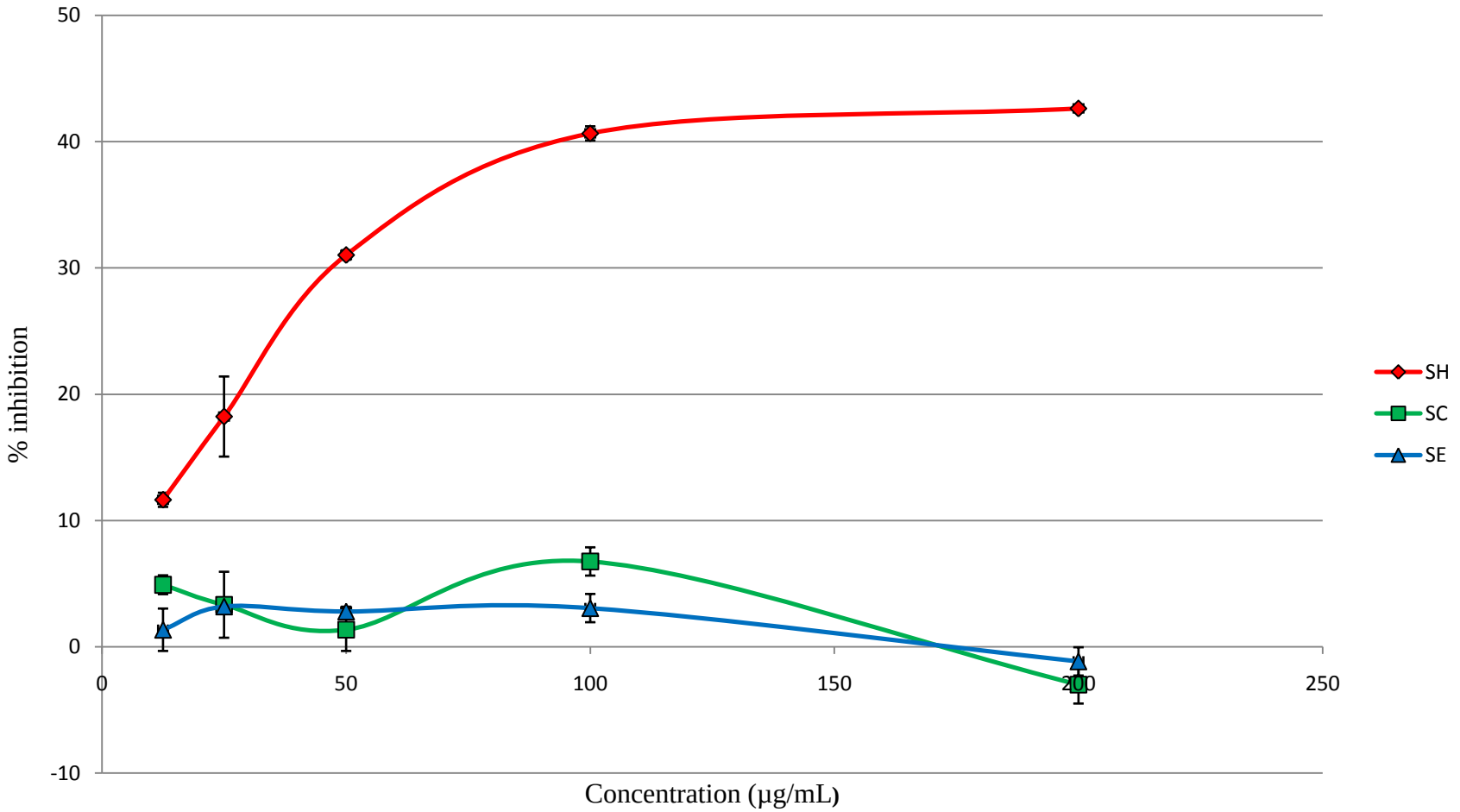


Figure 6.1: Inhibition of protein denaturation by *P. glaucus* stem extracts.

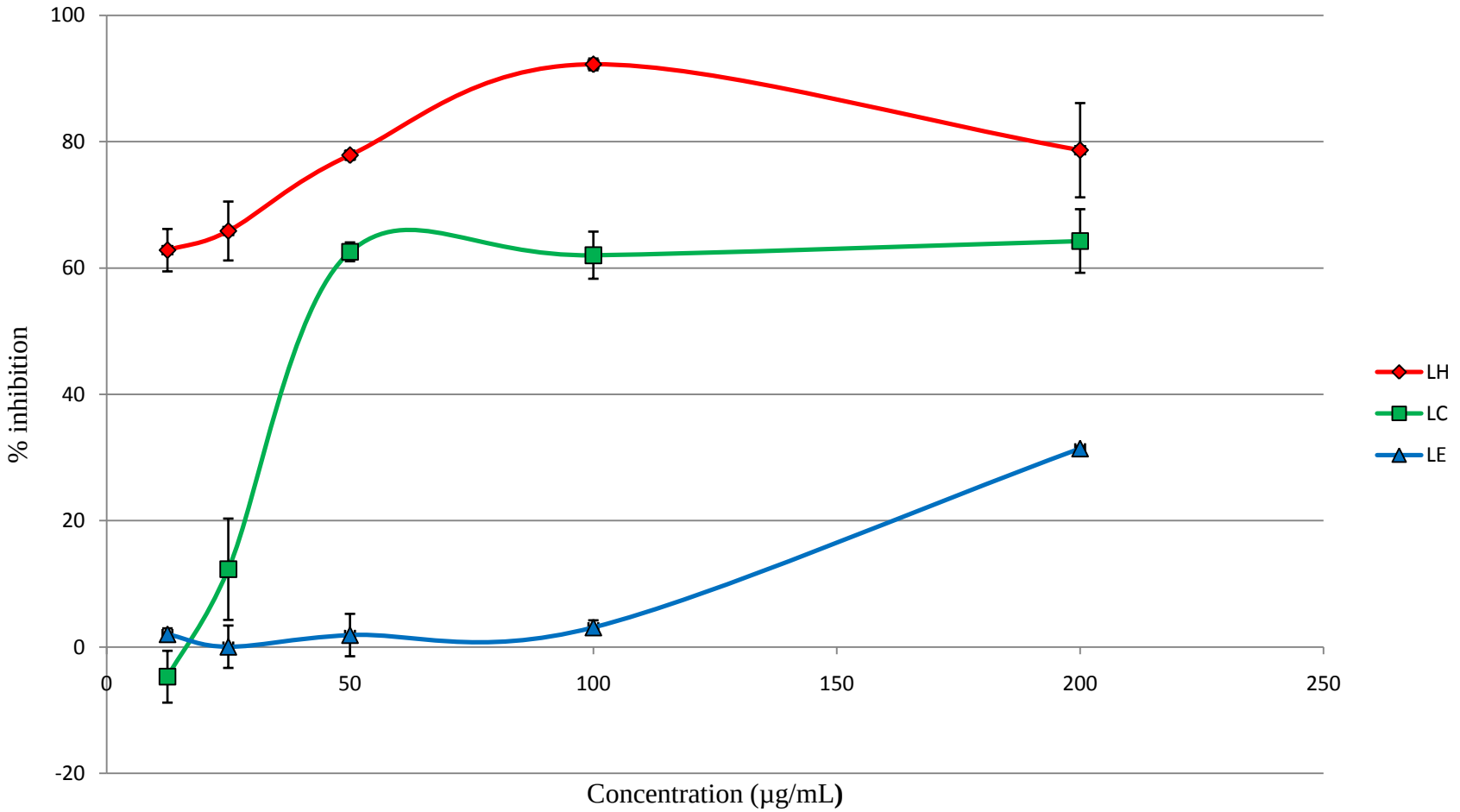


Figure 6.2: Inhibition of protein denaturation by *P. glaucus* leaf extracts.

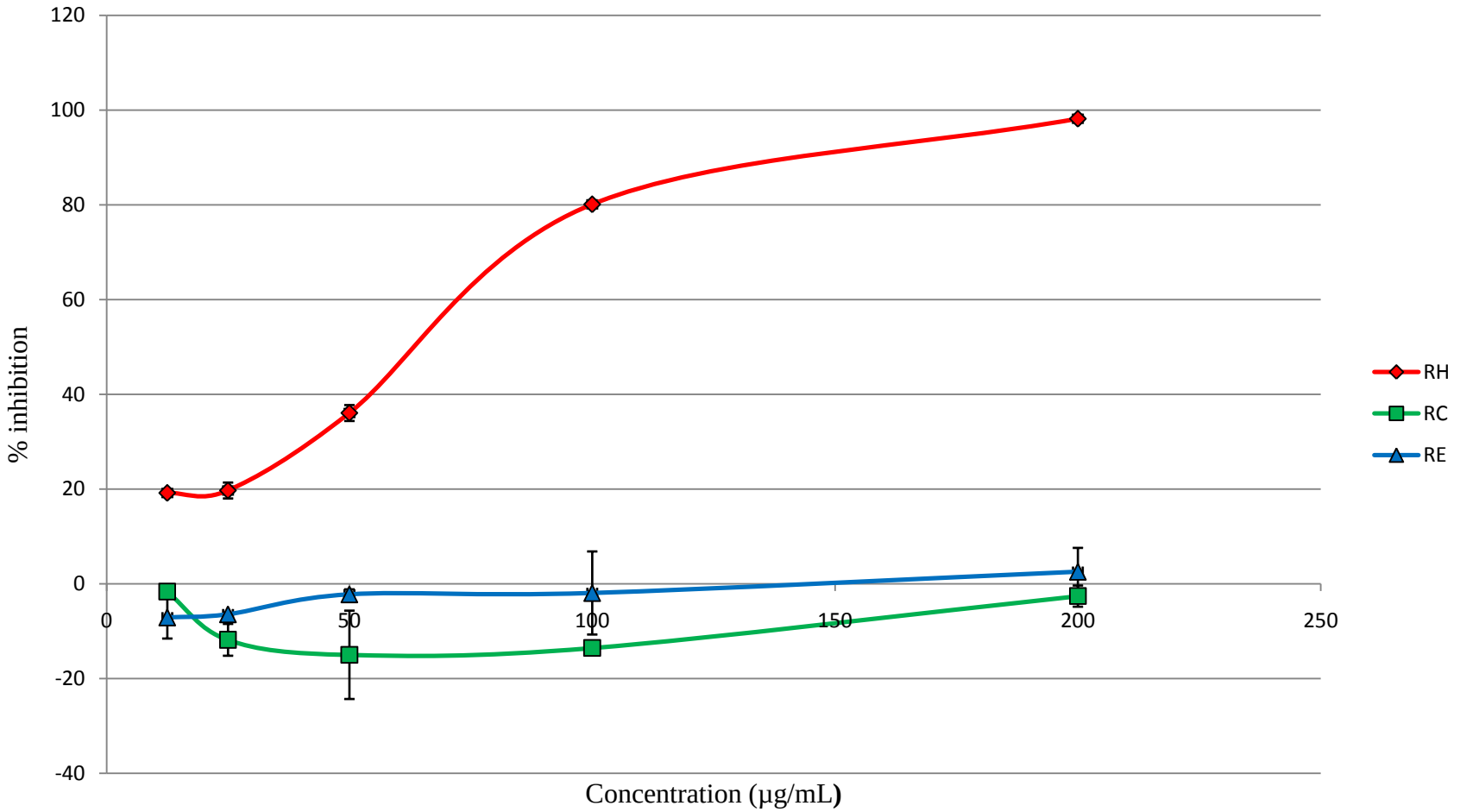


Figure 6.3: Inhibition of protein denaturation by *P. glaucus* root extracts.

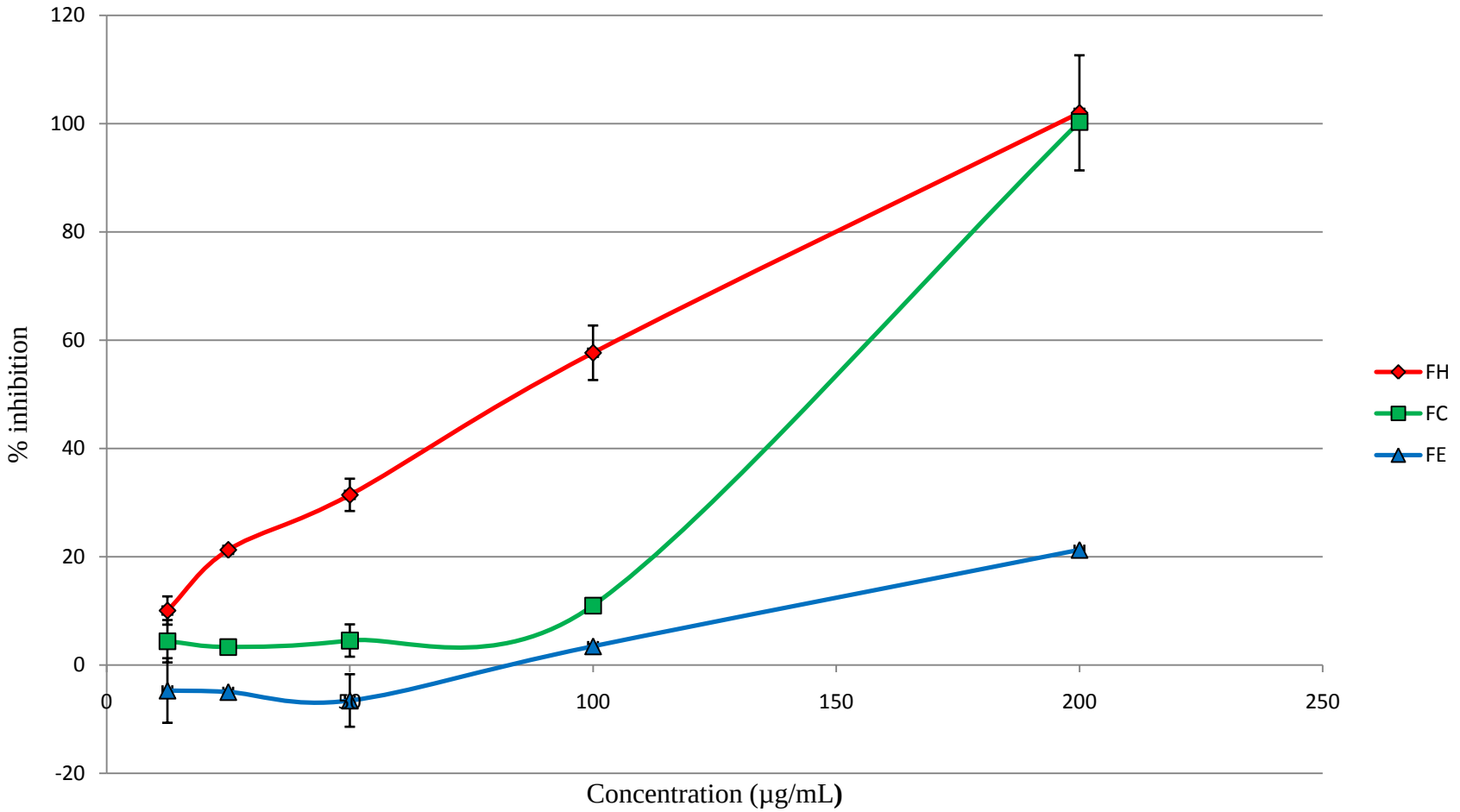


Figure 6.4: Inhibition of protein denaturation by *P. glaucus* fruit extracts.

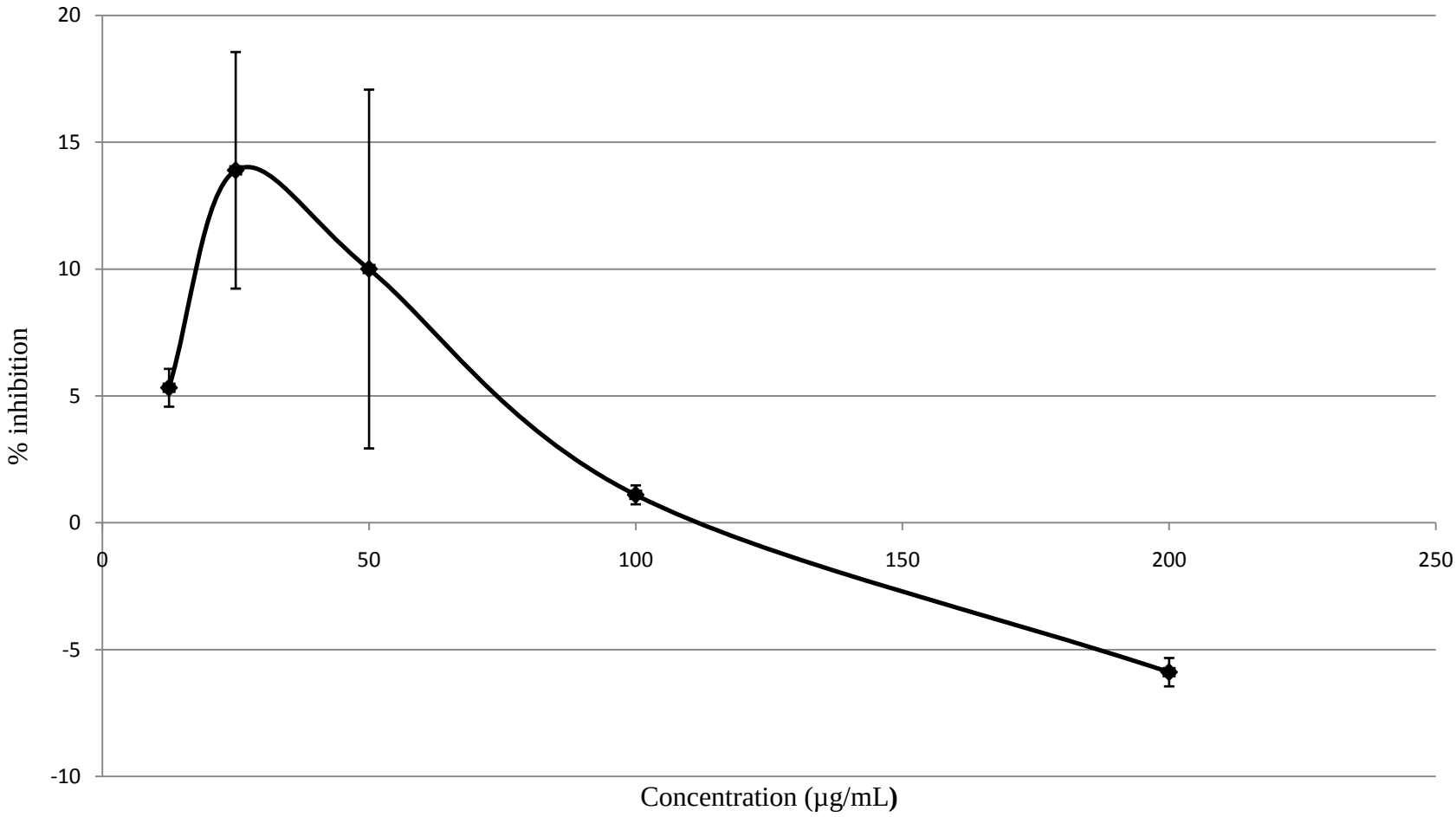


Figure 6.5: Inhibition of protein denaturation by *P. glaucus* stem alkaloid extract.

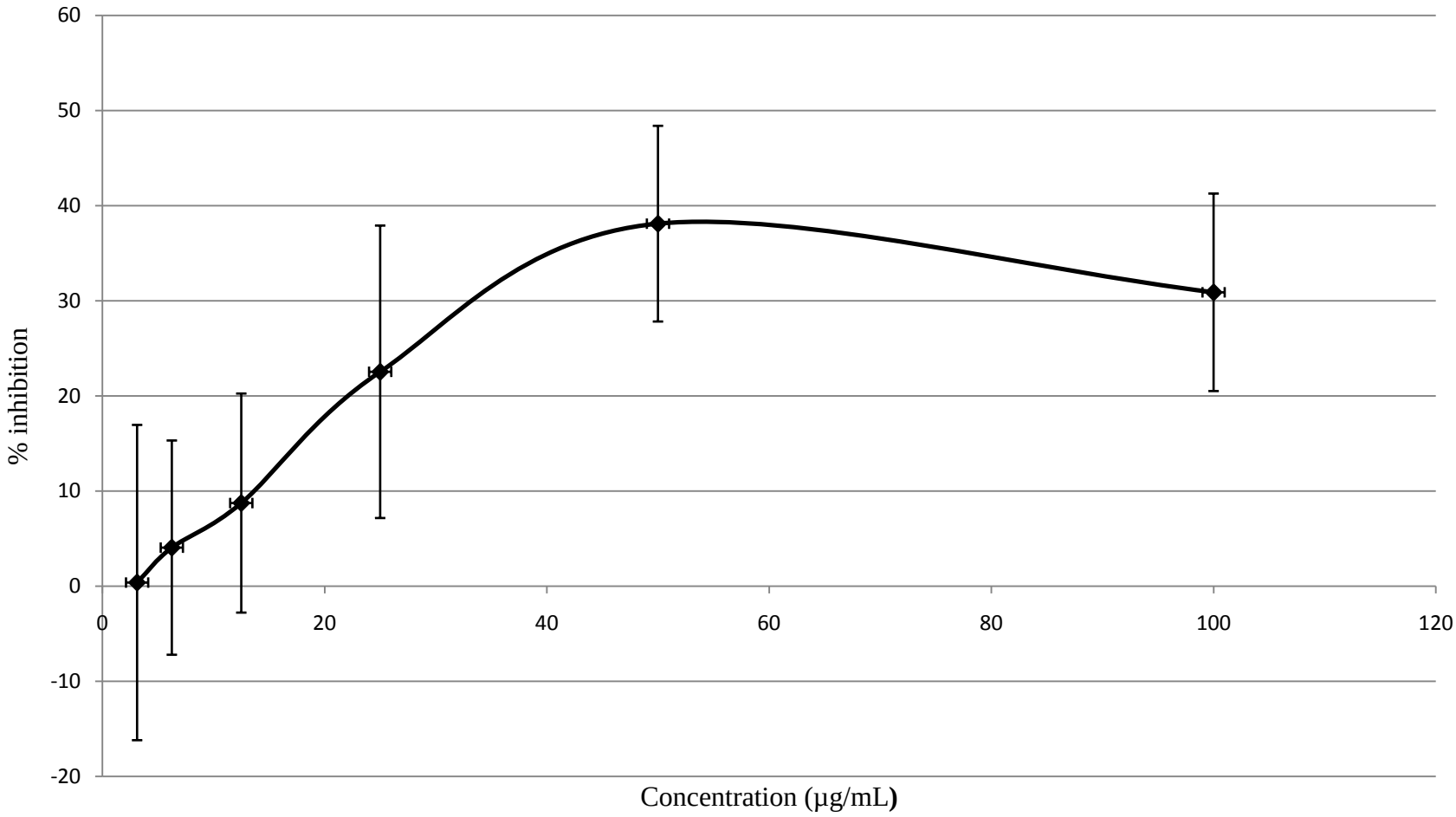


Figure 6.6: Inhibition of protein denaturation by periglaucine A.

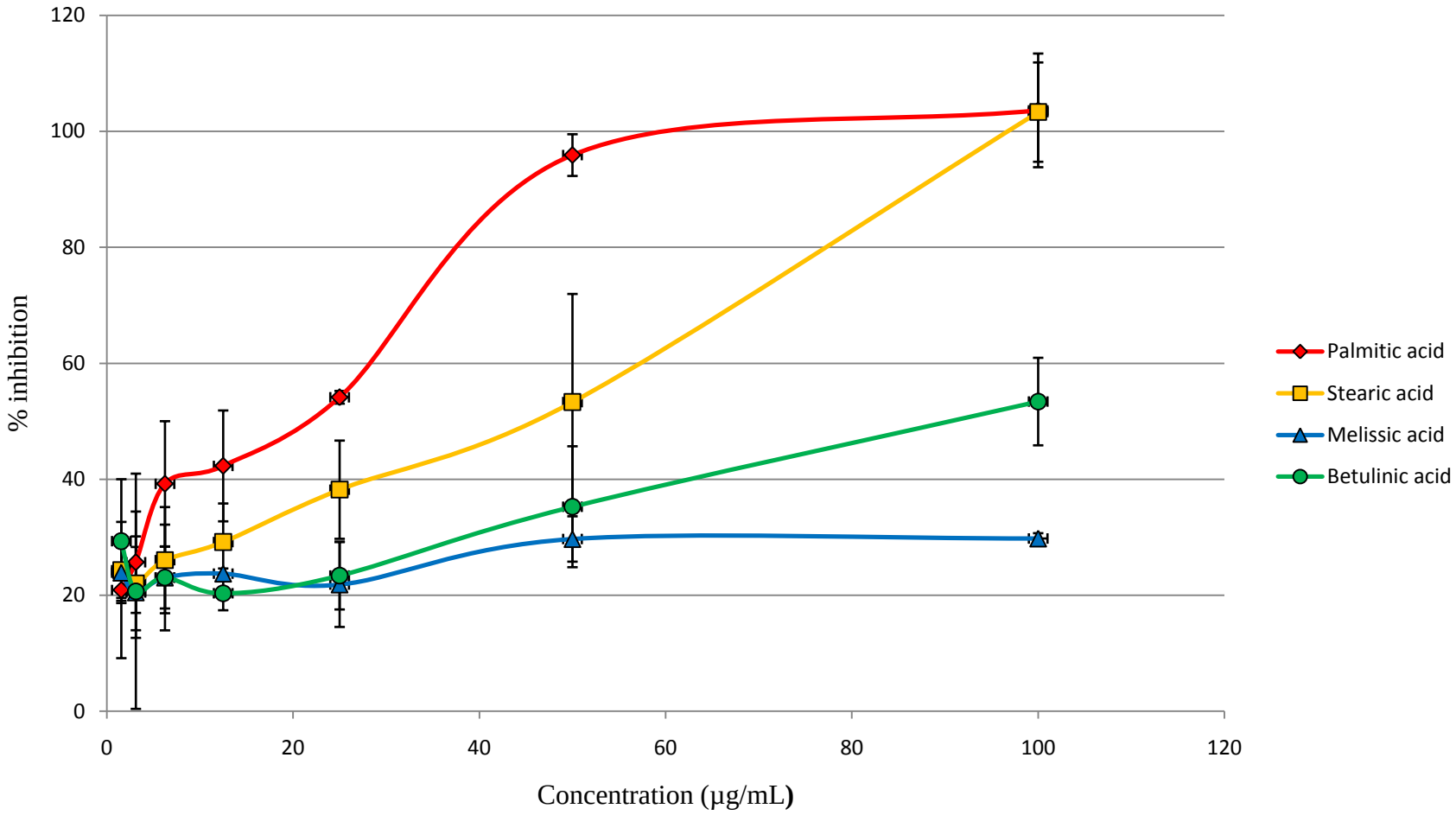


Figure 6.7: Inhibition of protein denaturation by palmitic, stearic, melissic and betulinic acid.

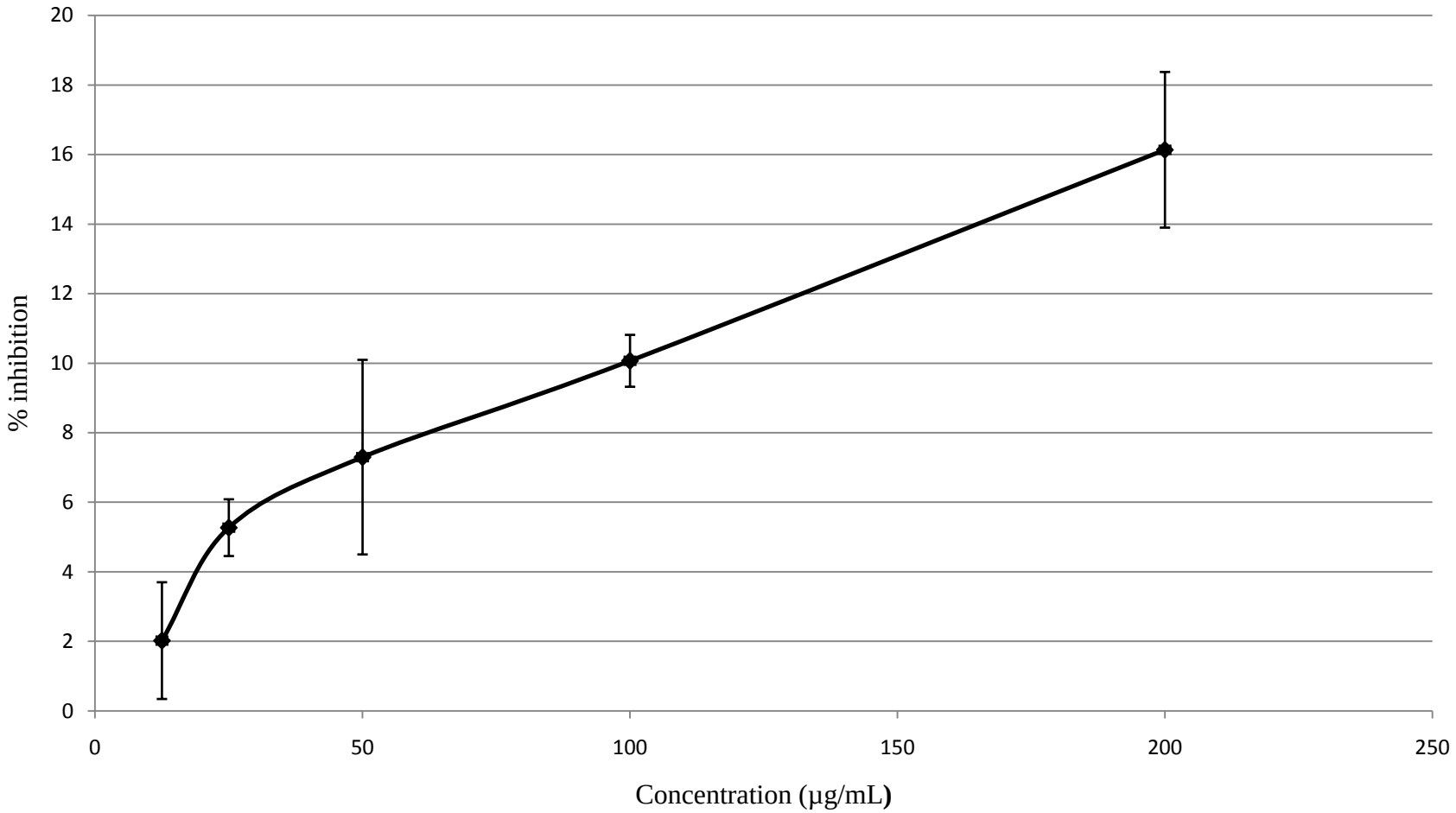


Figure 6.8: Inhibition of protein denaturation by diclofenac.

6.3.2: HRBC membrane stabilization assay

6.3.2.1: Heat induced lysis of HRBCs

The stem chloroform and ethanol extracts were able to prevent lysis of red blood cells (RBCs) induced by heat by approximately 50% regardless of concentration (**Figure 6.9**), while the hexane extract increased red blood cell lysis with increasing concentration. The leaf chloroform and ethanol extracts showed some degree of protection from lysis at the lower concentrations, however this decreased with increasing concentration with the leaf ethanol extract enhancing lysis at 200 µg/mL (**Figure 6.10**). The leaf hexane showed increasing enhancement of RBC lysis between the concentrations of 12.5 and 100 µg/mL but this enhancement was lower at 200 µg/mL. The root ethanol extract showed similar activity to the stem chloroform and ethanol extracts, showing inhibition of RBC lysis regardless of concentration (**Figure 6.11**). The root chloroform extract, although it inhibited lysis at lower concentration, with increasing dose, the protectivity decreased until it began to enhance lysis. The root hexane extract enhanced lysis of RBCs and this enhancement could not be correlated with concentration of the extract. The fruit ethanol extract inhibited lysis of RBCs in a dose dependant manner, however the chloroform and hexane extracts provided protection from lysis only at lower concentrations, enhancing lysis at 200 µg/mL (**Figure 6.12**). The stem alkaloid extract (**Figure 6.13**) was able to inhibit lysis of RBCs by approximately 30% at 100 and 200 µg/mL.

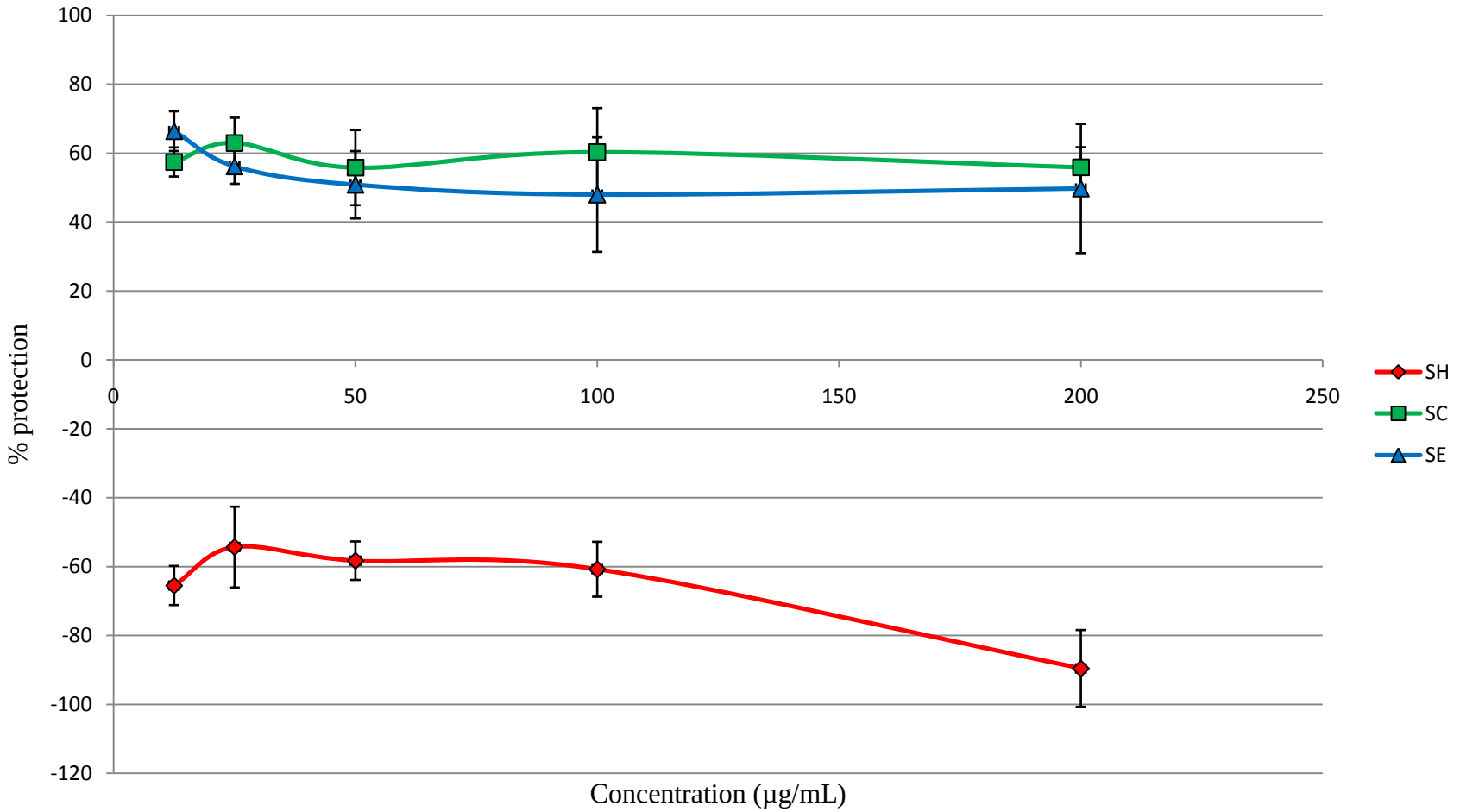


Figure 6.9: Membrane stabilizing activity of the stem extracts of *P. glaucus* under stress by heat.

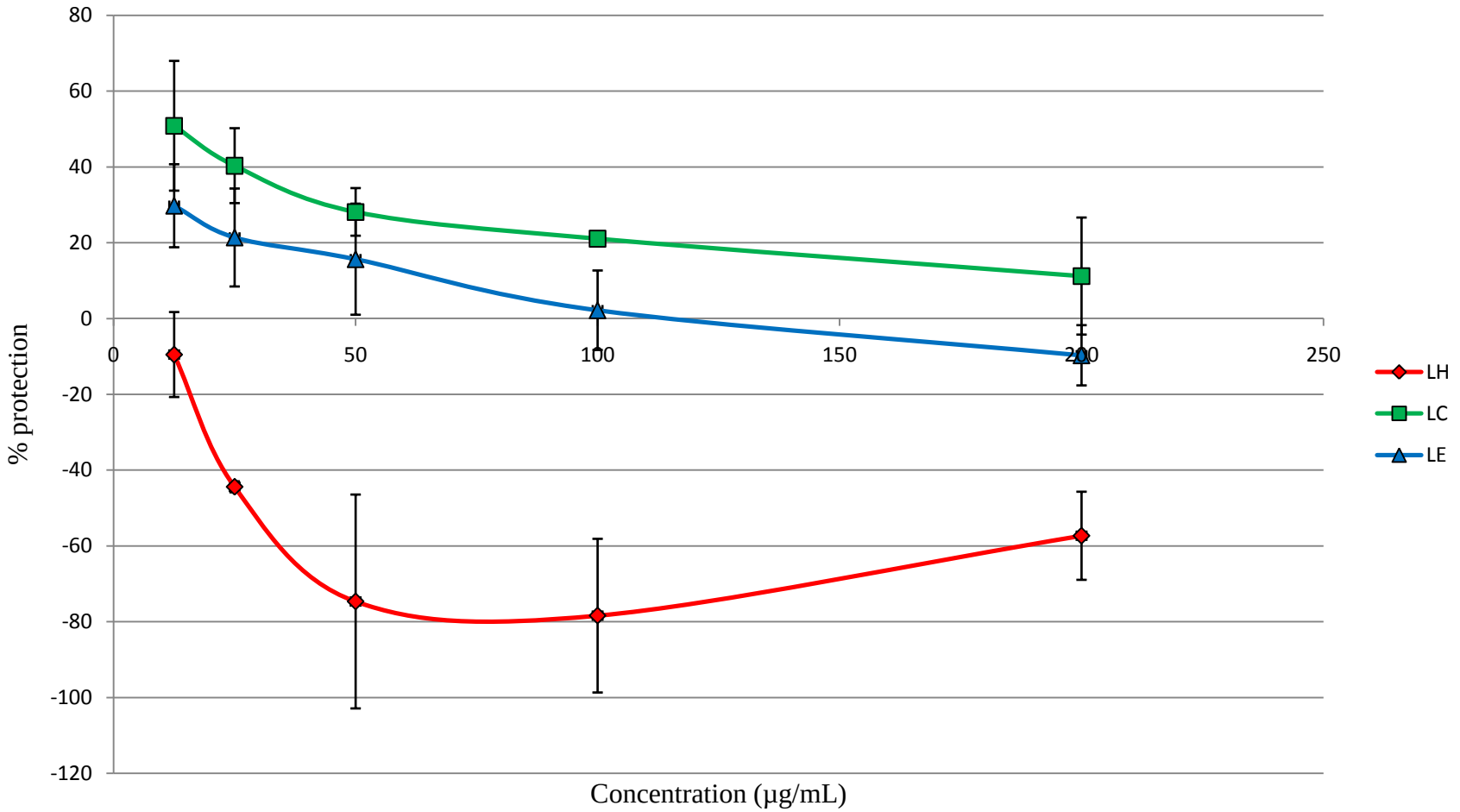


Figure 6.10: Membrane stabilizing activity of the leaf extracts of *P. glaucus* under stress by heat.

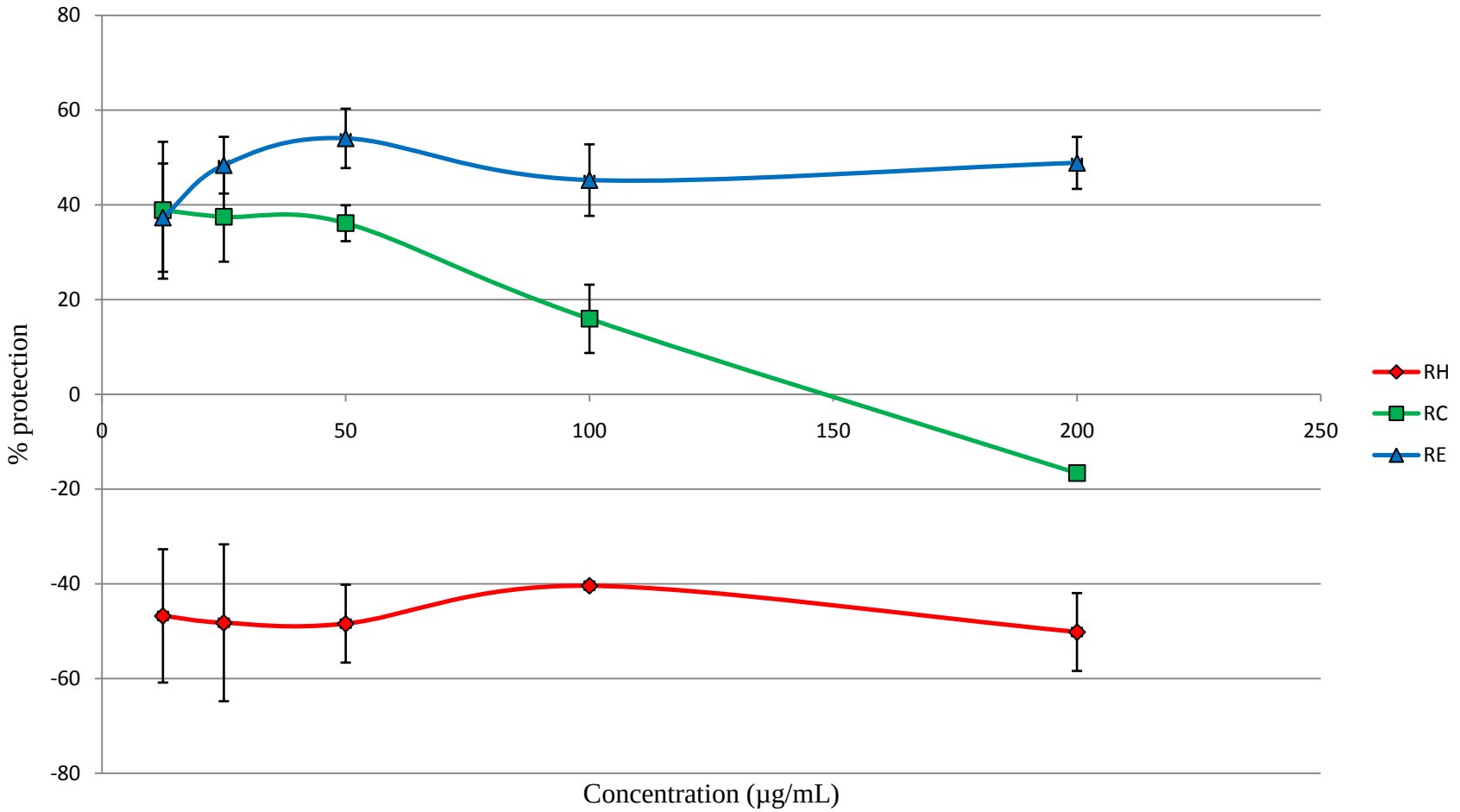


Figure 6.11: Membrane stabilizing activity of the root extracts of *P. glaucus* under stress by heat.

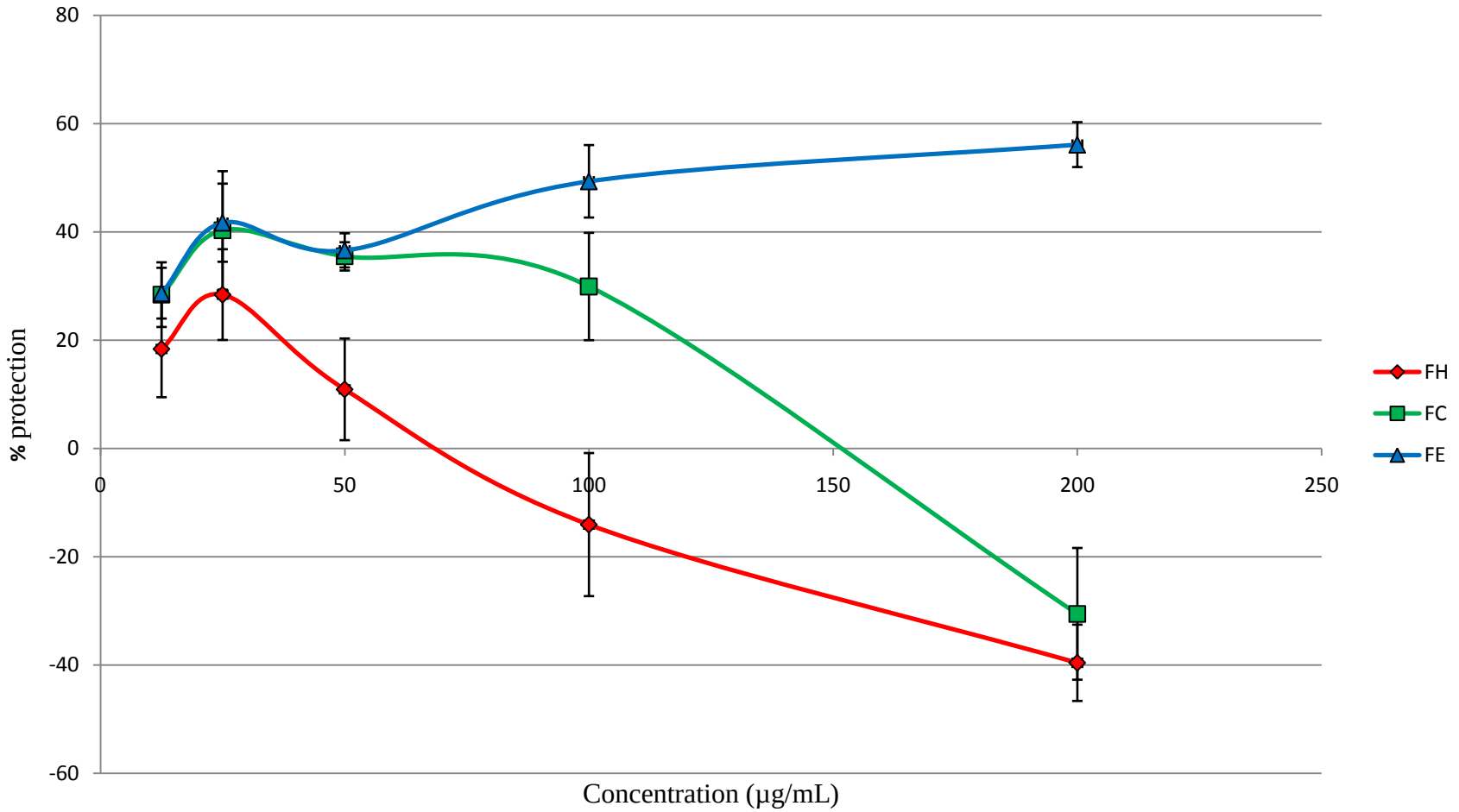


Figure 6.12: Membrane stabilizing activity of the fruit extracts of *P. glaucus* under stress by heat.

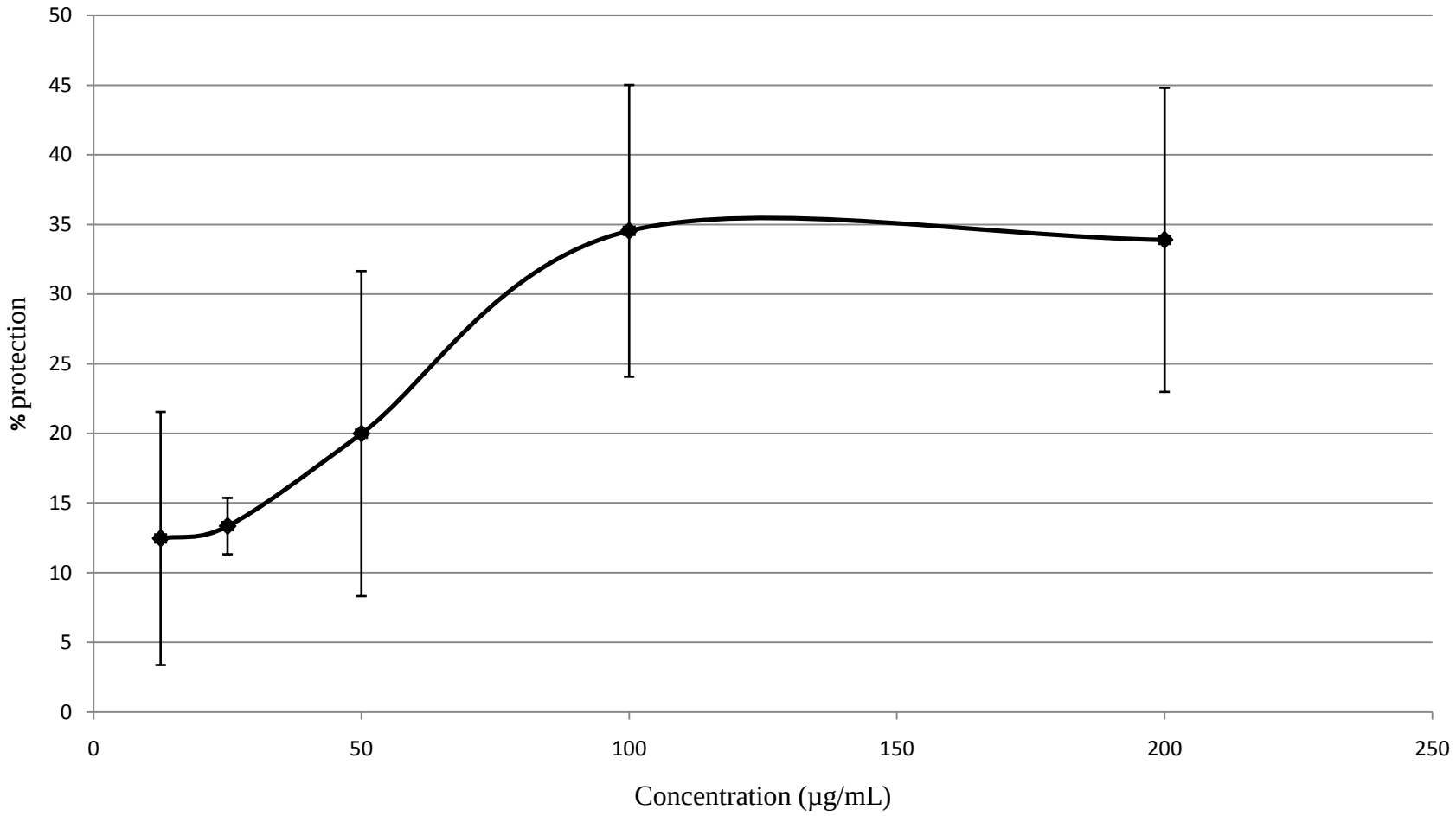


Figure 6.13: Membrane stabilizing activity of the stem alkaloid extract of *P. glaucus* under stress by heat.

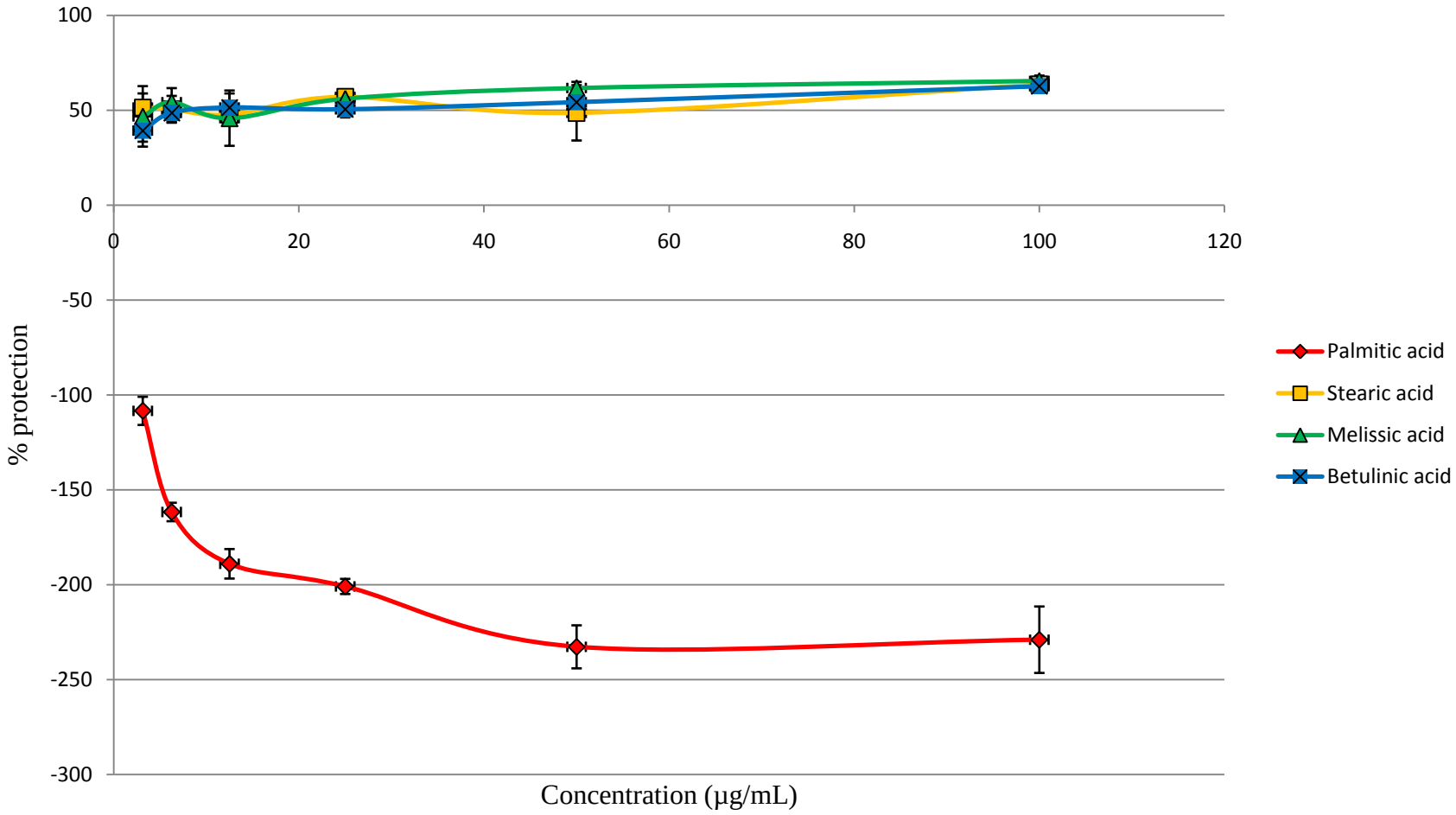


Figure 6.14: Membrane stabilizing activity of fatty acids and betulinic acid under stress by heat.

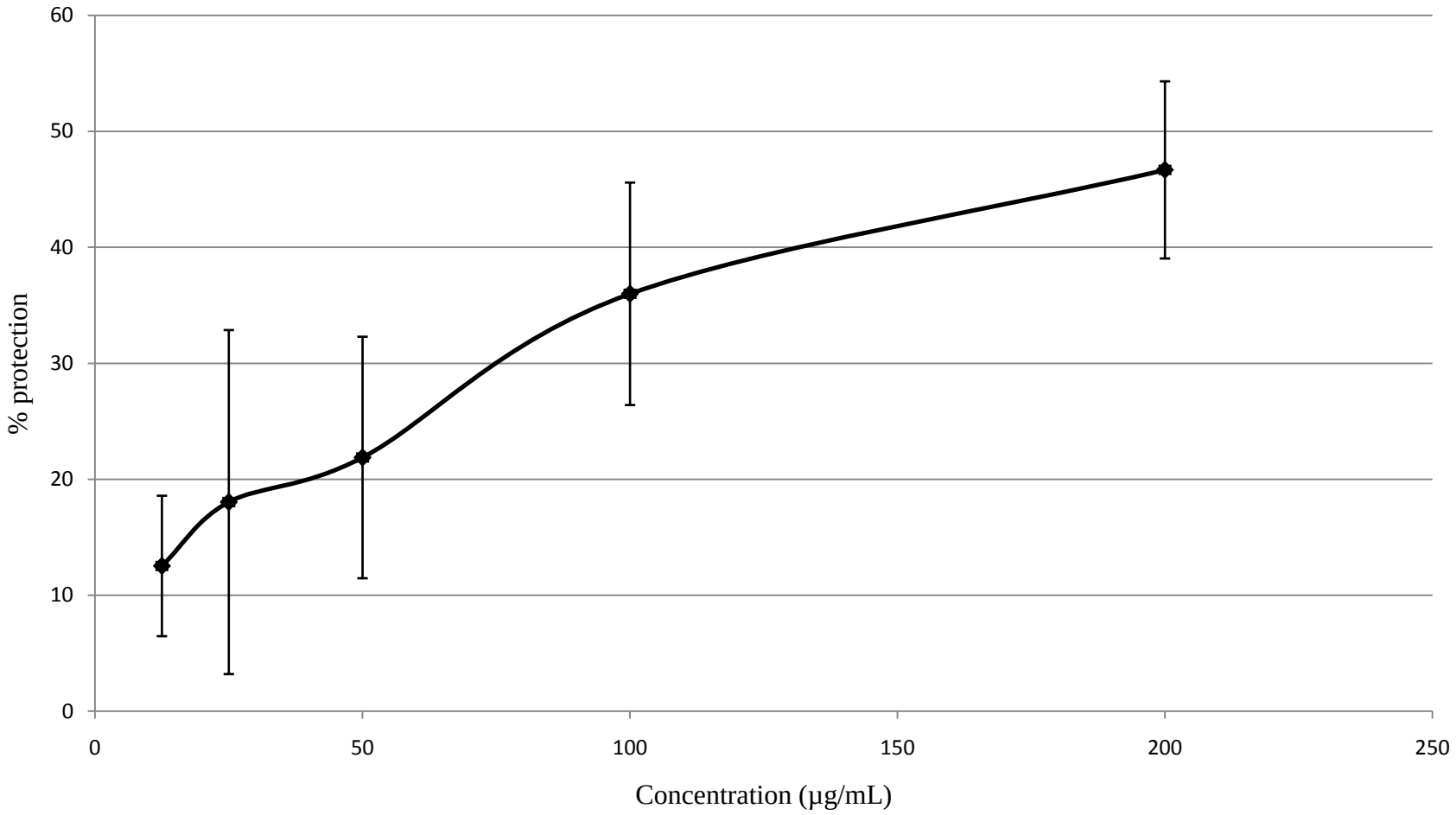


Figure 6.15: Membrane stabilizing activity of diclofenac sodium under stress by heat.

6.3.2.2: Hypotonicity induced lysis of HRBCs

When lysis was induced by hypotonicity, all stem extracts (**Figure 6.16**), the root chloroform and root ethanol extracts (**Figure 6.18**), all fruit extracts (**Figure 6.19**) and diclofenac (**Figure 6.22**) showed a dose dependant ability to prevent lysis of RBCs. The leaf chloroform and hexane extracts (**Figure 6.17**) showed optimal inhibition of lysis at a concentration of 100 µg/mL while the stem alkaloid extract (**Figure 6.20**) was most effective at preventing lysis at the lower concentrations, with its prevention of lysis being least effective at the highest concentrations.

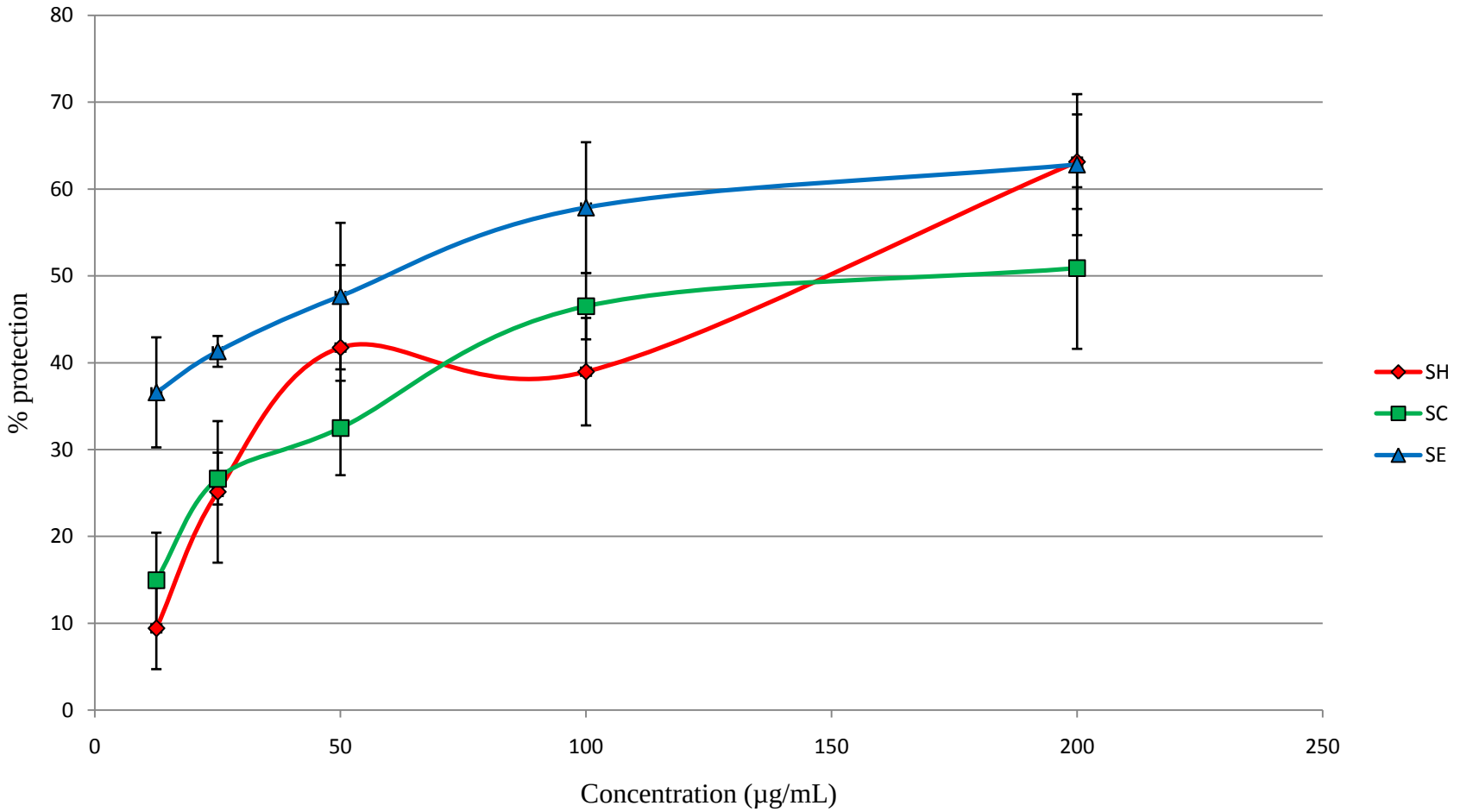


Figure 6.16: Membrane stabilizing activity of the stem extracts of *P. glaucus* under stress by hypotonicity.

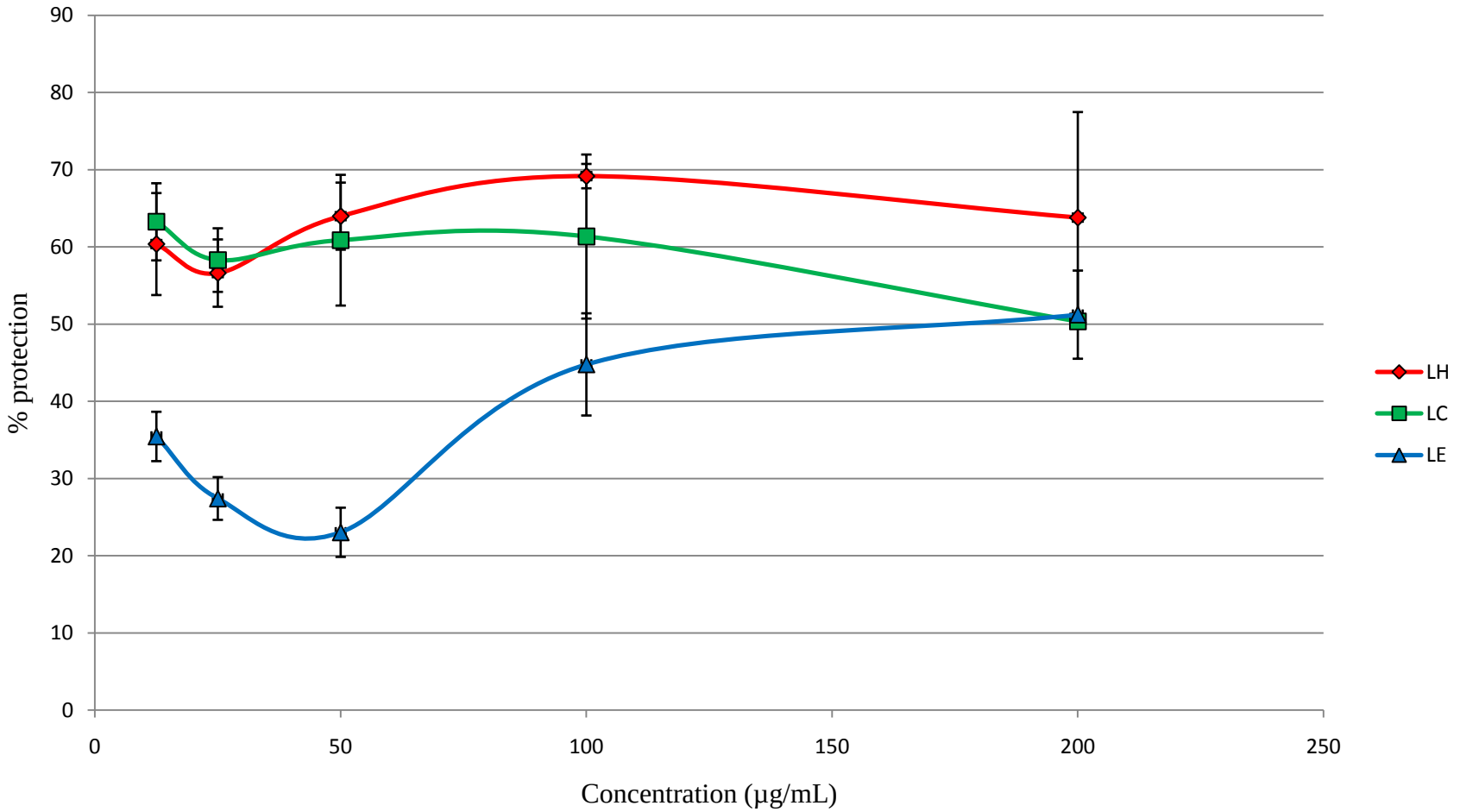


Figure 6.17: Membrane stabilizing activity of the leaf extracts of *P. glaucus* under stress by hypotonicity.

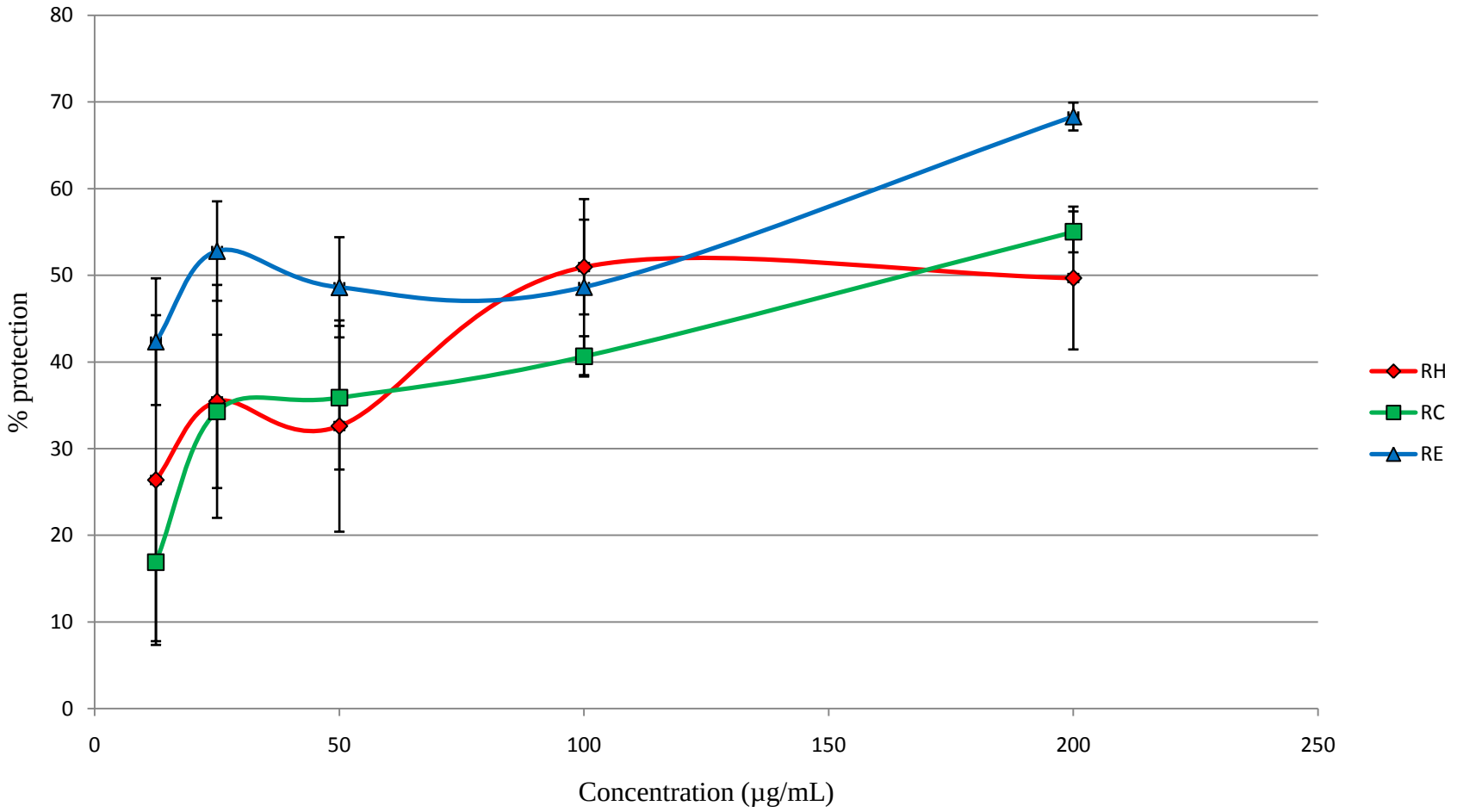


Figure 6.18: Membrane stabilizing activity of the root extracts of *P. glaucus* under stress by hypotonicity.

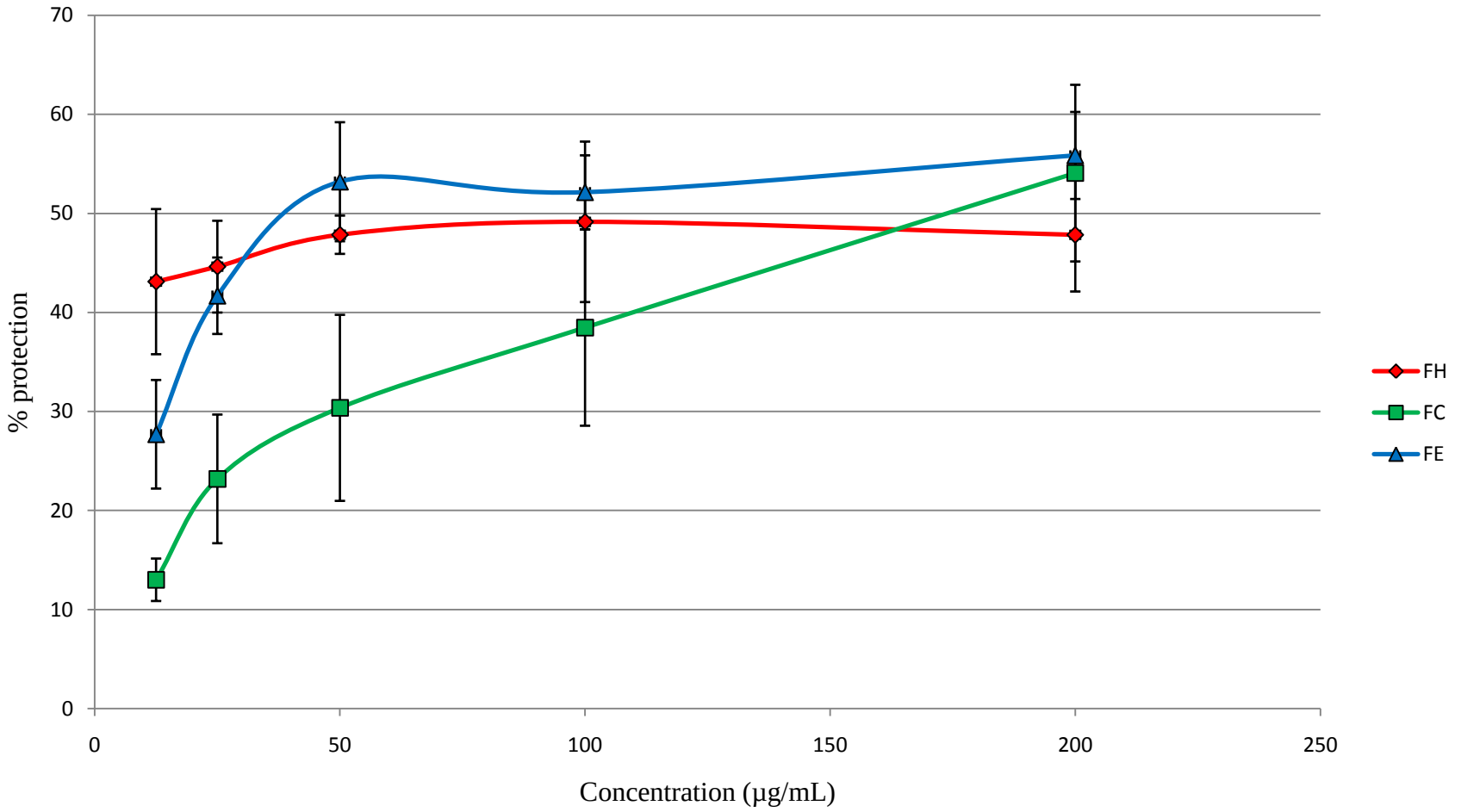


Figure 6.19: Membrane stabilizing activity of the fruit extracts of *P. glaucus* under stress by hypotonicity.

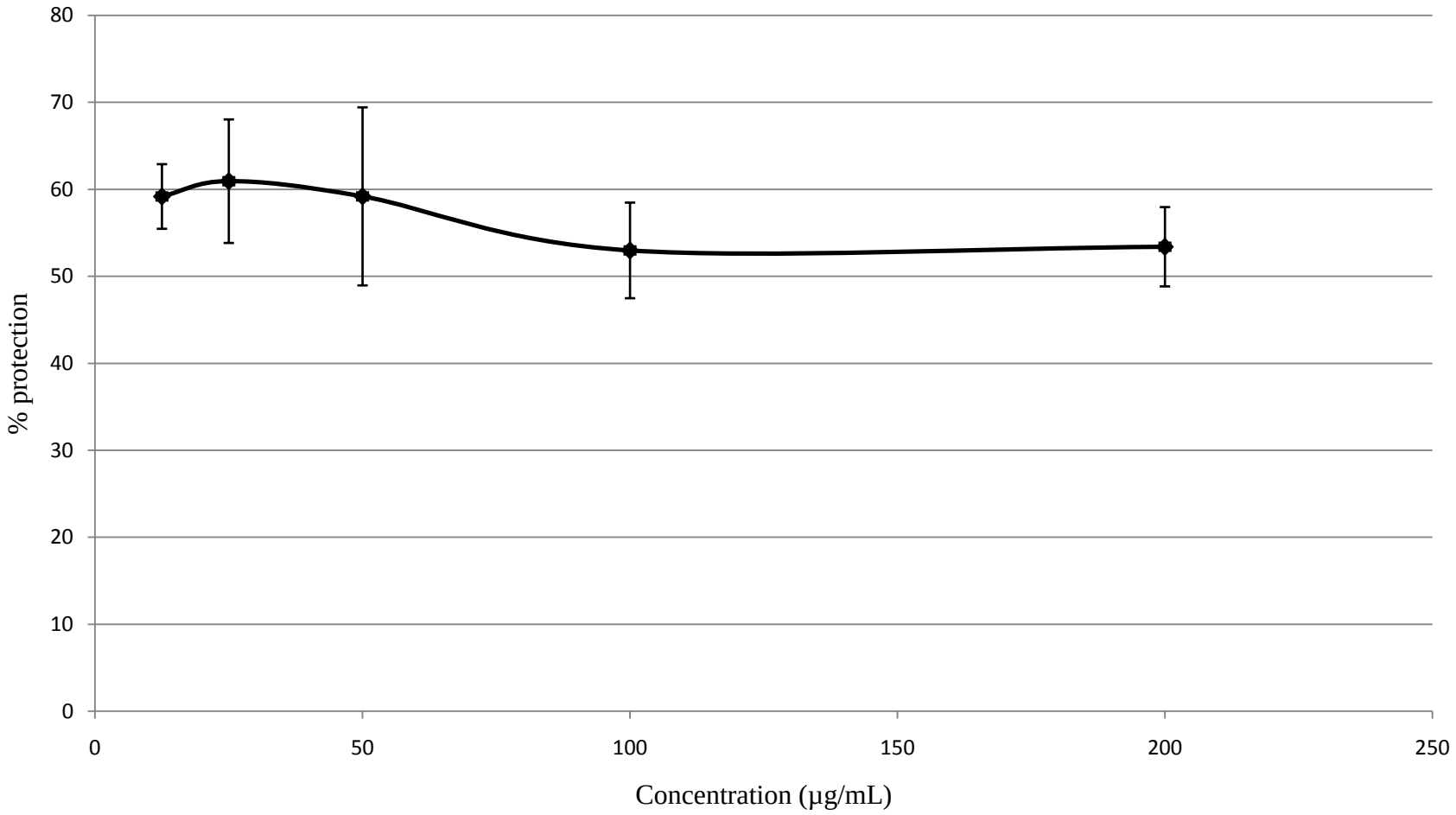


Figure 6.20: Membrane stabilizing activity of the stem alkaloid extract of *P. glaucus* under stress by hypotonicity.

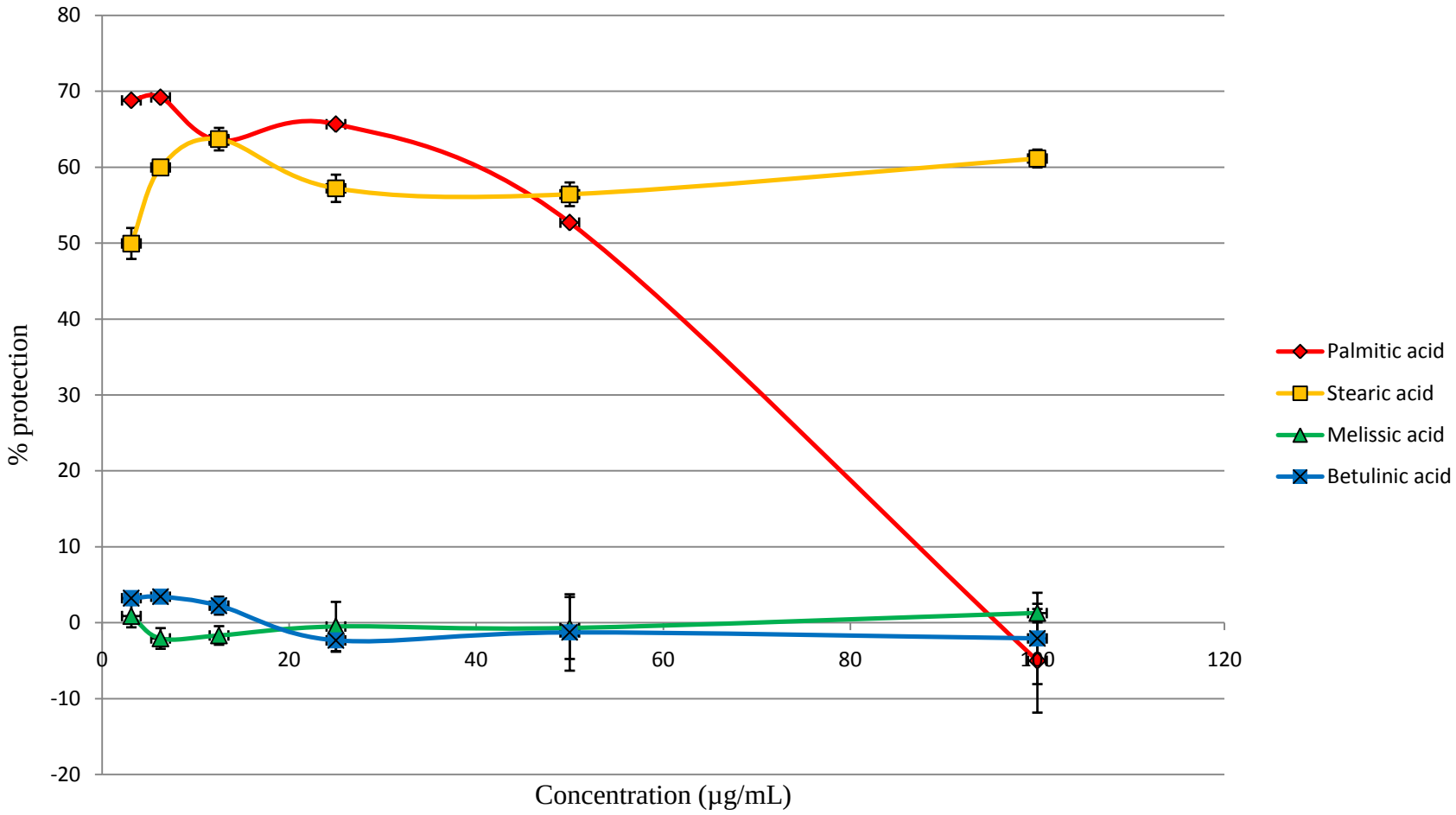


Figure 6.21: Membrane stabilising activity of fatty acids and betulinic acid under stress by hypotonicity.

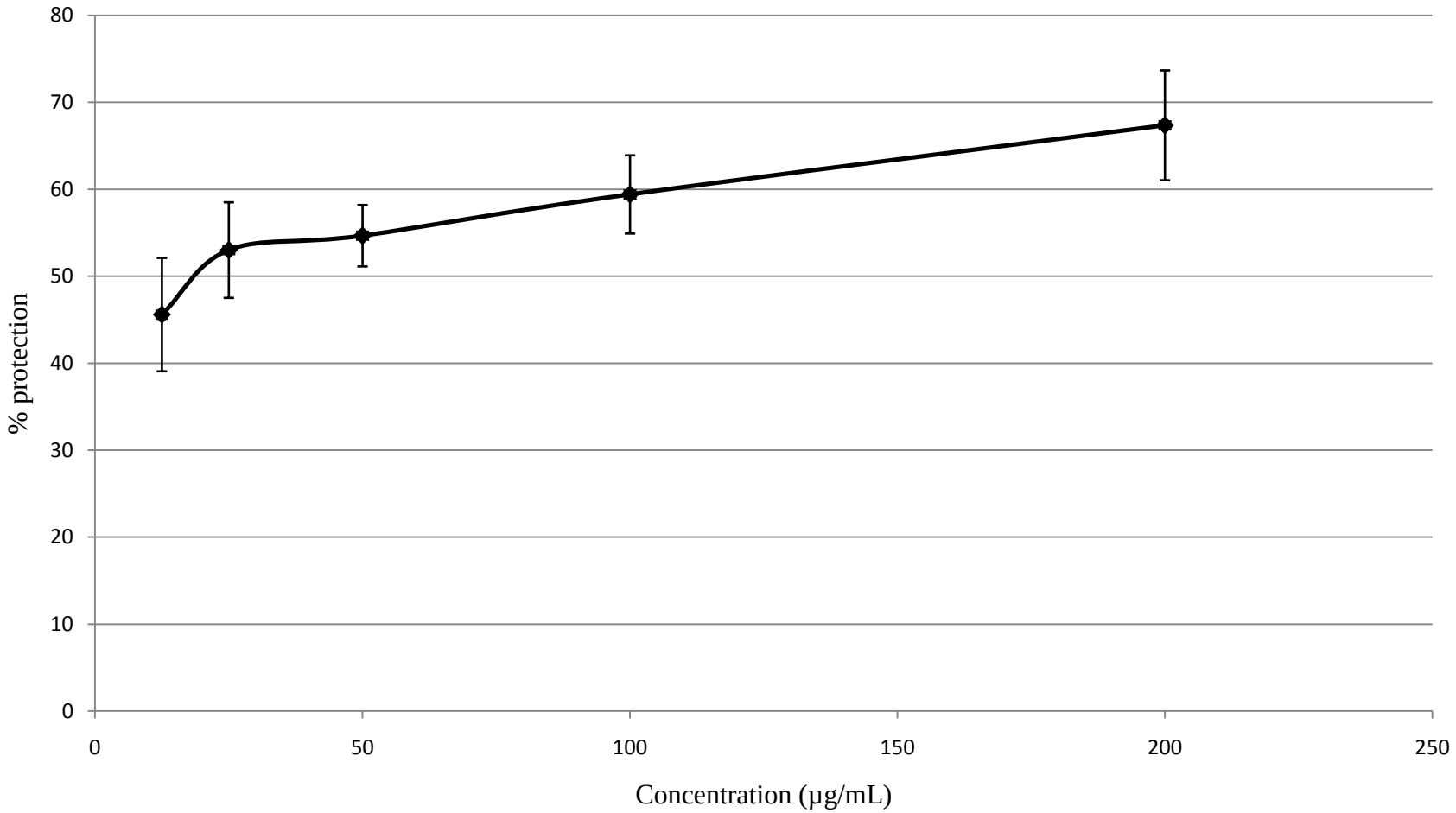


Figure 6.22: Membrane stabilizing activity of diclofenac sodium under stress by hypotonicity.

6.3.3: COX-1 and COX 2 inhibition assays

Table 6.1 shows that of the chloroform extracts showed strong inhibitory activity against COX-1 and COX-2 with the lowest inhibitory activity towards COX-1 being 97.7% inhibition by the fruit chloroform extract and all chloroform extracts completely inhibiting COX-2 at 500 µg/mL. The hexane extracts generally exhibited the lowest inhibitory activity towards the enzymes with the exception of the leaf hexane extract against COX-2 and the root hexane extract against both enzymes, which inhibited the enzymes by greater than 90%. The stem alkaloid extract also showed strong inhibitory activity towards the enzymes, inhibiting COX-1 and COX-2 by 78.10 and 96.80% respectively.

Periglaucine A inhibited COX-1 and COX-2 by 15.34 and 40.80% respectively (**Table 6.2**) while betulinic, palmitic, stearic and melissic acid appeared to enhance COX activity, with the fatty acids causing a significant apparent increase in activity.

Table 6.1: COX-1 and COX-2 inhibitory activity of *P. glaucus* at a concentration of 500 µg/mL.

	Enzyme inhibition (%)	
Sample at 500 µg/ml	COX-1	COX-2
Stem hexane	64.60 ± 0.23	63.08 ± 0.01
Stem chloroform	100.00	100.00
Stem ethanol	82.88 ± 1.29	100.00
Leaf hexane	55.06 ± 2.73	100.00
Leaf chloroform	100.00	100.00
Leaf ethanol	100.00	100.00
Root hexane	97.12 ± 0.69	95.60 ± 0.91
Root chloroform	97.96 ± 0.38	100.00
Root ethanol	53.88 ± 6.78	81.84 ± 0.77
Fruit hexane	26.85 ± 10.01	47.96 ± 3.32
Fruit chloroform	97.70 ± 0.55	100.00
Fruit ethanol	91.40 ± 1.93	99.21 ± 0.73
Stem alkaloid	78.10 ± 1.97	96.80 ± 0.73
Diclofenac	64.20 ± 3.16	100.00

Values for % inhibition that were over 100% are presented as 100%. Samples were tested in triplicate (n=3).

Table 6.2: COX-1 and COX-2 inhibitory activity of pure compounds at a concentration of 500 µg/mL.

	Enzyme inhibition (%)	
Sample at 500 µg/ml	COX-1	COX-2
Palmitic acid	-23.15 ± 1.61	-62.35 ± 13.52
Stearic acid	-22.02 ± 10.64	-47.19 ± 76.25
Melissic acid	-48.41 ± 7.37	-44.09 ± 9.46
Betulinic acid	-1.89 ± 18.92	-23.90 ± 16.12
Periglaucine A	15.34 ± 0.86	40.80 ± 22.86

Samples were tested in triplicate (n=3).

6.4: Conclusion

6.4.1: Protein stabilisation

Fatty acids, having both a hydrophobic tail and a carboxylic acid group that can form hydrogen bonds, could act as a chemical chaperone in the same manner as other chemicals used for protein stabilisation which may be why they displayed the ability to prevent denaturation of albumin by heat. The ability of fatty acids to maintain the stability of whey protein when exposed to heat has also been demonstrated in previous studies (Wijayanti et al. 2014).

Saturated fatty acids such as stearic acid can be taken up by the brain (Gozlan-Devillierre et al. 1976), some studies suggest that uptake of fatty acids may be by diffusion across the lipid bilayer (Hamilton & Brunaldi 2007) while others suggest a more specific and tightly regulated mechanism (Edmond 2001). Given the ability of some saturated fatty acids to stabilise proteins, they could possibly be used to prevent further protein aggregation which causes neurological disorders.

6.4.2: Membrane stabilisation

Five trends were seen in the HRBC membrane stabilisation assay, (1) a dose dependant increase in protective activity, (2) a dose dependant decrease in protective activity, an increase in protective activity followed by a drop and a subsequent (3) increase in protection, (4) decrease in protection or (5) initial increase in protection which falls at increasingly higher sample concentrations.

In the heat induced lysis assays, trend 1 was seen by the stem alkaloid extract, diclofenac, stearic, melissic and betulinic acid. Trend 2 was seen by the root, stem and leaf hexane extracts and palmitic acid. Trend 3 was seen by the root and fruit ethanol extracts. Trend 4 was seen by the leaf, root and fruit chloroform extracts, the stem and leaf ethanol extracts and the fruit hexane extracts. Trend 5 was seen by the stem chloroform extract. In the hypotonicity induced lysis assays, trend 1 was seen by the stem ethanol and chloroform extracts, fruit hexane, chloroform and ethanol extracts, root chloroform extract and diclofenac. Trend 3 was seen by the stem hexane, leaf ethanol and root ethanol extracts and stearic acid. Trend 4 was seen by the stem alkaloid extract and palmitic and betulinic acid. Trend 5 was seen by the leaf hexane, leaf chloroform and root hexane extracts.

It is possible that the initial increase in protection from lysis is dose dependant, however, at higher concentrations of the sample, a different effect is taking place which causes a different response. Samples which showed trend 1 may be simply stabilising membrane stability through a single step, samples showing trend 2 are causing additional stress towards the cells, enhancing rupture. Trends 3 to 5 show biphasic behaviour which may be due to a more complex interaction taking place. The initial dose dependant increase in protection followed by the unexpected drop in protection is similar to the dose dependant increase in substrate phosphorylation with increasing kinase concentration, which, at a certain point, causes double phosphorylation, leading to a drop in the levels of single phosphorylated substrate (Suwanmajo & Krishnan 2013). In the HRBC membrane stabilisation assay, compounds in the extract may be acting on components of the erythrocytes to cause stabilisation but at a certain concentration, may begin to cause an effect that counteracts the protective activity. The threshold at which the drop occurred in this assay would be dependent on the concentration of the compound(s) responsible for this activity in each of the extracts. The regained increase in protective activity may be due to a separate mechanism that is dose dependant while the subsequent decrease in activity exhibited by some samples may be due to a steady increase in the counteractive mechanism initiated after reaching the concentration that caused the first dip in protective activity. Differences in the affinity of the compounds of the extracts towards components of the erythrocytes which may affect membrane stability may also cause biphasic behaviour.

This effect may indicate an interaction with proteins in the cell or within the cell membrane as the concentration of proteins relative to the concentration of chemicals that interact with them can cause a counterintuitive response in some cases, for example, a solution containing a low concentration of albumin will require a greater quantity of tannins to cause aggregation than a solution containing a high concentration of albumin and the resulting aggregates will be different (Haslam 1989).

Interestingly, the extracts produced different response curves when erythrocytes were treated with different stressors. The root ethanol extract was the only extract which produced the same kind of response curve in both the heat and hypotonicity membrane stabilisation assays. This result strongly implies that different mechanisms are involved in the stabilisation of erythrocyte membranes under conditions of excessive heat and hypotonic environment,

however, the exact effect of heat and hypotonicity on erythrocytes and the induction of lysis is not fully understood and further work would have to be undertaken to fully understand the effect that was observed in the membrane stabilisation assays.

6.4.3: COX-1 and COX-2 inhibition

One particular class of compounds that are found in plants, the polyphenols, tend to have a strong ability to inhibit a number of enzymes, causing the enzyme to precipitate or complexing with the enzyme. Since polyphenols are found in all plants, some degree of inhibitory activity can be expected from a plant extract that is rich in polyphenols. The alkaloid fraction showed strong inhibitory activity towards both COX-1 and COX-2. The root and stem extracts, which contained detectable alkaloids, also inhibited enzyme activity significantly, but due to the low yield of alkaloid in the crude extracts, it is likely that other compounds in these fractions contributed more to the inhibitory activity than the alkaloids.

The fatty acids and betulinic acid appeared to enhance COX activity. Fatty acids can act as a substrate of the COX enzymes. This particular assay works by measuring the production of resorufin which is produced after the COX enzyme converts arachidonic acid to a hydroperoxy endoperoxide (PGG_2) and the peroxidase component, 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP) reduces this endoperoxide to its corresponding alcohol (PGH_2), resorufin. Since other fatty acids are able to bind to the active site of the COX enzymes (Dong et al. 2016; Vecchio et al. 2010), the reaction of the fatty acids with COX may produce an compound that also emits at the wavelength of 585-595 nm after further reduction. The binding of some fatty acids may also alter the enzymatic activity of COX by binding to the E-cat or E-allo sites of the enzymes, this can enhance or reduce the activity of COX inhibitors (Dong et al. 2016). COX-1 is able to bind to free fatty acids, however COX-2 is also able to bind to other substances, such as endocannabinoids, anandamide and arachydonoylglycine (Fowler 2007; Patsos et al. 2010; Patsos et al. 2005; Van Dross 2009). It is unknown whether the COX enzymes were able to bind with any other compounds in the extracts or what products would be made from their reaction. The lower selectivity of COX-2 may explain why greater enhancement of activity was observed in the enzyme assay when incubated with stearic and palmitic acid whereas COX-1 normally only binds favourably with certain free fatty acids (Rouzer & Marnett 2009). The difference in the substrates that can be bound by the COX enzymes may give rise to a different quantity of the same substrate being bound and converted into a product by the different isoforms. Betulinic acid was not able to

inhibit the COX enzymes in this study, but has been found to reduce COX-2 levels in other studies (Lin et al. 2015; Tsai et al. 2011).

Since the hexane extracts were the most likely to contain fatty acids which could act as substrates for COX, this may be why a lower inhibitory activity was seen. Unless the enzyme was completely inhibited, the fatty acids in the hexane extract could have been converted into products which, when added with the reading given by the products of the substrate provided, could give an enhanced reading. Essentially, the extracts containing fatty acids may have simply provided the enzymes with another substrate.

The COX enzymes produce a wide variety of products that play important roles in various biological functions. The enzymes are targeted for inhibition as they regulate inflammation and pain, however, inhibiting the enzymes for these specific purposes has wider consequences such as thrombosis and disruption in the production of the protective mucous lining of the stomach which can lead to gastrointestinal bleeding. The inhibition of an enzyme with such diverse biological activity has such wide consequences that more specific targets for reducing inflammation should ideally be sought. In order to more specifically target the problems that current NSAIDs hope to resolve, some researchers have focused their attention on PLA_2 inhibitors instead. PLA_2 is directly responsible for the release of arachidonic acid and other lipids from membrane phospholipids which then mediate an inflammatory response. The inhibition of PLA_2 would prevent disruption other normal physiological functions (Meyer et al. 2016).

Chapter 7: Cytotoxicity and anti-cancer activity of *P. glaucus*

7.1: Introduction

Cancer is a term used to describe a group of diseases in which cell growth has been deregulated and where the cells could possibly invade other tissues. Cancerous cells possess a variety of traits which may include an ability to induce angiogenesis, ability to migrate and invade other tissues, immortality, ability to grow independently, an unresponsiveness to growth suppressive signals and ability to evade programmed cell death.

The most common types of cancer diagnosed in 2012 were lung, breast, colorectal, prostate and stomach cancer respectively, with men being more likely to develop cancer than women. In 2012, about 14.1 million new cases of cancer and 8.2 million deaths were reported globally (Cancer research UK), this is expected to rise to 21.7 million new cases and 13 million deaths by 2030 (American Cancer Society and IARC).

7.1.1: Development of cancer

Cancer arises due to mutation of a certain set of genes that are responsible for regulating the cell cycle and the cells response to damage. It is inevitable that, over time, cells will accumulate mutations. Each time a cell divides, the process of DNA replication itself will lead to the incorporation of mutations throughout the genome as no polymerase can replicate DNA perfectly (Berg et al. 2002).

Mutations can also be accumulated through damage to DNA from external sources, such as UV damage and chemical damage. Cells have adapted different ways of repairing DNA, such as excision repair, and in cases where double strand DNA breaks occur, such as from damage cause by x-rays, the cell may attempt to match up the correct strands of DNA through homologous recombination. However, DNA repair is not always carried out perfectly, and the likelihood of gaining mutations increases the more DNA damage it receives (Mathews et al. 2013).

It normally takes a long period of time to accumulate a sufficient number of mutations in a set of genes that will lead to the formation of cancerous cells. As such, cancer generally tends to affect more of the older population compared to the younger population (Hesketh 2013).

7.1.2: Causes

Since cancer is a disease caused by genetic mutation, anything that causes genetic mutation can potentially contribute to the development of cancer. Replication of DNA in dividing cells can lead to the incorporation of mutations, cell metabolism creates ROS as a by-product which can cause DNA damage. Organisms are constantly receiving damage to their DNA from normal cellular processes, much of which, under normal circumstances, they can repair. There are many other things that can make an organism more susceptible to developing cancer, such as exposure to mutagenic chemicals, radiation, an unhealthy lifestyle, infection by bacteria and viruses, hormones and genetics (Hesketh 2013).

Lifestyle is the most contributing factor to the development of cancer with the smoking of tobacco being the biggest cause of cancer (Van Zandwijk & Fong 2015). Individuals with a high percentage of body fat can also be at greater risk of developing cancer due to the fact that adipose cells release hormones which affect cell growth. Alcohol consumption will increase the risk of developing cancer as it is metabolised into mutagenic products, it can increase the cells susceptibility to damage and effects hormones. Individuals who are sedentary are at increased risk of certain cancers as a healthy level of activity helps to regulate hormones and maintain healthy bowels. These contributing factors mean that cancer is preventable, to a certain degree, by making changes to one's lifestyle (Hesketh 2013).

7.1.3: Key genes and pathways in cancers

Mutations can be accumulated throughout the genome, but only mutations in certain genes will lead to the development of cancer, namely oncogenes and tumour suppressor genes. Generally, the over activity of an oncogene and the under activity or deactivation of a tumour suppressor gene can lead to the development of cancer.

Oncogenes and proto-oncogenes promote cell growth, to cause cancer they can be mutated or over expressed. The over expression of oncogenes can prevent cell death where a cell would normally undergo programmed cell death due to an alteration in critical cell functions. Oncogenes encode growth factors, transcription factors and other enzymes that are involved in cell signalling, proliferation and cell cycle regulation.

Over expression can occur when the oncogene is translocated to a different position where an upstream promoter causes greater expression than normal. Over expression can also be caused by amplification of the gene, where the gene is copied multiple times and, as a result, is expressed may times more than normal. A mutation in the gene can cause the encoded protein to increase in activity or become unregulated (Hesketh 2013).

Some proto-oncogenes include RAS and MYC. RAS is a family of proteins that are small GTPases which are involved in cell signalling of growth, differentiation and survival. Mutation of RAS is found in approximately 20-30% of all cancers, making this gene a target for some cancer treatments (Bos 1989). MYC encodes a transcription factor which is involved in the regulation of the cell cycle, apoptosis and transformation. MYC is found mutated or translocated in many cancers and since it regulates a significant percentage of genes, the overexpression of this gene is able to cause significant disruption to normal cellular functions (Hesketh 2013).

The WNT and MAPK/ERK signalling pathways are found disrupted in many cancers. There are a number of different proteins involved in the WNT signalling pathways which, if mutated or overexpressed, can contribute to the development of cancer. The MAPK/ERK signalling pathway involves a number of proteins, including RAS and MYC, that work to transmit a signal from a cell surface receptor to the cell nucleus. A mutation in the proteins involved in this pathway can lead to unregulated growth of cells and thus the development of cancer and this pathway is the target of a number of anti-cancer drugs which are able to act by inhibiting the pathway (Thompson & Lyons 2005).

Tumour suppressor genes act by downregulating the expression of genes that are involved in the progression of the cell cycle, preventing cell division in the presence of DNA damage, initiating apoptosis when the cell has undergone irreparable damage, controlling cell adhesion and DNA repair. When a tumour suppressor gene is unable to function correctly due to mutation and becomes deactivated or its activity hampered, this can lead to uncontrolled cell proliferation (Hesketh 2013).

Some tumour suppressor genes include TP53, PTEN, BRCA1, BRCA2 and RB. The tumour suppressor gene that is most commonly found to be mutated or missing in cancers is TP53 which encodes 15 known protein isoforms. These proteins regulate gene expression by

binding to DNA where they work to activate genes involved in DNA repair, growth arrest and apoptosis initiation. TP53 is activated as a response to stress such as DNA damage, oxidative damage and deregulated oncogene expression, by protein kinases (Hesketh 2013). p53 can be affected not only by mutations in the gene encoding it, but also by pathogens such as human papillomavirus (HPV) (Thomas et al. 1999).

RB1 encodes pRB, which prevents the cell cycle from progressing from G1 phase by binding to E2F transcription factors. When pRB is expressed, the cell will remain in G1 phase. This gene is found to be mutated in familial retinoblastoma, where the mutation is inherited from a parent or mutated early in life, it is also mutated in some sporadic forms of cancer (Goodrich 2006). pRB is the target of proteins whose expression is increased by HPV (Goodrich 2006), as such, HPV infection can lead to the formation of cancer.

PTEN is a gene which is often found to be lost in cancers. It negatively regulates the Akt/PKB pathway, so promotes apoptosis, arrests the cell cycle and stops angiogenesis. Mutation of this gene can lead to the Akt/PKB pathway becoming unregulated, promoting cell survival, cell cycle progression and angiogenesis (Carnero 2010).

The genes BRCA1 and BRCA2 are responsible for a number of functions including repairing double strand DNA breaks by homologous recombination, transcription and transcriptional regulation. If DNA damage is irreparable, they can initiate apoptosis. If either of the genes is mutated, this means that DNA damage will not be repaired properly and will therefore lead to a much greater chance of developing mutations in other oncogenes or tumour suppressor genes (Wu et al. 2010).

Tumour suppressor genes are involved in suppressing growth and repairing DNA damage, as such, if just one of the tumour suppressor genes is mutated, this compromises the stability of the genome, making it more likely that further mutations will be accumulated (Hanahan & Weinberg 2011).

7.1.4: Genetic predisposition

Although sporadic mutations are most commonly the cause of cancers, there are cases where people carry a genetic mutation which they acquired from a very early stage in life (*in utero*)

or from their parents, which makes them more susceptible to developing cancer (Fearon 1997).

Some examples of genes whose mutation can lead to certain diseases or increase chances of developing certain types of cancer are listed in **Table 7.1** below.

Table 7.1: Genes and their links to cancer.

Gene	Implication in disease
TP53 (Tumour suppressor gene 53)	Li-Fraumeni syndrome: Leads to the early onset of cancer which can be of different varieties. Multiple cancers can develop throughout an affected individual's lifetime (Srivastava et al. 1990).
ATM (Ataxia telangiectasia mutated)	Ataxia telangiectasia: Increased risk of certain cancers, in particular lymphoma and leukaemia (Armata et al. 2007).
APC (Adenomatous polyposis coli)	Familian adenomatous polyposis: Causes the growth of a high number of polyps in the bowel which can then develop into cancer (Waller et al. 2016).
BRCA1 and BRCA2 (Breast cancer 1 & 2)	Breast cancer: Mutations in either of these genes leads to a greater chance of developing breast cancer. It can also increase the chances of developing prostate cancer (Levy-Lahad & Friedman 2007).
RB1 (Retinoblastoma gene 1)	Retinoblastoma: A large proportion of children who develop retinoblastoma have a faulty copy of RB1 (Pandey 2014).
PTEN (Phosphatase and tensin homolog)	Cowden syndrome: Causes the growth of hamartomas in numerous parts of the body and increases the

	risk of breast, endometrial, thyroid and kidney cancers (Eduard Yakubov 2016).

Genetic testing can be done on individuals who suspect they may have inherited a genetic mutation that predisposes them to cancer, which may help to make them aware of their risk of developing certain types of cancers and possibly encourage healthier lifestyle choices. After a person is diagnosed with cancer they may also have genetic testing done which will help to determine the prognosis and optimal treatments (Plichta et al. 2016).

7.1.5: Current treatments

Commonly used treatments for cancer that are currently used include chemotherapy, radiotherapy, surgery, targeted therapy and immunotherapy.

Surgical removal of a tumour can be carried out given that the tumour is accessible and the surgical procedure does not carry a very high risk. This can sometimes be used to cure the disease, remove part of a tumour or ease pressure and this improve quality of life.
Chemotherapy is a common treatment option with a large number of cancer patients receiving this form of treatment. The principle upon which chemotherapy is based is that normal cells are better able to repair damage than cancer cells and that cancer cells are more rapidly dividing that most normal cells. Chemotherapy drugs are carefully dosed so that they are effective at treating cancer but not intolerably toxic to the patient.

Some chemotherapy involves inflicting damage upon cells so that cells which are unable to repair the damage will receive such a great volume of damage to their genome that they will apoptose and the cells that will be most likely unable to repair the damage and thus receive the most damage will be cancer cells. Alkylating agents are an example of a type of drug that works in this way (Payne & Miles 2008).

Other chemotherapy drugs work by damaging cell that are actively dividing, inhibiting DNA/RNA synthesis or becoming incorporated into newly synthesised DNA/RNA and causing DNA damage that triggers cell death. Antimetabolites are an example of a type of drug that works in this way and their ability to exert their effect is limited by the stage of cell

cycle that the cell is in. Since they affect DNA/RNA synthesis, they only act on cells that are in the S-phase (Payne & Miles 2008).

Inhibition of topoisomerases 1 or 2 can exert an anticancer effect. These enzymes are involved in reducing the stress on DNA by creating single or double strand breaks during the unwinding of DNA and tightening of supercoils which accompany the unwinding process. Inhibition of these enzymes can lead to uncontrolled breaks and prevents DNA transcription (Payne & Miles 2008).

Antimicrotubule agents can be used to halt cell division by preventing the formation or disassembly of microtubules. After a cell has replicated its DNA, microtubules are responsible for cell division, although microtubules are important in many other cellular functions. Since antimicrotubule agents will prevent proper assembly of microtubules, although a cell may be able to replicate its DNA, the cell will not be able to divide afterwards, thus cell proliferation will be prevented (Payne & Miles 2008).

Some common side effects of chemotherapy include nausea and vomiting, gastrointestinal disturbances, fatigue, hair loss and mucositis (Lotfi-Jam et al. 2008). Nausea and vomiting, in particular, can lead to patients refusing or postponing treatment (Mustian et al. 2011). Due to the damaging, mutagenic nature of chemotherapy drugs, it is possible for chemotherapy to result in the development of a secondary neoplasm (Petru & Schmähl 1991; Remer 1982). Chemotherapy drugs are also teratogenic and can lead to significant damage in the first trimester of pregnancy (Koren et al. 2013).

Chemotherapy is not always successful in curing cancer, the 5-year survival rate for adults with malignancies who had received chemotherapy alone was estimated at 2.3% and 2.1% in Australia and the USA respectively which suggests that chemotherapy, when used alone, does not contribute significantly to survival (Morgan et al. 2004). Other methods have been found to yield a more optimistic prognosis.

Radiotherapy involves targeting ionising radiation at the site of the cancer and reduces damage to normal tissues by using multiple beams from different angles that intersect at the target site. Ionising radiation causes DNA damage and formation of free radicals which leads to cell death (Choi & Cho 2016). Side effects of radiation therapy include tissue damage,

damage to blood vessels and connective tissue, sores, intestinal discomfort, swelling and infertility, depending on the area being treated.

Targeted therapies, including immunotherapy, target a faulty protein or enzyme which is found specifically in cancer cells and is required in order for the cancer cell to persist. Targeted therapy is currently undergoing much research and development but is expected to be more effective than chemotherapy and cause less damage to normal cells (Sawyers 2004). Some other treatments include stem cell therapy which involves transplanting stem cells into a patient whose cells have been destroyed by chemotherapy, radiotherapy or disease (Sharp & George 2014), and hyperthermia involves the use of heat to enhance chemotherapy treatment, photodynamic therapy uses photosensitizing agents and light to destroy cancer cells (Kobayashi 2011). These treatments are used much less commonly compared to chemotherapy and standard surgery.

Current treatments provide many patients with the hope of being cured of cancer, however, there is much improvement that is required for a more effective treatment for cancer to be developed. Treatments such as chemotherapy can damage normal cells as well as the cancer cells and lead to many unpleasant side effects.

7.1.6: Plants as treatments

Herbs used for the treatment of cancer can work in a variety of ways to treat cancer, some herbs used are toxic or can be toxic if consumed frequently. There are large numbers of other plants which have been shown to exhibit an anticancer effect through a variety of different mechanisms including inhibition of telomerase, apoptosis of cancer cells and by boosting the immune system.

Much research has been done on the potential use of certain herbs as anticancer agents and some of this research has led to the development of new chemotherapy drugs, such as the vinca alkaloids, vincristine and vinblastine from the periwinkle plant (*Catharanthus roseus*). These particular alkaloids are spindle toxins, so act by disrupting microtubule formation which prevents cells division (Wilson 1986). Although herbal medicines are commonly believed to have fewer negative side effects than traditional chemotherapy drugs, chemicals derived from plants can be just as potent and toxic as synthetic medicines. Some traditional

medicines have been shown to improve the quality of life of cancer patients (Wu et al. 2016) and improve survival (Tröger et al. 2013), while some have been shown to produce a number of adverse effects (DiPaola et al. 1998; Ben-Arye et al. 2016).

Traditional medicines are used by a large number of people for the treatment of cancer and other diseases across the world (Ekor 2014; Yin et al. 2013). Many researchers have studied the anticancer activity of numerous traditional medicines, however clinical studies are lacking. Yet, a significant portion of cancer patients will turn to these medicines and some will use them in combination with traditional chemotherapy. This combined use of drugs is a concern for health practitioners as the potential drug interactions are not known (Gratus et al. 2009).

Despite the lack of proper studies done on the efficacy and safety of these remedies, over 65% of cancer patients use herbal medicines in some populations (Ernst 2000).

7.1.7: Apoptosis pathways

Apoptosis can be described as a series of morphological changes in a cell that result in cell death. It is a tightly controlled, programmed form of cell death which can be part of the normal development of an organism or can be induced by certain factors such as damage.

After apoptosis is initiated, the organelles will begin to be degraded by caspases. The cell will shrink due to the breakdown of the cytoskeleton, making the cytoplasm appear dense and packing organelles together tightly. Pyknosis, the condensation of chromatin, occurs and the nuclear envelope becomes discontinuous, the DNA becomes fragmented and breaks apart into nucleosomal units. The cell membrane will begin to form blebs (irregular buds) and the cell will finally break apart into apoptotic bodies which are phagocytosed (Elmore 2007).

Apoptosis can be activated by the intrinsic or extrinsic pathways. The intrinsic pathway can be activated by lack of growth factors, DNA damage, heat and radiation exposure and involves the mitochondria. The extrinsic pathway can be activated by death ligands or stresses such as exposure to cytotoxic drugs or radiation. This pathway involves the activation of pro-apoptotic receptors (death receptors) on the cell surface by pro-apoptotic ligands (death ligands). The binding of the ligand to the receptor causes the receptors to cluster together to form a death-inducing signalling complex (DISC) activate caspase-8 which in turn activates a sequence of caspases that lead to apoptosis (Fulda & Debatin 2006).

Although certain chemicals are able to induce apoptosis, they may not necessarily produce this effect in all cells. Glucocorticoids, for example, are able to promote the growth of some cells, but cause other to apoptose (Nieto et al. 1992; Ninomiya et al. 2014).

p53 expression is induced by stress, so irradiation and chemotherapy drugs generally induce cell cycle arrest and apoptosis through a p53-dependant pathway. p53 expression can also induce expression of Fas, a protein in the TNF receptor family, which allows more Fas/FasL binding, leading to cell death (Hotchkiss et al. 2000).

7.1.8: Determining cytotoxicity

The cytotoxicity of substances can be determined by a variety of *in vitro* methods. The MTT assay is one commonly used method which measures the ability of cells to convert the salt, MTT, into purple formazan crystals (Mosmann 1983). The quantity of formazan that is produced is dependent on cell metabolism. As such, if there is low toxicity, cell survival will be greater and more formazan will be formed, whereas if there is high toxicity, cell survival will be lower and there will be fewer healthy cells to metabolise the salt into formazan. Additionally, if the substances increase metabolism and do not induce cell death, more formazan will be produced and *vice versa*. It is possible that the compounds in an extract may exert different degrees of toxicity as well as affect metabolism. Observation of the morphology of the cells is therefore an important indicator of the reliability of the MTT assay as a measure of cytotoxicity, so that effects on metabolism can be taken into account, as well as possible interactions of samples with MTT which can lead to false positive results (Maioli et al. 2009; Rollino et al. 1995).

7.1.9: Cell lines used for studying cancer in vitro

There is a vast collection of cell lines that are available for culturing in the laboratory. Cells grown for the purposes of research are often immortalised, immortal cells can be passaged indefinitely. Non-immortalised cells can be passaged a limited number of times before their ability to divide becomes poorer as the cells become senescent. Since normal cells are not immortal, immortalised cells are genetically and biologically different to cells found in a living organism (Yeager 1999).

In the present study, two cell lines will be studied to determine the cytotoxic activity of *P. glaucus* extracts and known components, HK1 (Huang et al. 1980), a nasopharyngeal carcinoma and MRC5, lung epithelial cells.

Although nasopharangeal carcinoma is often linked to the Epstein-Barr Virus (EBV), HK1 is an EBV-free, human carcinoma. The EGFR signalling pathway and beta catenin signalling is enhanced (Szeto et al. 2014) and this upregulation of EGFR is thought to be responsible for the sensitivity of this particular cell line to gefitinib (Ma et al. 2010). Quercetin has also been shown to inhibit the growth of HK1 (Daker et al. 2012).

MRC-5 is a fibroblast cell line derived from the normal, human lung tissues of a healthy, 14 week old foetus (Jacobs et al. 1970). This cell line is used for the production of vaccines, cytotoxicity testing and as a host for viral transfection, as a results it is mentioned in a large number of publications that cover a variety of topics.

The cytotoxicity of the extracts towards the normal and carcinoma cell lines may give some indication as to the toxicity of *P. glaucus* towards cancer cells and its effect on normal cells.

7.2: Materials and methods

7.2.1: Chemicals and reagents

DMSO was purchased from RCI Labscan (Thailand), RPMI 1640, FBS and PBS were purchased from Thermofisher, quercetin and MTT were purchased from Sigma Aldrich (USA), periglaucine A was purchased from ChemFaces (China), palmitic acid, stearic acid, melissic acid and betulinic acid were purchased from Guokang Biotechnology (China).

7.2.2: Cell lines and cell culture media

The cell lines HK1 and MRC-5 were used in the cytotoxicity assays. MRC-5 was obtained from ATCC (CCL-171) while HK1 was donated by Prof. Tsao from the University of Hong Kong. The cell lines were cultured in RPMI 1640 supplemented with 10% FBS at 37 °C in a humidified incubator with 5% CO_2.

7.2.3: MTT assay

Plant extracts were prepared by dissolving in DMSO and RPMI 1640 to make a stock solution which had a final concentration of 0.1% DMSO. This stock solution was serially diluted to make different concentrations of the extracts which ranged from 200 to 6.25 µg/mL.

The MTT assay was performed as described by Mosmann (1983), with modifications. HK1 or MRC-5 cells were grown in RPMI 1640 with foetal bovine serum (FBS). Freshly trypsinised cells were seeded into a 96 well plate, at 5,000 cells per well and 100 µL of medium per well. The cells were left for 24 h before removing the media and adding the plant extract at different concentrations. Untreated cells were used as a negative control and cells treated with quercetin were used as the positive control. Cells were incubated with the extracts for 72 h after which the extract was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) added. The MTT was prepared by dissolving the MTT salt in phosphate buffered saline (PBS), making a final concentration of 5 mg/mL. This stock solution was diluted 1:10 into R_0 RPMI. 100 µL of this medium, containing MTT, was added to each well and incubated at 37 °C for 4 h, after which 75 µL of medium was removed from each of the wells. 50 µL of DMSO was added to each well and mixed thoroughly with a pipette before incubating for 10 min. Each experiment was run in triplicate of triplicates (n=9). Each sample was mixed again and the absorbance read at 540 nm using a microplate reader.

7.3: Results

7.3.1: Anti-cancer activity against HK1

The results of the anticancer activity assay with HK1 (**Figures 7.1-7.8**) gave results that would suggest a variety of actions. The inverted "U" shape of the curves generated by the stem hexane, leaf chloroform, root hexane and root chloroform extracts would suggest that the extracts have a growth promoting activity, but at higher concentrations, initiate cell death. The stem chloroform extract appeared to promote proliferation. The ethanolic extracts all reduced proliferation, which suggests that the compounds of chemotherapeutic value are mainly polar compounds. The results of the stem alkaloid extract implied a possible hormetic response by the cells, as proliferation was significantly enhanced at lower concentrations, but not as greatly at higher concentrations. This same type of response was seen when the cells were treated with periglaucine A, strongly suggesting that this response is due to the

alkaloids. The fatty acids and betulinic acid all elicited a dose dependant reduction in proliferation of HK1 cells. The samples which exhibited the greatest toxicity towards HK1 were quercetin, betulinic acid and the root ethanol extract, for which IC_{50}s of 8.65, 31.5 and 60.23 µg/mL were obtained, respectively (**Table 7.2**).

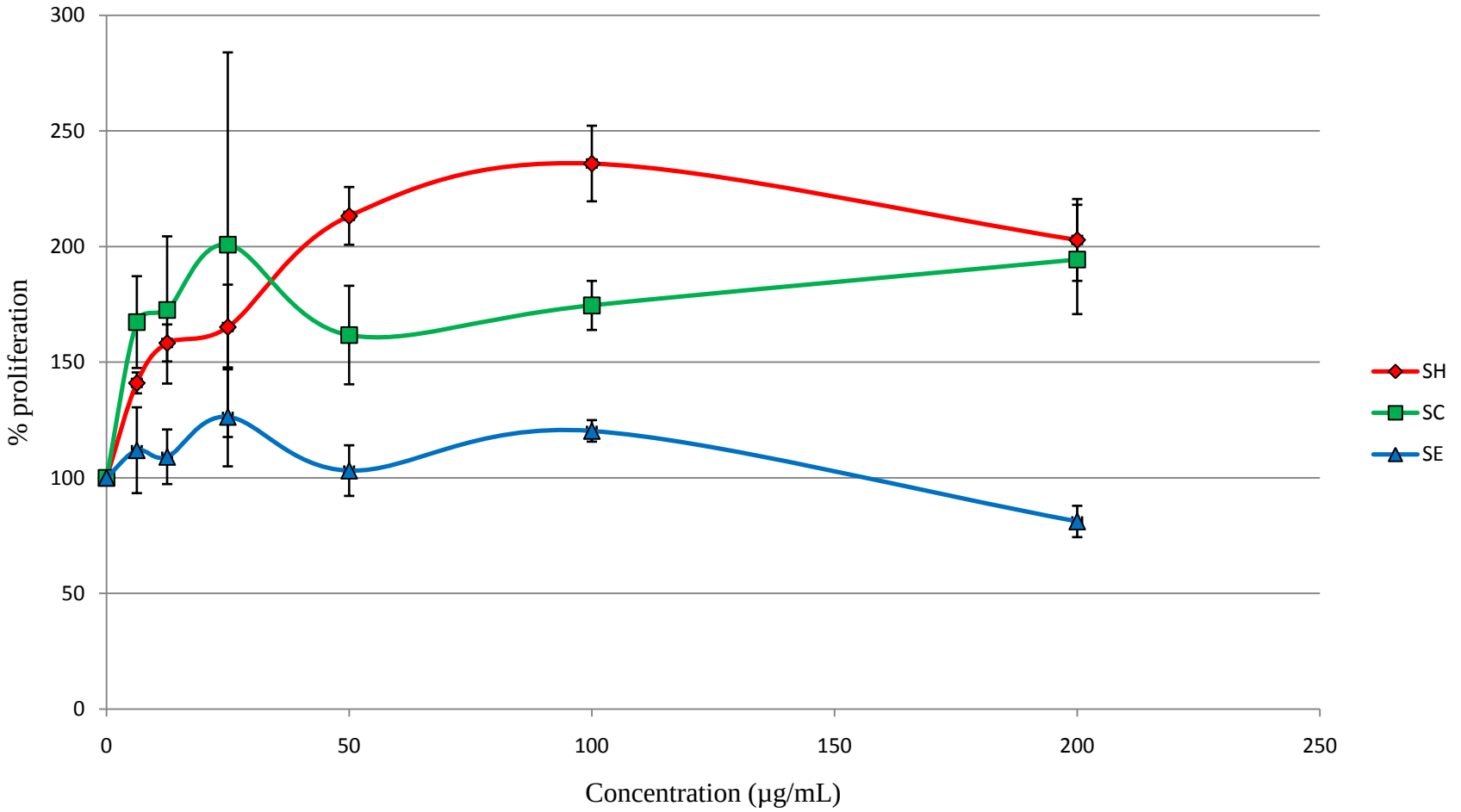


Figure 7.1: Activity of *P. glaucus* stem extracts against HK1.

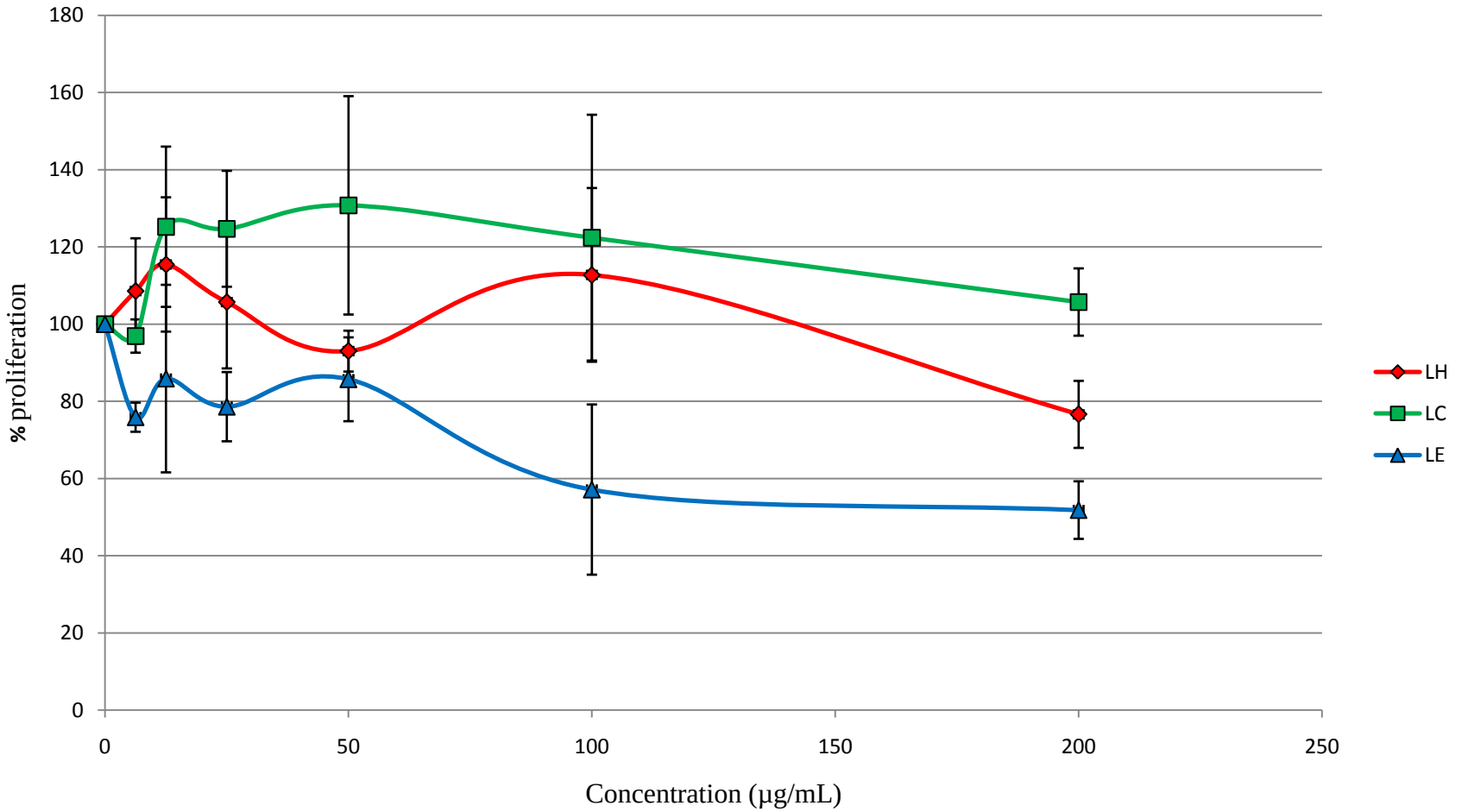


Figure 7.2: Activity of *P. glaucus* leaf extracts against HK1.

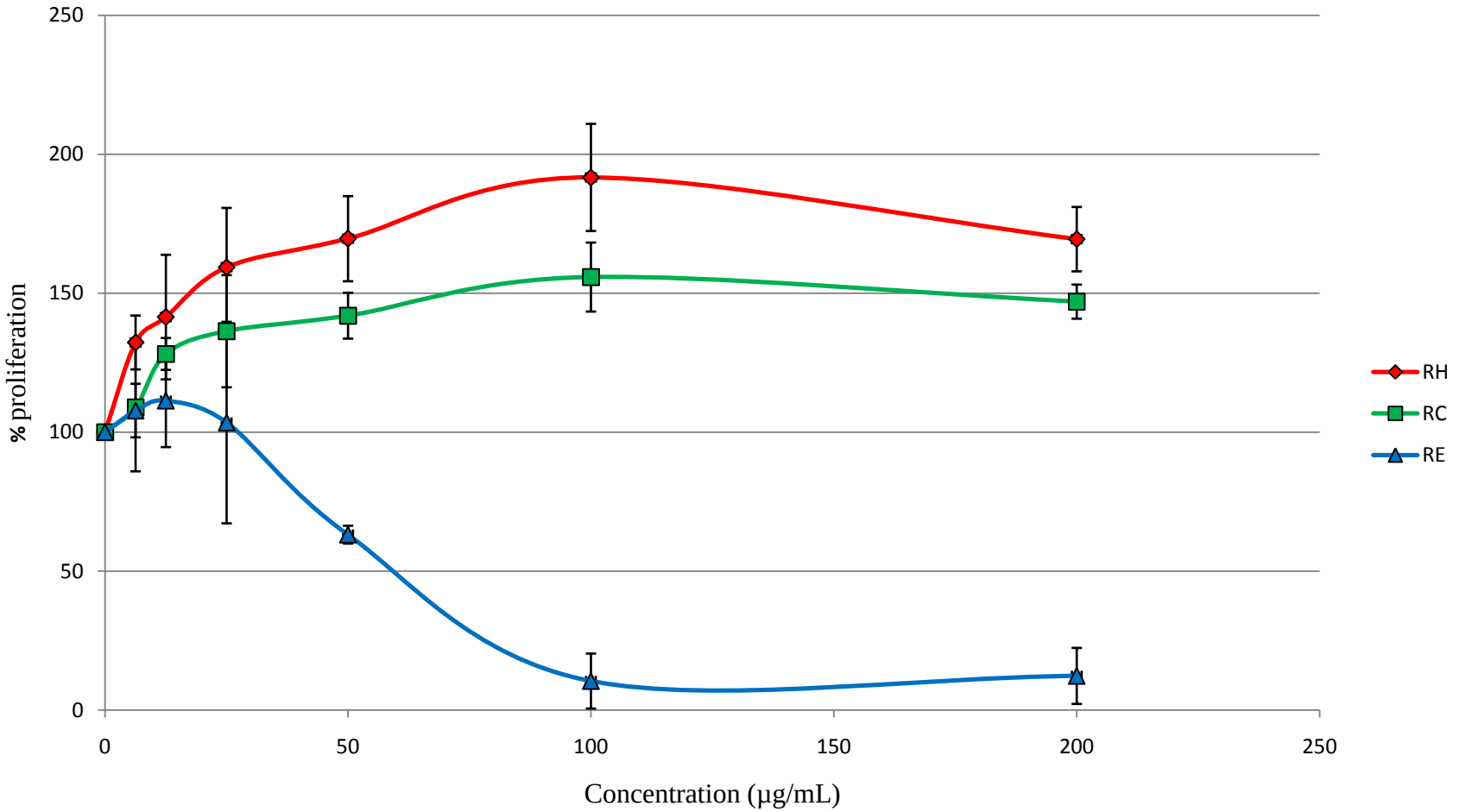


Figure 7.3: Activity of *P. glaucus* root extracts against HK1.

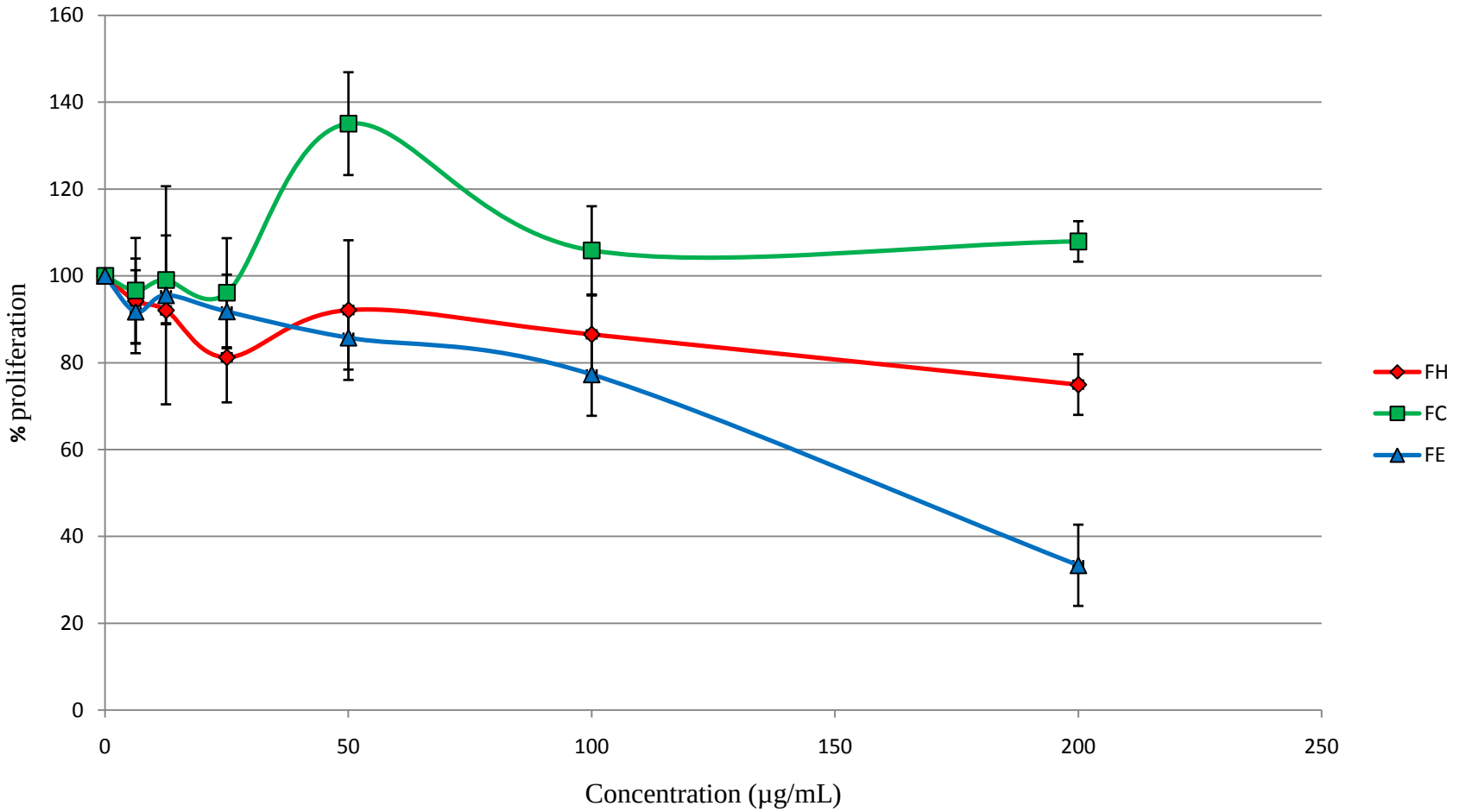


Figure 7.4: Activity of *P. glaucus* fruit extracts against HK1.

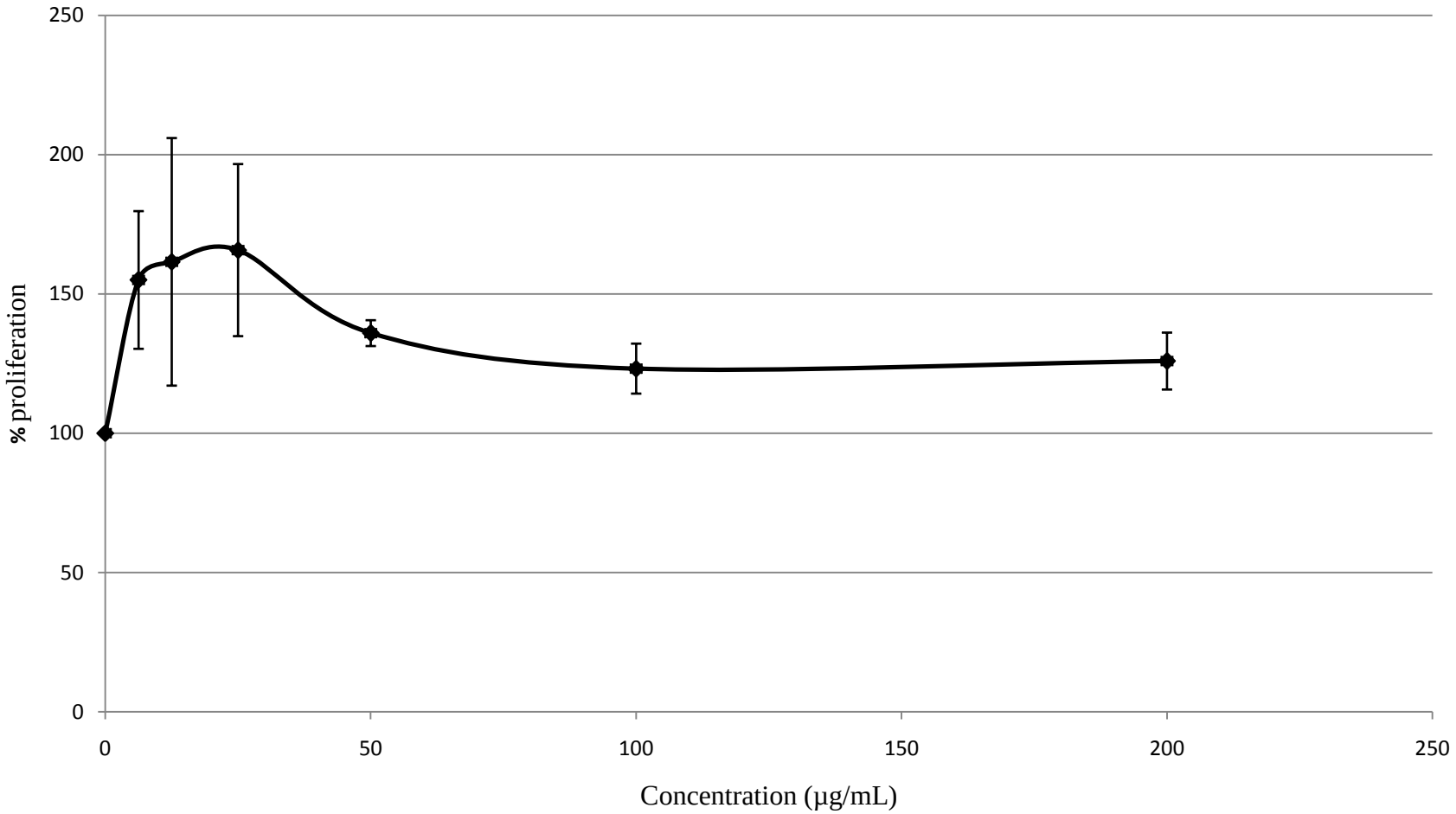


Figure 7.5: Activity of *P. glaucus* stem alkaloid extract against HK1.

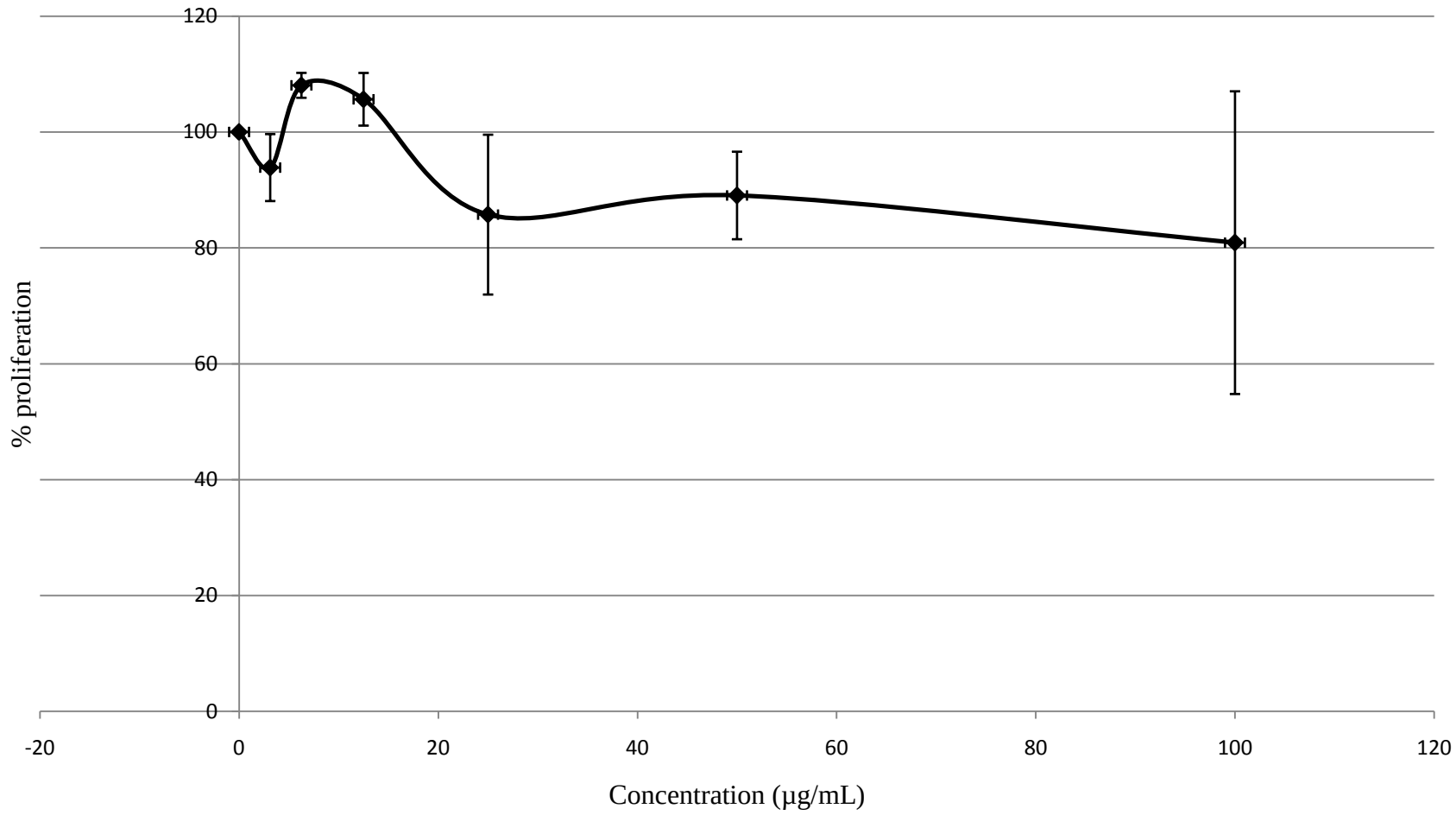


Figure 7.6: Activity of periglaucine A against HK1.

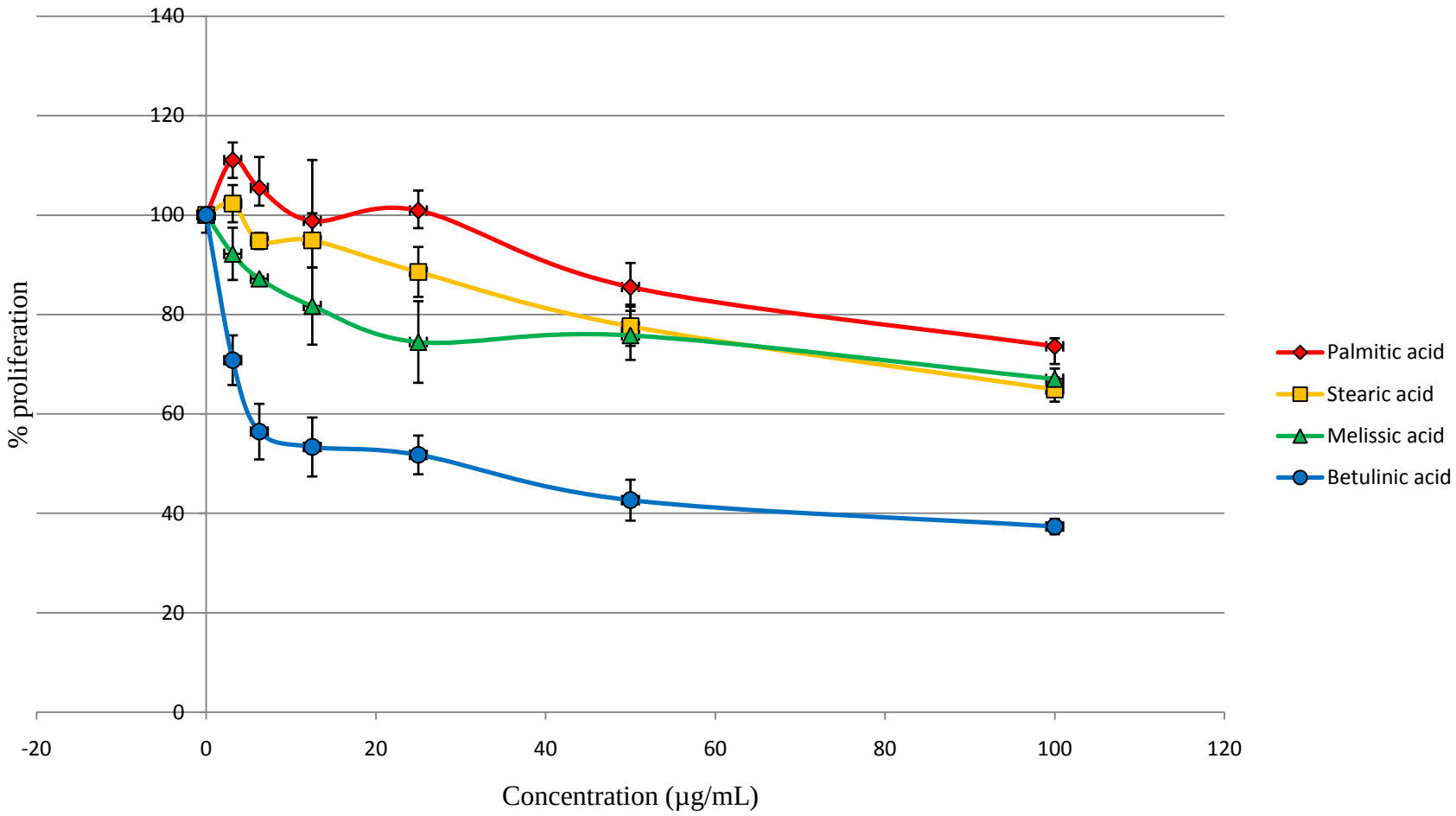


Figure 7.7: Activity of fatty acids and betulinic acid against HK1.

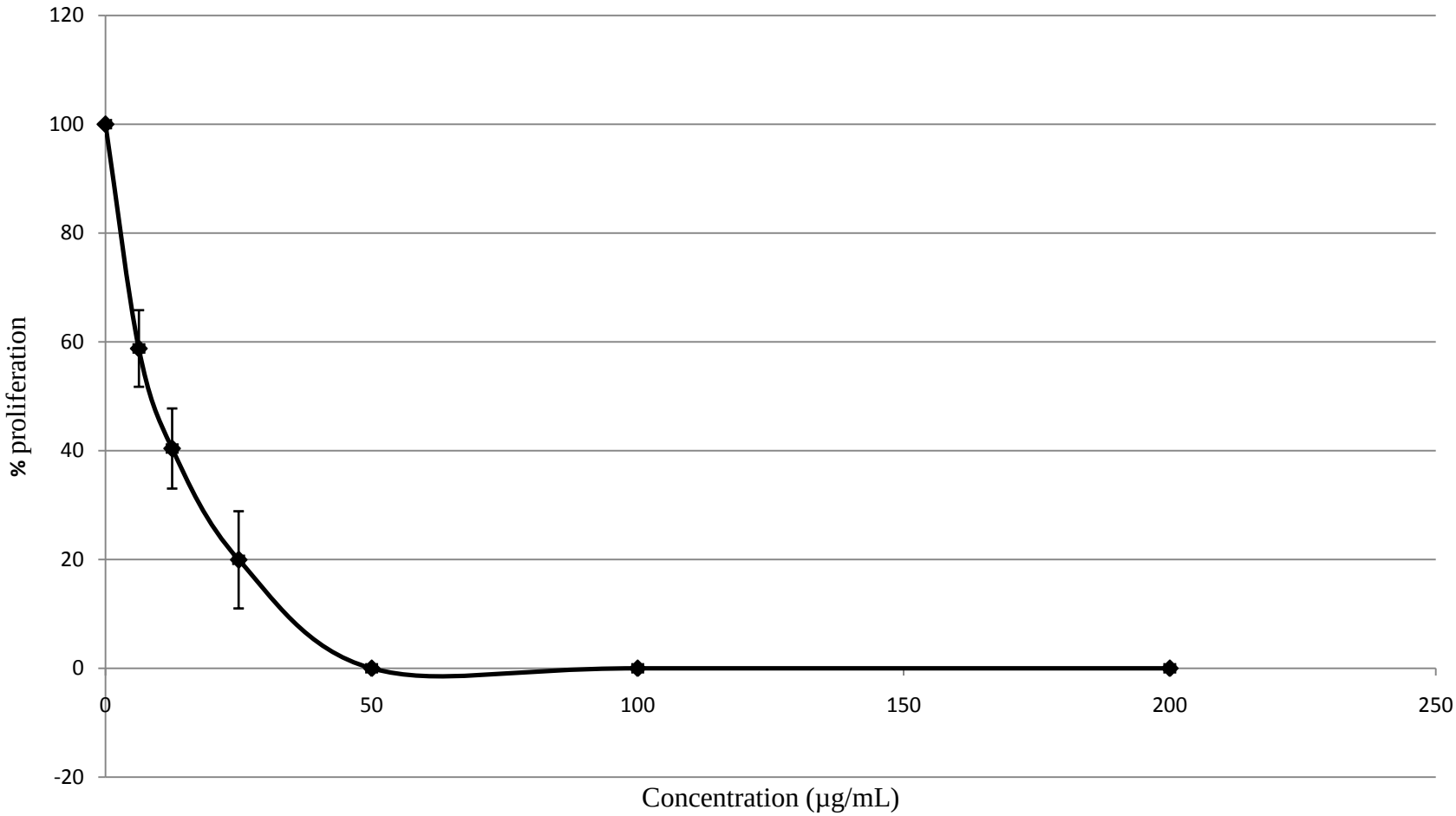


Figure 7.8: Activity of quercetin against HK1.

Table 7.2: IC_{50} of samples tested against HK1.

Sample	IC_{50} (µg/mL)*
Root ethanol	60.23
Betulinic acid	31.5
Quercetin	8.65

*The IC_{50} was generated using the equation of a linear trend line where the ln of the concentration was plotted against % proliferation. Only samples which gave an IC_{50} of less than 200 µg/mL are shown.

7.3.2: Cytotoxic activity against MRC-5

The results of the cytotoxicity assays using MRC-5 are shown in **Figures 7.9-7.15** and **Table 7.3**. All of the ethanolic extracts with the exception of the fruit ethanol extract were toxic towards MRC-5. All of the remaining extracts increased proliferation markedly, with the stem chloroform, root hexane and fruit chloroform extracts enhancing proliferation in a dose dependant manner, but showing a reduction in this activity at 200 µg/mL. The stem alkaloid extract induced a similar, possibly hormetic, response as was seen towards HK1, and this was seen again in the graph showing the response of MRC-5 to periglaucine A. Melissic and betulinic acid both reduced proliferation of MRC-5 with increasing concentration, however, palmitic and stearic acid increased proliferation with the greatest increase at 6.25 µg/mL. The root ethanol extract had the lowest IC_{50} (20.71 µg/mL) followed by the leaf ethanol (25.15 µg/mL) and stem ethanol (51.96 µg/mL) extracts as shown in **Table 7.3**. An IC_{50} could not be obtained from the data generated by the other samples.

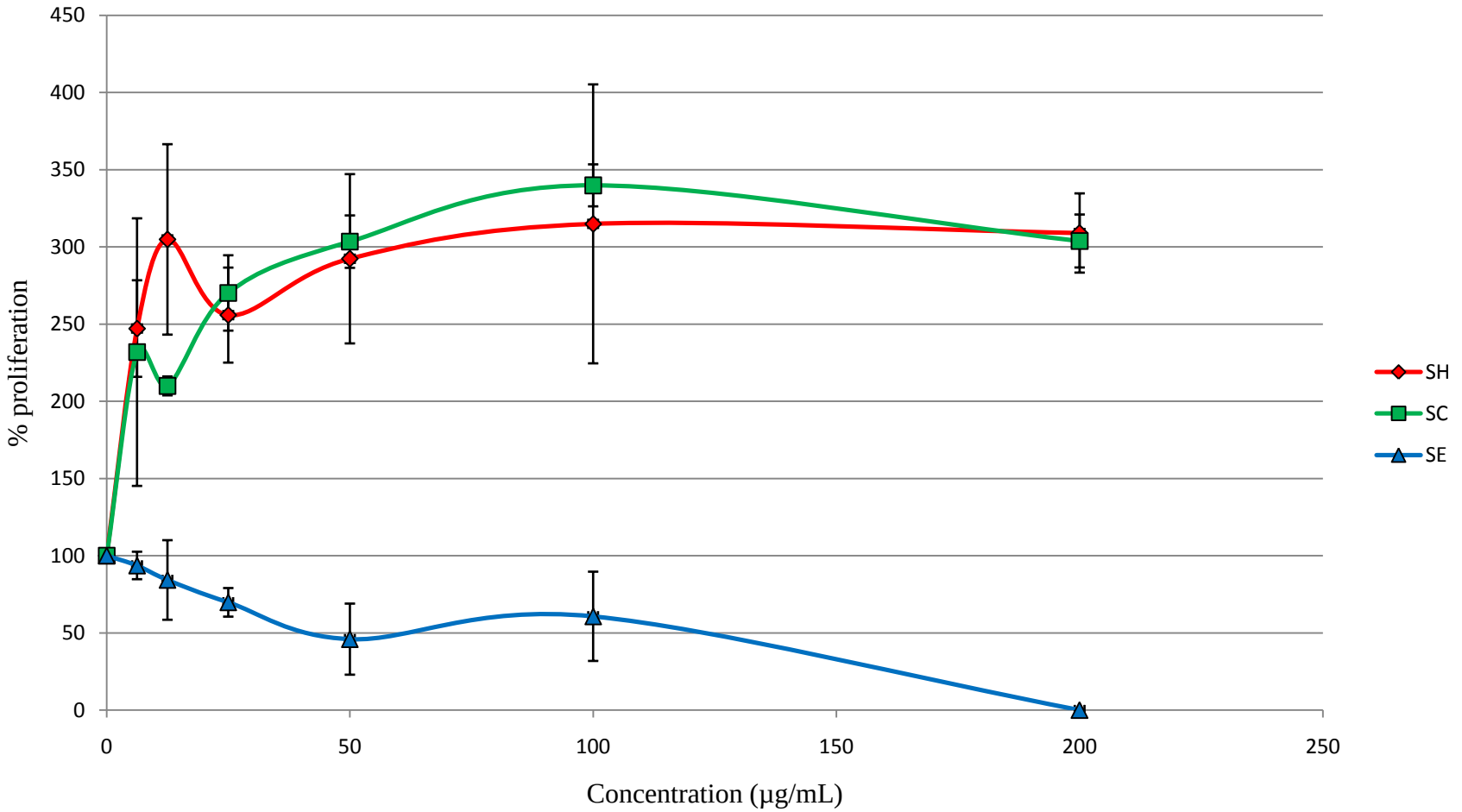


Figure 7.9: The effect of *P. glaucus* stem extracts on MRC-5 growth.

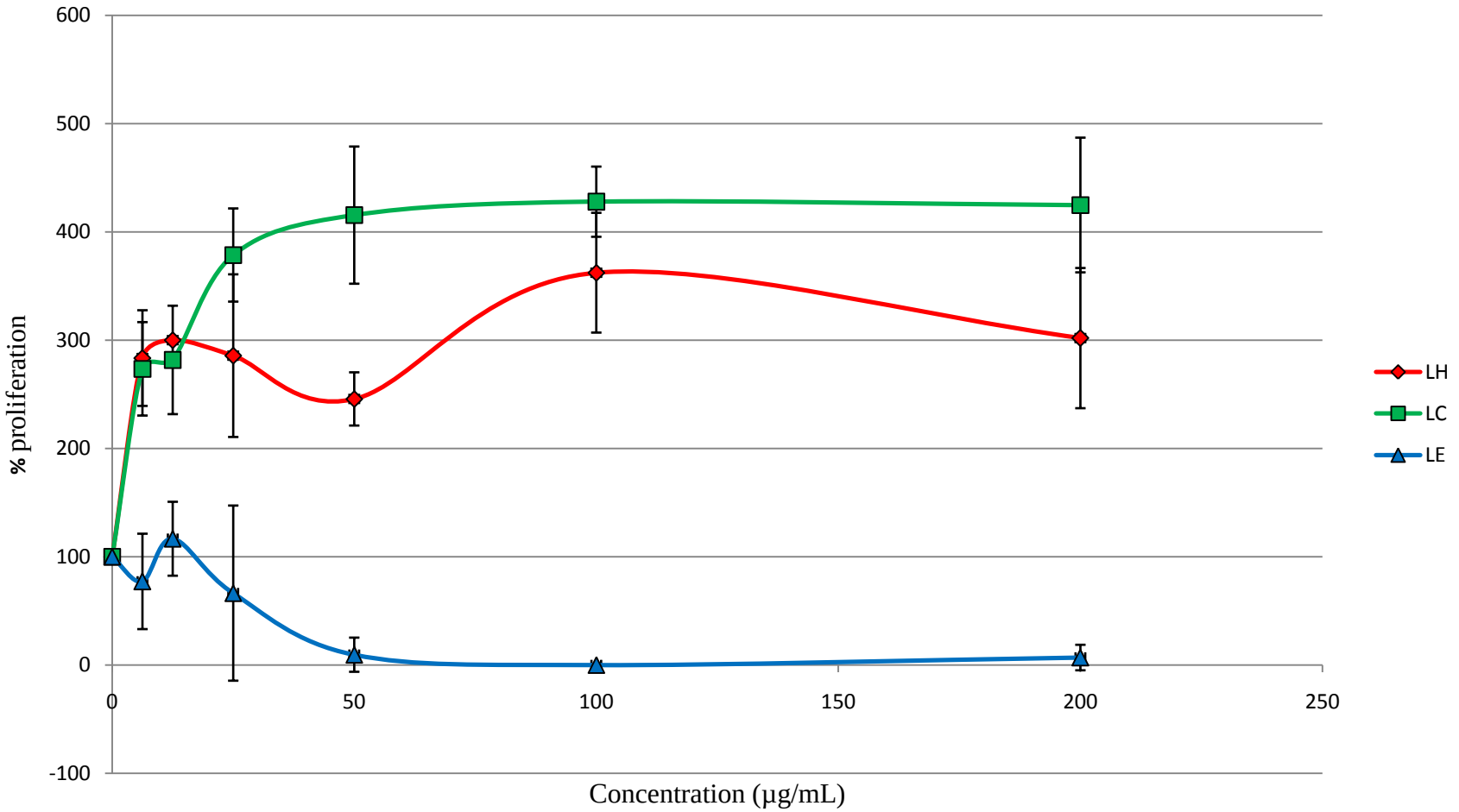


Figure 7.10: The effect of *P. glaucus* leaf extracts on MRC-5 growth.

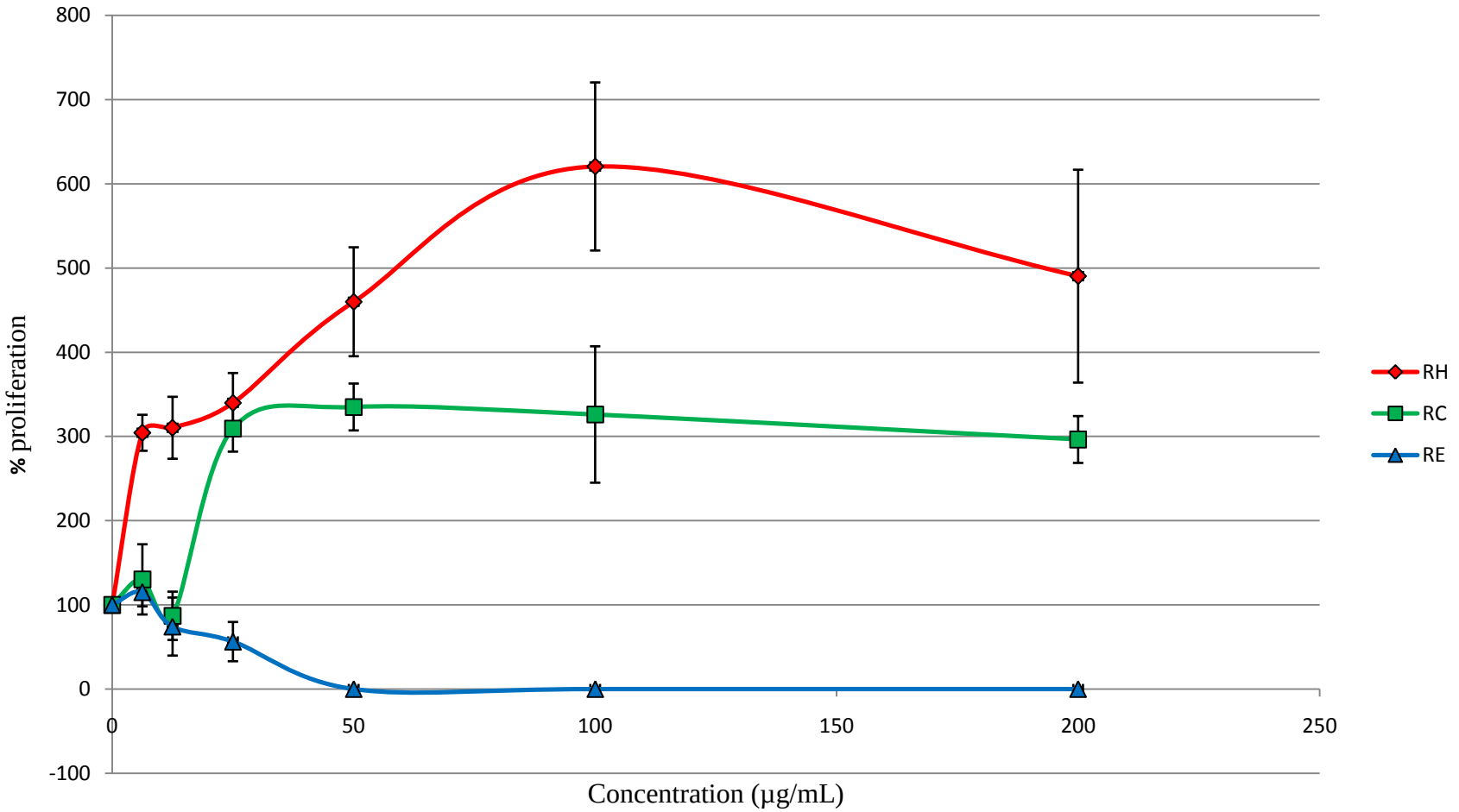


Figure 7.11: The effect of *P. glaucus* root extracts on MRC-5 growth.

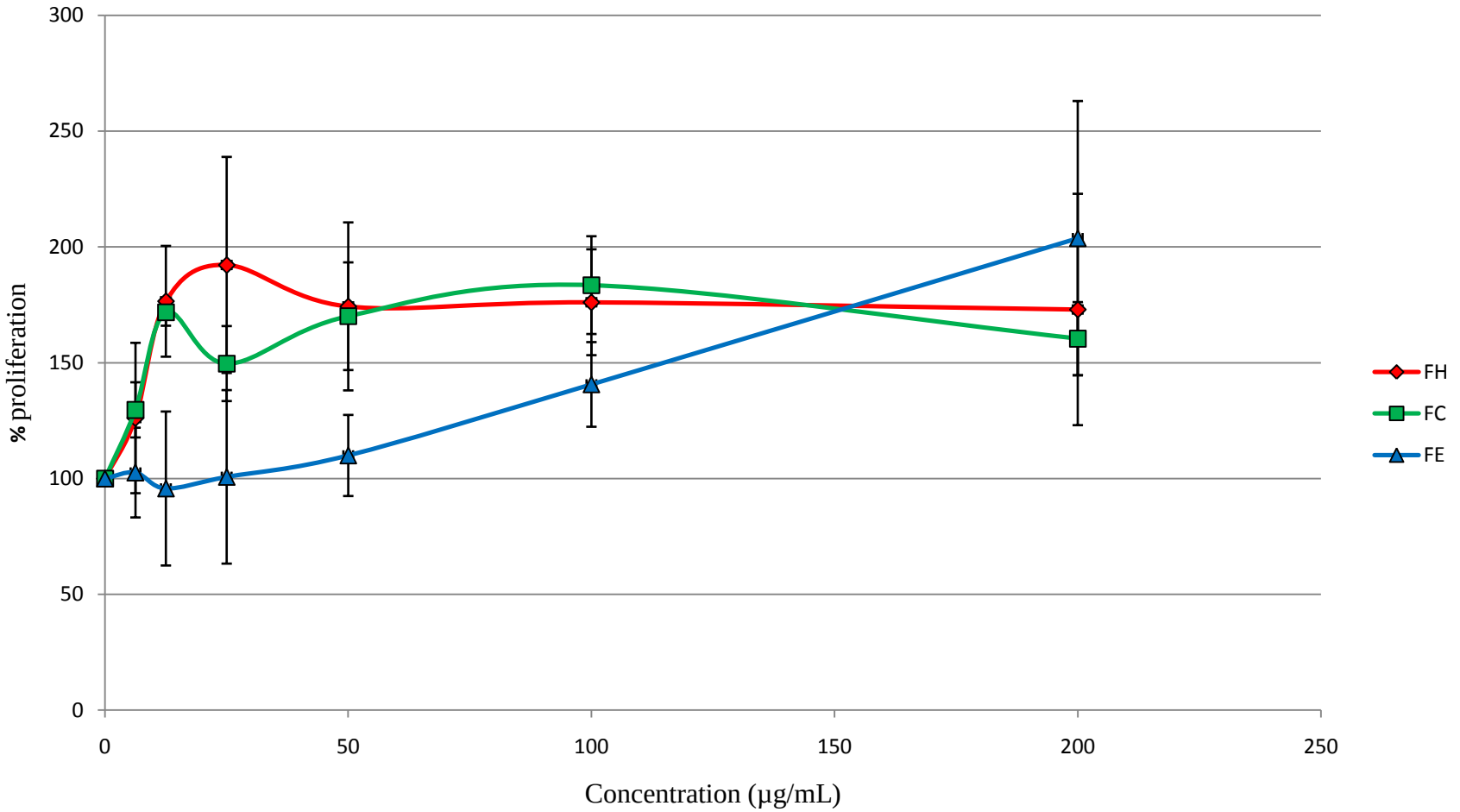


Figure 7.12: The effect of *P. glaucus* fruit extracts on MRC-5 growth.

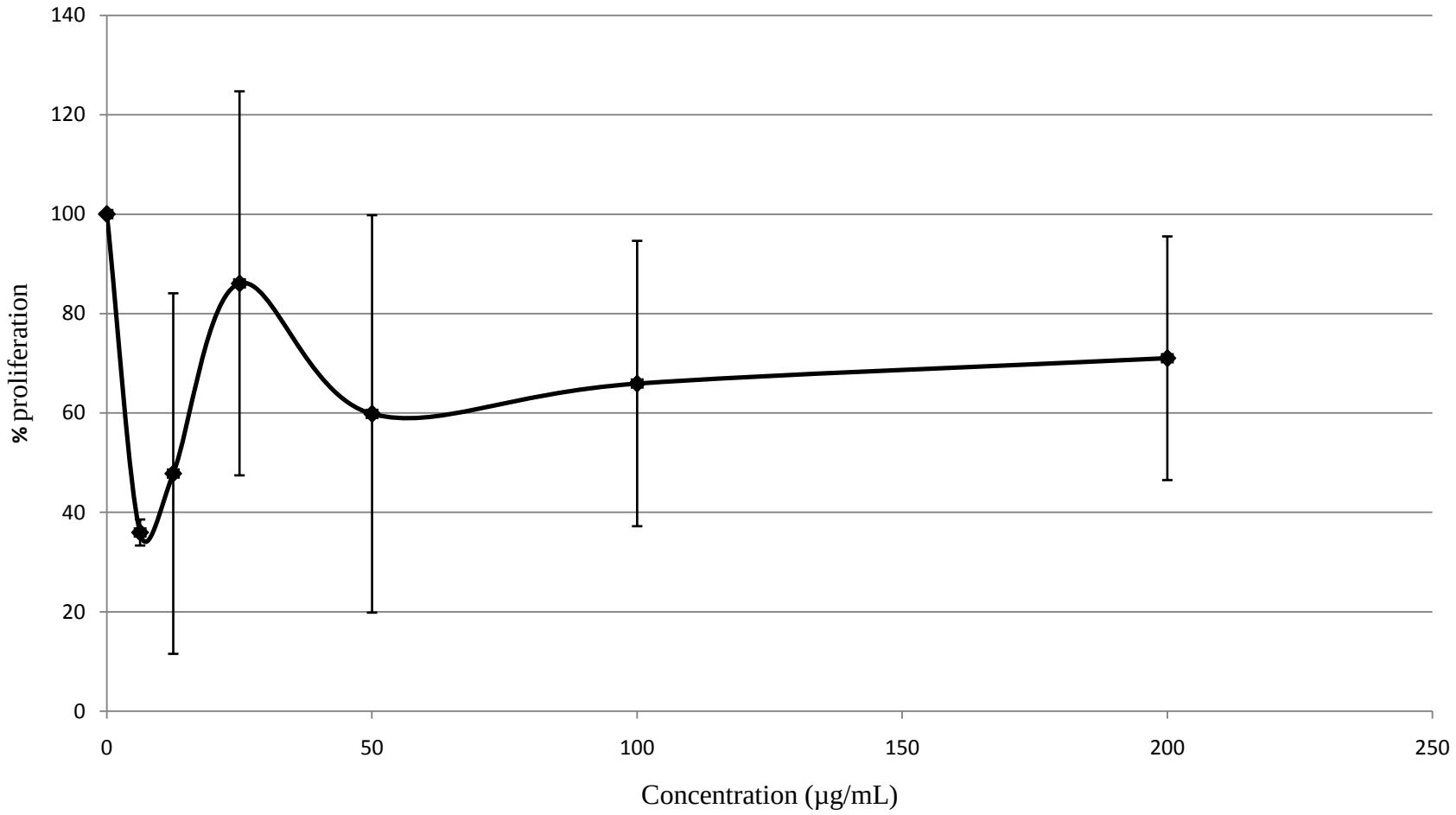


Figure 7.13: The effect of *P. glaucus* stem alkaloid extract on MRC-5 growth.

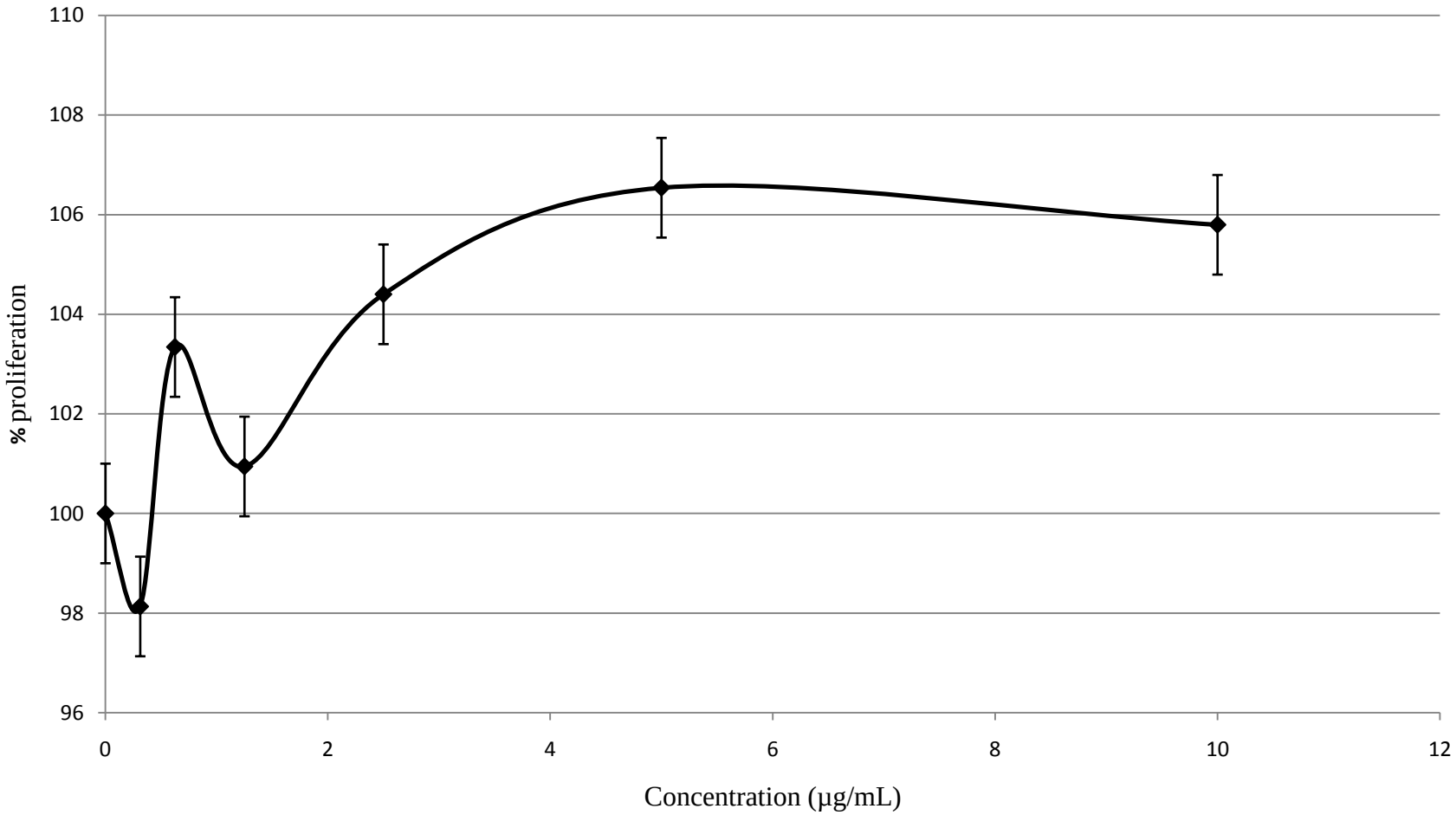


Figure 7.14: The effect of periglaucine A on MRC-5 growth.

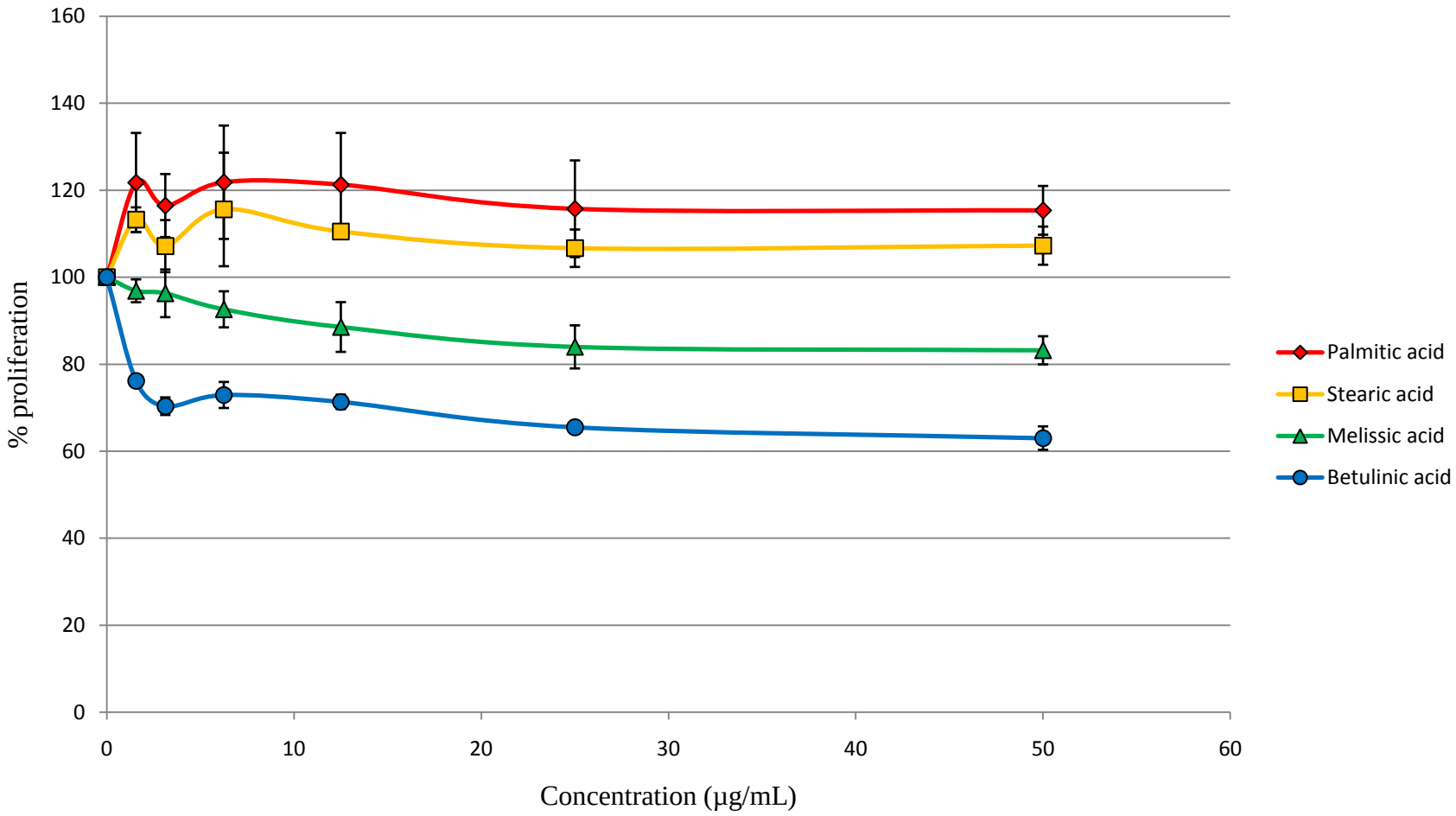


Figure 7.15: The effect of fatty acids and betulinic acid on MRC-5 growth.

Table 7.3: IC_{50} of samples tested against MRC-5.

Sample	IC_{50} (µg/mL)*
Stem ethanol	51.96
Leaf ethanol	25.15
Root ethanol	20.71

*The IC_{50} was generated using the equation of a linear trend line where the ln of the concentration was plotted against % proliferation. Only samples which gave an IC_{50} of less than 200 µg/mL are shown.

7.4: Conclusion

A number of the samples elicited a hormetic response in the cells. Hormesis is an effect seen by cells in response to toxins or other stressors where the cells are stimulated by low doses of the sample, but inhibited by higher doses (Gems & Partridge 2008). The most characteristic example of this response was seen by the alkaloid extract and periglaucine A. Some of the responses of the cells to the samples also appeared to produce a biphasic response, with an initial hormetic response followed by a different trend of either enhanced growth, to a point, as seen by the hexane and chloroform extracts, or reduction in growth, as seen by the leaf ethanol extract.

Many of the extracts appeared to enhance proliferation, with proliferation enhancement reducing at the highest concentration. This trend was confirmed by observation under microscope. The enhancement in proliferation according to observations by microscopy, although increasing with dose and decreasing at the highest dose, was not as exaggerated as the MTT results may have suggested. This implies that there may have also been an increase in the metabolic rate of the cells. The ethanolic extracts of the stems, leaves and roots were the most toxic towards both HK1 and MRC-5. The polar compounds are therefore most likely responsible for toxicity, while the less polar compounds promote cell proliferation and possibly enhance metabolism.

The most therapeutically useful drugs would be ones that are less toxic to normal cells than cancer cells. Based on the results gathered from the MTT assays, the leaf hexane, leaf ethanol, fruit hexane and fruit ethanol extracts and periglaucine A, betulinic acid, palmitic acid, stearic acid and melissic acid all exhibited greater cytotoxicity and inhibition of growth towards HK1 compared to MRC-5. The stem ethanol, root ethanol and stem alkaloid extracts were more toxic towards MRC-5 than HK1, making these the least attractive samples for use as potential chemotherapeutic agents for the treatment of nasopharyngeal cancer. Different cells lines can respond differently to chemicals, the results gathered from different cells have different value in predicting their performance in a clinical trial (Voskoglou-nomikos et al. 2003), as such, further testing would have to be done to determine the activity of these samples against other cell lines. Many of the samples did not show a simple dose response relationship when incubated with the cell lines, the assay should be repeated with a wider range of concentrations and further tests, such as proteomic tests could be carried out to determine what response the cells are having towards to samples.

Chapter 8: The wound healing activity of *P. glaucus*

8.1: Introduction

Trauma to tissues can lead to bleeding and the possibility of infection, as such, the process of repairing damage begins shortly after it is received. The processes involved are clotting of the blood (Haemostasis), where platelets accumulate at the injured site and release chemicals that promote clotting. Fibrin is activated and forms a mesh which binds platelets together and forms a mesh that prevents further blood loss. The site becomes inflamed and damaged or dead cells and bacteria are phagocytosed by white blood cells. Platelet derived growth factors are released which promote the migration and division of cells. New blood vessels will be developed (angiogenesis), collagen is deposited, granulation tissue is formed (fibroplasia) and epithelialization and wound contraction occur. As the repairing tissue matures, it is remodelled and collagen is realigned, any cells that are no longer needed for the healing process undergo apoptosis (Elnar & Ailey 2009).

A number of factors, such as lack of moisture, inflammation, diabetes, malnutrition, infection and immunosuppression, can negatively impact the healing process (Guo & Dipietro 2010).

8.1.1: Growth factors

A number of growth factors expressed by cells at the site of injury and are involved in wound healing (Werner & Grose 2003), as summarised in **Table 8.1**:

Table 8.1: Growth factors and their functions.

Growth factor	Function
Epidermal growth factor (EGF)	These are produced by platelets and macrophages, they stimulate the secretion of collagenase by fibroblasts and promote keratinocytes and fibroblast proliferation.
Transforming growth factor (TGF)	There are a number of TGFs which can promote angiogenesis, stimulate collagen production, inhibit collagen degradation and promote keratinocyte and fibroblast

	proliferation and migration.
Platelet derived growth factor (PDGF)	PDGFs attract macrophages and fibroblasts to the site, thus promoting the synthesis of collagen and proteoglycan.
Fibroblast growth factor (FGF)	Promotes the formation of granulation tissue, reepithelialisation, matrix formation and angiogenesis.
Vascular endothelial growth factor (VEGF)	Promotes the formation of granulation tissue and angiogenesis.
Keratinocyte growth factor	Stimulates migration, proliferation and differentiation of keratinocytes.
Colony stimulating factors	Colony stimulating factors can stimulate granulocyte proliferation and/or macrophage proliferation.
Interleukins	There are a number of different interleukins that can activate neutrophils, promote inflammation and reepithelialisation, induce collagen and proteoglycan synthesis, recruit neutrophils and fibroblasts to the site.
Tumour necrosis factor alpha (TNF-α)	Promotes inflammation and reepithelialisation.

The therapeutic use of these growth factors and cytokines have been investigated and PDGF and EGF have been found to have beneficial effects on wound healing (Lynch et al. 1987; Hardwicke et al. 2008).

8.1.2: Plants and natural products that improve wound healing

Natural products including plant extracts have been used for wound healing in traditional medicine historically and to this day. Generally, these medicines possess antimicrobial, anti-inflammatory and reepithelialisation and collagen formation enhancing chemicals which act to promote the wound healing process (Thakur et al. 2011).

Honey is a product, used historically, for treating wounds. This activity has been attributed to a number of properties possessed by honey, such as its antimicrobial activity which is partly

owed to its hydrogen peroxide content, its anti-inflammatory activity and osmotic properties (Mandal & Mandal 2011).

Turmeric is also used to treat wounds. In particular, this plant is used to treat wounds and it possesses antimicrobial and anti-inflammatory activity (Maheshwari et al. 2006). Most plants or other natural products that are used to treat wounds and encourage healing possess some degree of anti-inflammatory, antioxidant and antimicrobial activity.

8.1.3: Pathogens and wound healing

Natural products used for wound healing often possess some degree of antimicrobial activity (Ghuman et al. 2016), pathogens can cause infection and further damage to tissues, so antimicrobials will ensure that the healing process is not hampered by the deleterious effects of microbes (Guo & Dipietro 2010). The anti-inflammatory activity helps to ease pain, swelling and redness in the affected area, which may not necessarily help with the healing process but will relieve discomfort. Antioxidants may help to reduce the damage done to cells such as keratinocytes and monocytes by protecting them from damage by oxygen radicals and have been found to facilitate healing (Martin 1996).

Despite their benefits, crude preparations of natural products can be harmful under some circumstances. The introduction of natural products in their crude form can potentially introduce pathogens into the wound site (Suslow et al. 2003) and due to the large numbers of chemicals in a crude preparation, some may cause irritation or an allergic reaction.

8.1.4: Studies on herbs that enhance wound healing

Herbs can be used to enhance cell proliferation which can improve proliferation of stem cells, stimulate the immune system and promote hair growth, although enhanced proliferation can also have some negative effects such as promoting the development of cancer (Hasan & Sorkhy 2014).

The use of herbs to stimulate stem cell proliferation for the purposes of wound healing has been investigated with studies reporting an increase in proliferation of bone marrow mesenchymal stem cells, bone marrow stromal cells and bone cell proliferation which could

possibly speed up the healing time of fractures and keratinocyte cell proliferation can be stimulated by various extracts (Hasan & Sorkhy 2014).

8.2: Materials and methods

8.2.1: Chemicals and reagents

DMSO was purchased from RCI Labscan (Thailand), RPMI 1640, FBS and PBS were purchased from Thermofisher.

8.2.2: Wound healing assay

The wound healing assay was carried out as described by Lee et al. (2000), with modifications. MRC-5 was cultured in flasks with a surface area of 25 cm^2 as confluent monolayers. The monolayers were incubated in the absence of serum for 16 h to synchronise the cell cycle of the cell population. The monolayers were wounded in a line across the flasks surface with a standard 200 µL tip attached to a modified, sterile Pasteur pipette. The wounded monolayers were washed with serum free media twice and incubated with *P. glaucus* extracts at a concentration of 100 µg/mL or 10% FBS, in serum free RPMI 1640 media. The wound surface area unoccupied by cells was recorded at specified time points using a digital camera connected to an inverted microscope. Images were analysed using ImageJ 1.50i. Wound healing was calculated as the coverage of the wound relative to the initial wound area. The experiment was run in triplicate (n=3).

8.3: Results

The results of the wound healing assay for the stem, leaf, root and fruit extracts that were tested for their wound healing activity are shown in **Figures 8.1 - 8.4** and were selected based on their enhancement of cell proliferation in the cytotoxicity assay in **Chapter 7**. The fruit extracts showed the greatest wound healing activity when compared to the other extracts, with the fruit chloroform and hexane extracts covering 28.0 and 24.2% of the wound after 24 h respectively. The fruit ethanol and leaf hexane both covered approximately 18.5% of the wound in 24 h. The leaf chloroform and root hexane extracts did little to promote cell migration across the wound site, covering only 4.4% and 4.6% of the wound after 24 h. The ability of the extracts to promote wound healing in this assay was much lower compared to

the control (**Figure 8.5**) consisting of 10% FBS, which resulted in a 79.4% coverage of the wound site after 24 h.

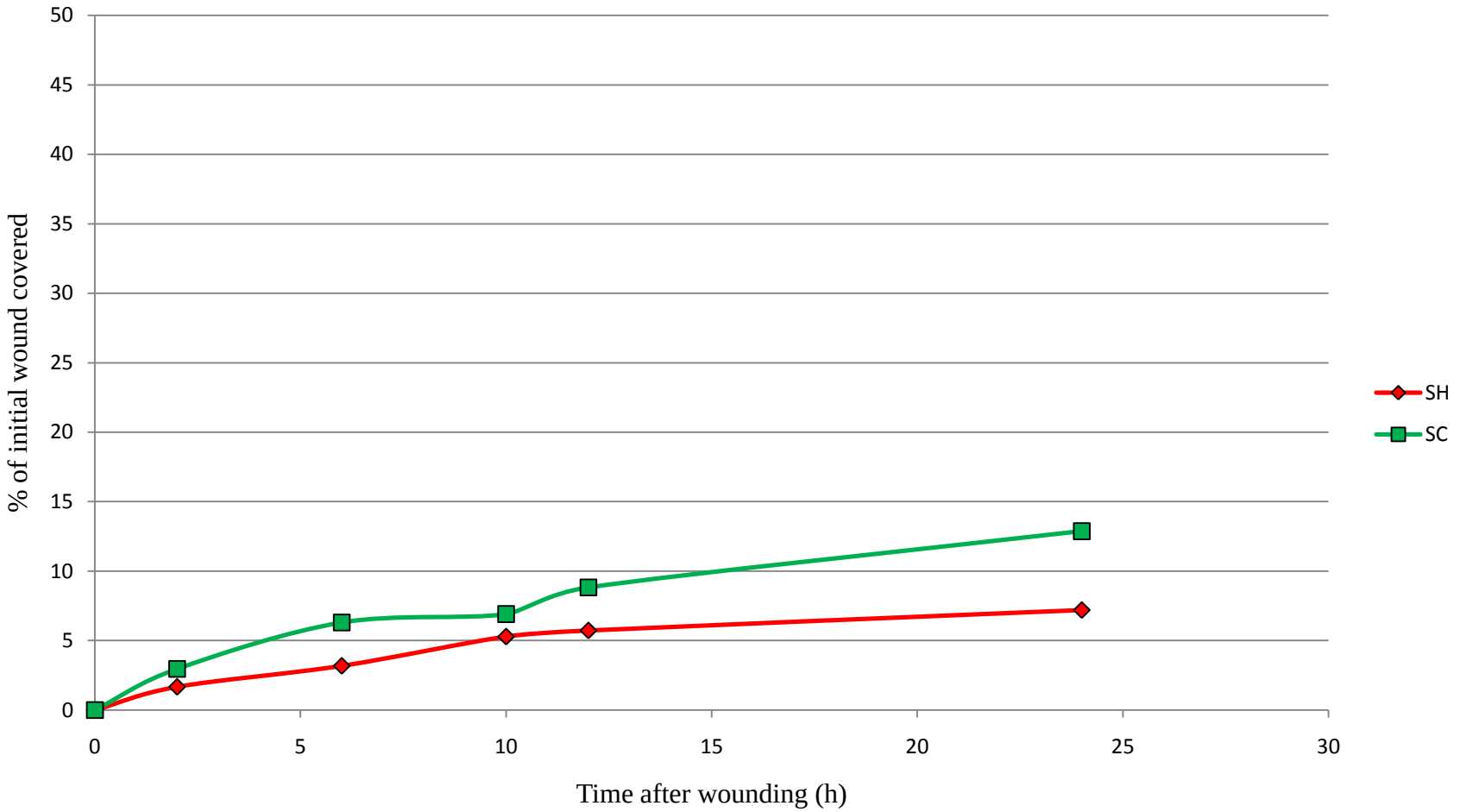


Figure 8.1: Wound healing activity of *P. glaucus* stem extracts.

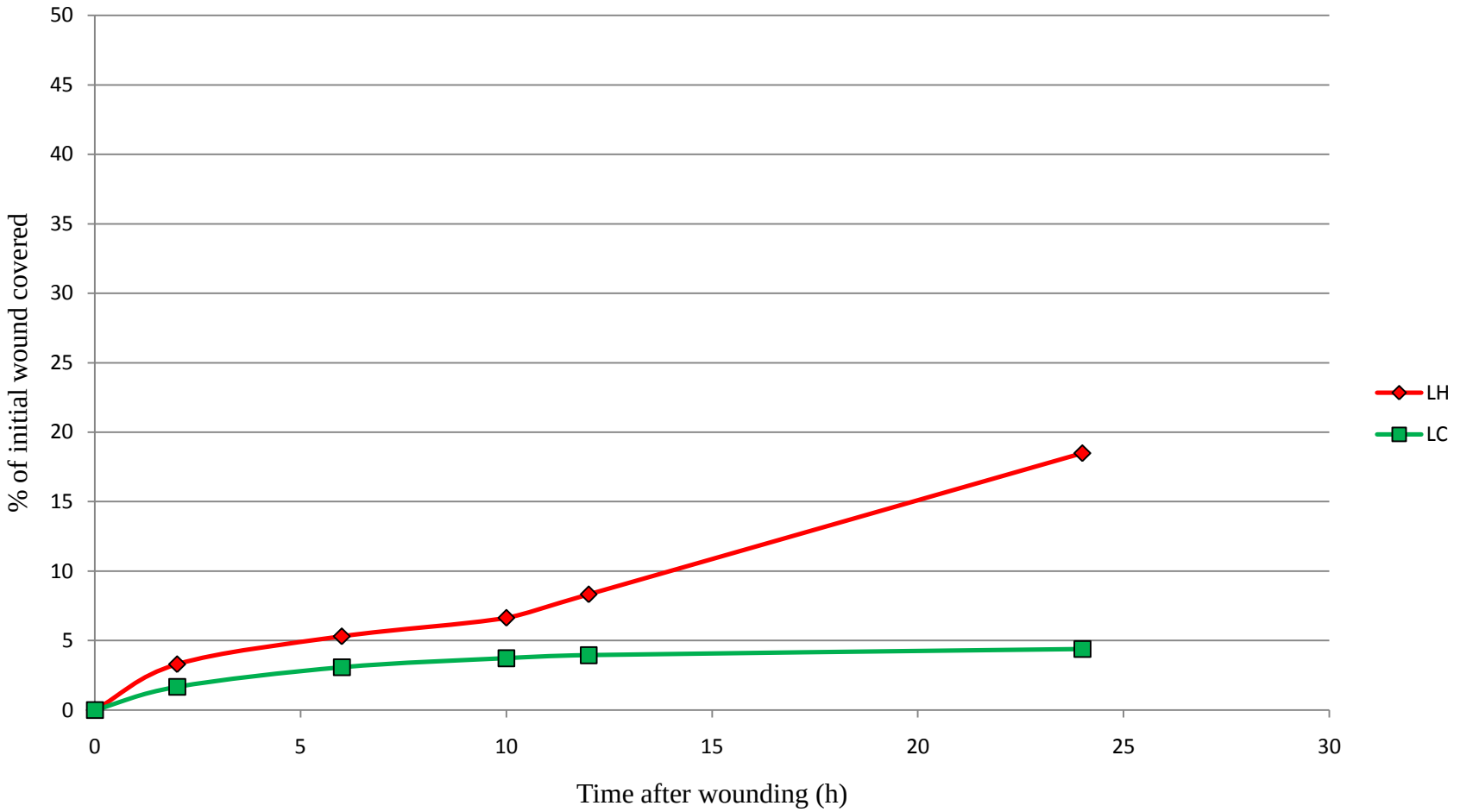


Figure 8.2: Wound healing activity of *P. glaucus* leaf extracts.

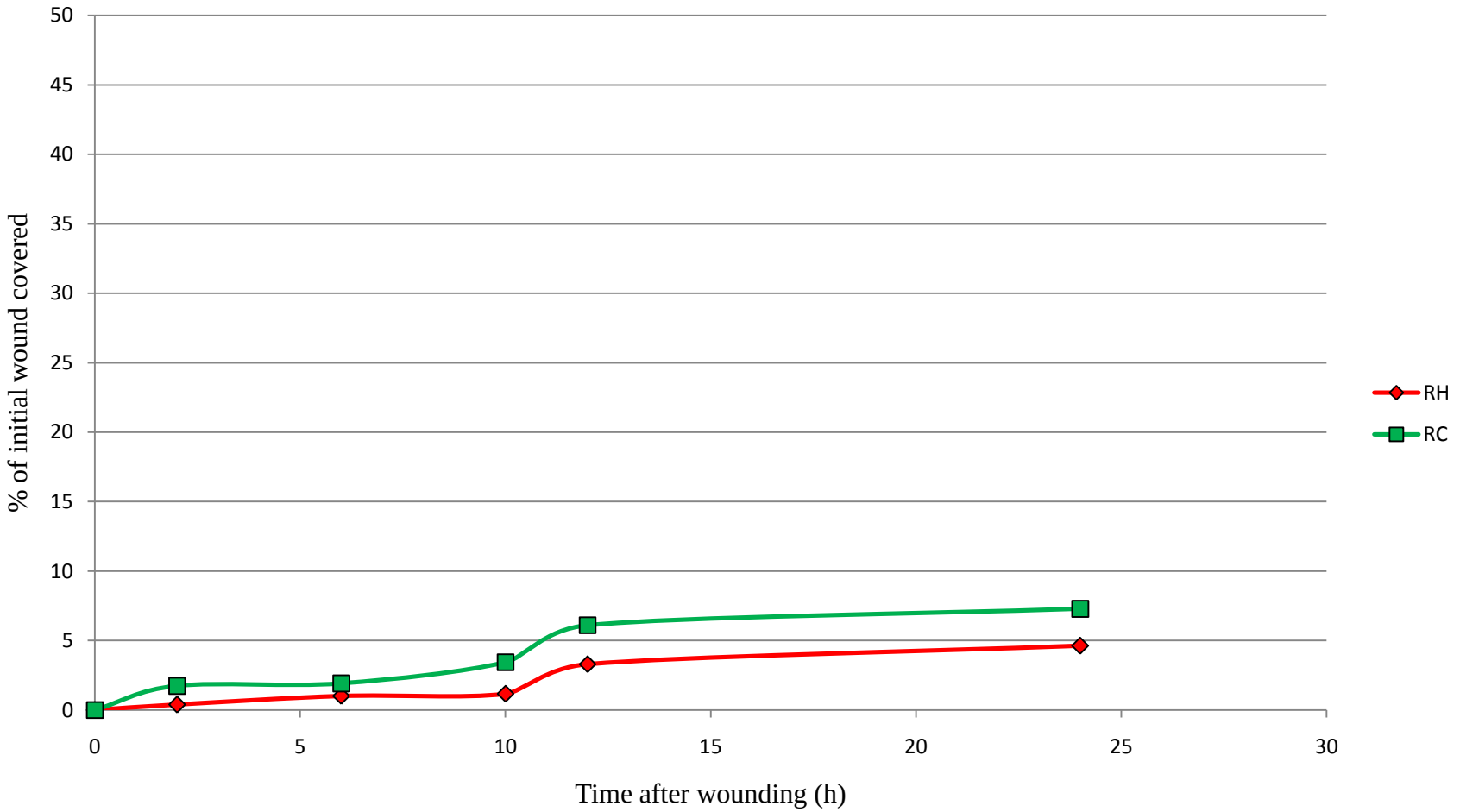


Figure 8.3: Wound healing activity of *P. glaucus* root extracts.

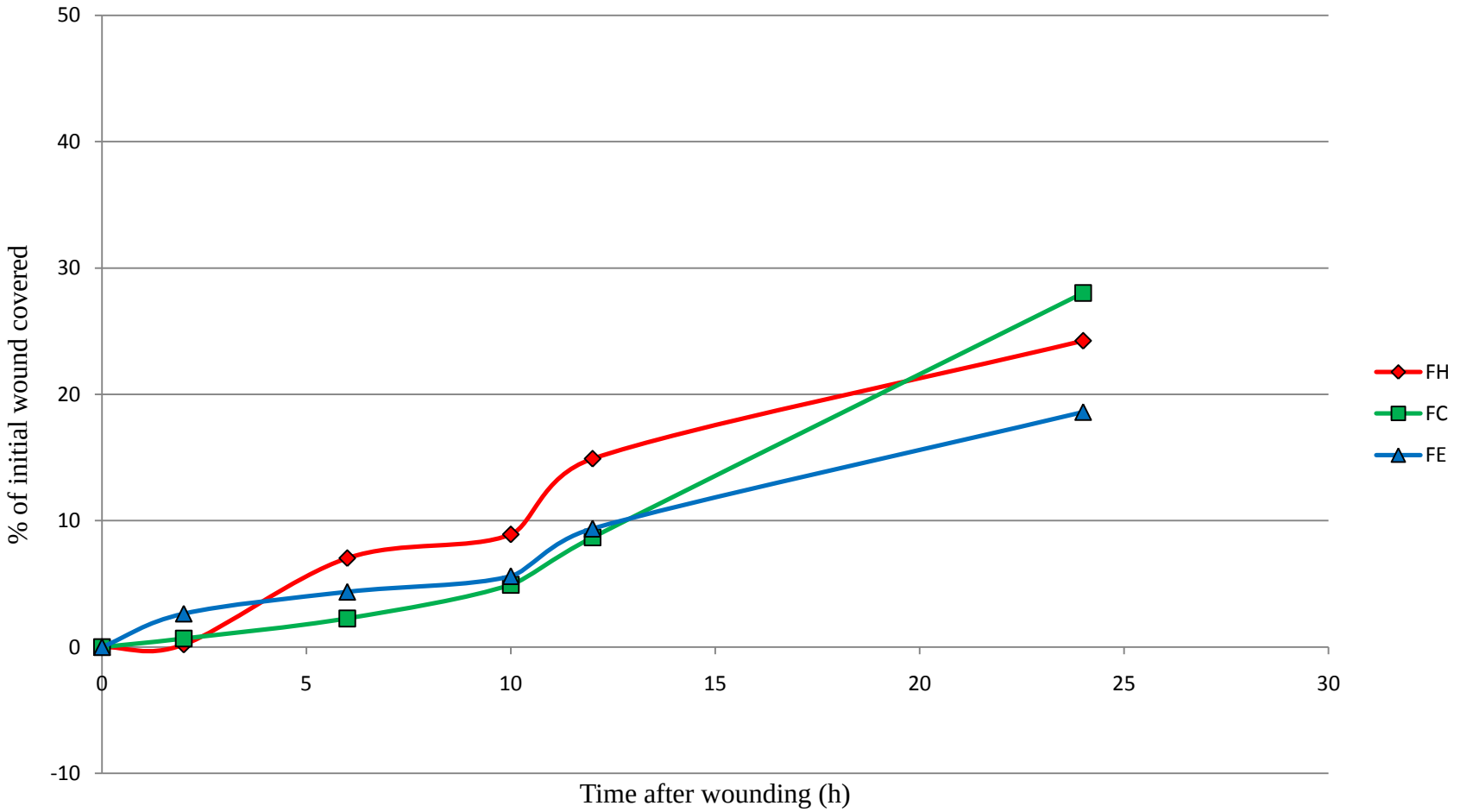


Figure 8.4: Wound healing activity of *P. glaucus* fruit extracts.

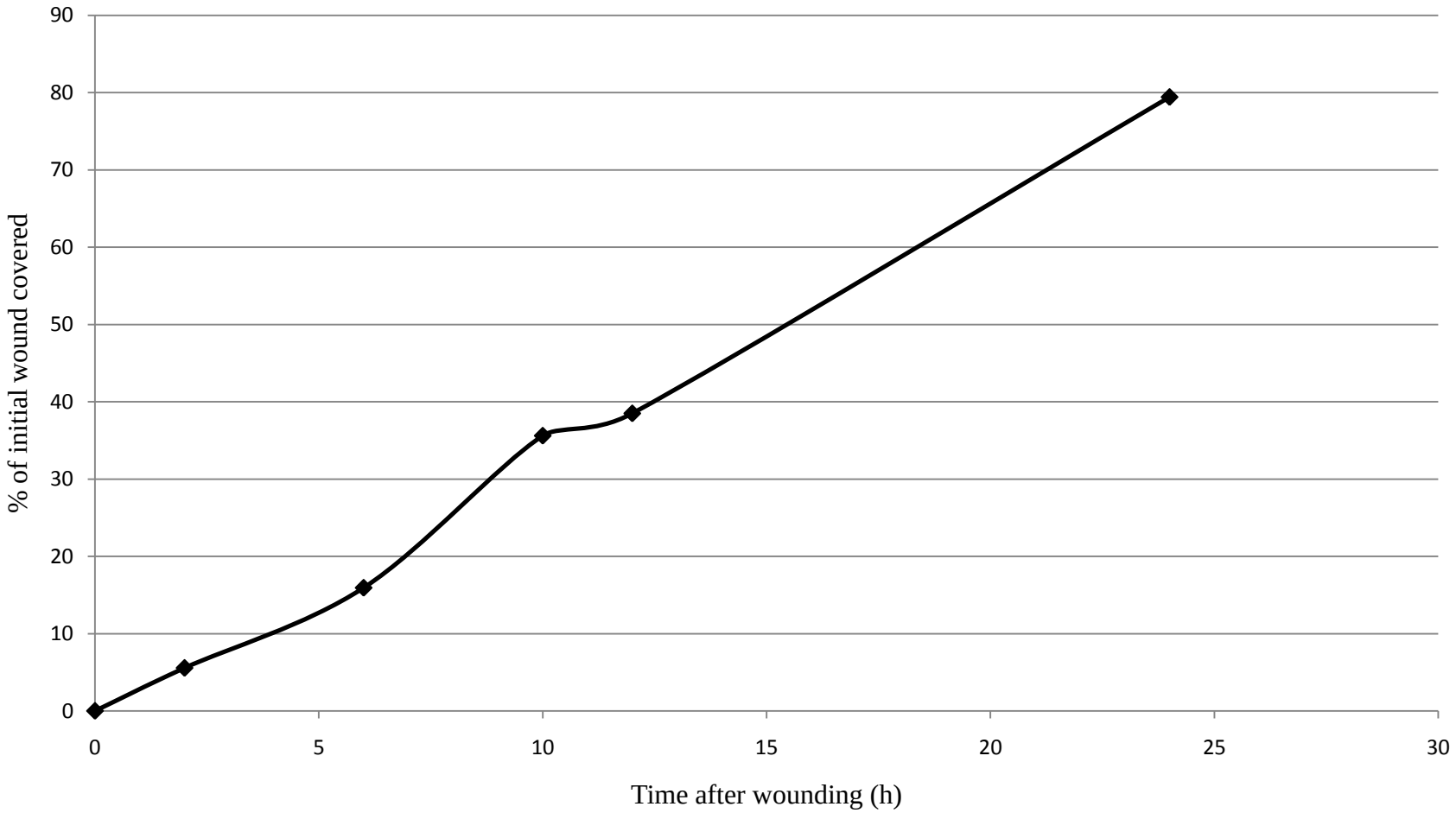


Figure 8.5: Wound healing activity of media containing 10% FBS.

8.4: Conclusion

This assay showed that FBS promoted wound healing to a greater extent than any of the extracts, yet greatly enhanced cell proliferation was observed by cells treated with the samples in the cytotoxicity tests. This may be due to the difference in the treatment of the cells, as the cells in the wound healing assay were starved of serum so that they would enter the G0/G1 phase whereas the cells were not starved of serum in the cytotoxicity assay and cells may have been in various stages of the cell cycle when treatment was commenced. Serum starvation can also cause stress to cells and be used to induce apoptosis, this may have affected the response of the cells to the samples (Pirkmajer & Chibalin 2011).

The fruit extracts showed the greatest wound healing effect, with the fruit chloroform extract showing the greatest effect.

The promotion of wound healing in this study was done under sterile conditions and no inflammatory response was induced. Therefore, this assay mainly tested the effect that the extracts had on the proliferation and migration of cells across the wound site and not the effect of wound healing under normal conditions which involve inflammation and possibly infection. Further studies would have to be conducted to generate a more accurate prediction of the wound healing potential of the samples.

Chapter 9: The antivenom activity of *P. glaucus*

9.1: Introduction

Snakes are carnivorous reptiles that live across many parts of the world. Most snakes are non-venomous, with approximately only 500, out of the 2,900 or so species of snakes, carrying venom. The families of snake that carry venom are Viperidae, Elapidae (including the sub-family Hydrophiidae) and the subfamily Atractaspidinae. Viperidae includes snakes such as the pit viper (*Crotalus horridus*) and western diamondback rattlesnake (*Crotalus atrox*), Elapidae includes the black mamba (*Dendroaspis polylepis*), the king cobra (*Ophiophagus hannah*) and Belcher's sea snake (*Hydrophis belcheri*) and Atractaspidinae includes the small-scaled burrowing asp (*Atractaspis microlepidota*).

9.1.1: Epidemiology

Millions of people are affected by snakebite each year, with it causing hundreds of thousands of deaths and resulting in even more amputations every year, mostly in Asia and Africa (Kasturiratne et al. 2008). Death by snakebite is most commonly due to envenomation and of the estimated 5.5 million people who are bitten each year, up to 1.8 million are envenomed (Kasturiratne et al. 2008). Considered a neglected disease by the WHO (World Health Organization 2007), it affects mostly poor populations and has received limited attention in terms of research and drug development.

9.1.2: Venom components and mechanism of action

Although a venomous snake may not envenomate upon biting, envenomation, when it does occur, is a serious health risk which can lead to a variety of deleterious effects on the body, possibly resulting in death. As well as the risk of envenomation the bite of a venomous snake also poses the risk of bacterial infection. The mouth of a snake contains a variety of pathogenic bacteria that could potentially lead to sepsis (Shek et al. 2009).

Snake venom consists of a number of different components that are intended to aid in immobilising prey and digesting them. The venoms of different snakes varies between

families, species and even between snakes of the same species (Boldrini-França et al. 2010; Casewell et al. 2014; Jayanthi & Gowda 1988; Menezes et al. 2006).

The components act to cause different effects, with proteolytic enzymes being responsible for digesting tissues by breaking peptide bonds in proteins, denaturing them. Such proteolytic enzymes include collagenases, which degrade collagen causing necrosis and arginine ester hydrolases, which target proteins rich in arginine residues for hydrolysis. Procoagulants and anticoagulants are found in snake venom. Procoagulants cause coagulation of blood and use up clotting factors, with clotting factors expended, anticoagulants may act to promote haemorrhaging. L-amino acid oxidase is thought to contribute to the toxicity of snake venoms as well as possibly maintaining the health of the venom glands of the snake. These enzymes can cause apoptosis and disrupt platelet function (Li et al. 1994; Suhr & Kim 1996). Phospholipases, such as phospholipase A (PLA), break lipids down into free fatty acids damaging cell membranes and causing apoptosis, which triggers inflammation. Acetylcholinesterase breaks down the neurotransmitter acetylcholine, which terminates the synaptic transmission caused by acetylcholine, resulting in paralysis. Paralysis may also be caused by α-neurotoxins in venom that act as acetylcholine antagonists, which bind at post synaptic membrane receptors (Koh et al. 2006). Fasciculins work by, instead of breaking down acetylcholine, degrading acetylcholinesterase which results in prolonged signalling by acetylcholine due to the neurotransmitter remaining at synaptic receptor. This leads to uncontrolled muscle spasms (Ranawaka et al. 2013). Phosphodiesterases break the phosphodiester bonds that make up the backbone of DNA and RNA and include DNase and RNase, they also include other enzymes that break phosphodiester bonds. NAD nucleotidase renders nicotinamide adenine dinucleotide (NAD) inactive, which disrupts cell metabolism, causing cell death (Tu 1977). Not all of these components are found in all snake venoms, and different families can, in some cases, be distinguished by the typical composition of their venom (Chippaux 1991).

The venom of the Russell's viper (*Daboia russelli*) has been found to have strong trypsin inhibitory activity, adenosine mono- and triphosphatase activity, haemorrhagic activity and PLA_2 activity (Mukherjee et al. 2000; Prasad et al. 1999; Saikia et al. 2012; Mukherjee 2008). The venom composition has also been noted to vary significantly depending on the age of the snake, becoming less potent with age (Tun-Pe et al. 1995).

The Saw-scaled viper (*Echis carinatus*) has venom containing the potent anticoagulant, echistatin (Garsky et al. 1989) as well as procoagulants (Franza & Finlayson 1975), PLA_2, serine proteases and L-amino oxidases (Casewell et al. 2009). It has been found to share sequences encoding C-type lectins that are similar to other vipers (Harrison et al. 2003).
The Western diamondback rattlesnake's venom (*Crotalus atrox*) has haemorrhagic toxins (Bjarnason & Tu 1978), hypotensive activity, nucleotidase, L-amino acid oxidase, phosphodiesterase, phospholipase A, proteolytic and depressive activity (Bonilla 1978). Snakes of different age and geographical location have been found to possess venom of different composition and potency (Minton & Weinstein 1986).

The venom of the Indian cobra (*Naja naja*) contains three finger toxins, a potent toxin that is found in snakes in the Elapidae family. This toxin exhibits a range of effects, such as neurotoxicity, cytotoxicity, anticoagulant activity and antiplatelet activity (Sintiprungrat et al. 2016; Utkin 2013). PLA2, cystein-rich secretory proteins, metalloproteases, protease inhbitors, nerve growth factors, and L-amino oxidase are also found in the venom of the Indian cobra (Sintiprungrat et al. 2016; Ali et al. 2013; Modahl et al. 2010; Mukherjee & Maity 2002; Mukherjee & Maity 1998). Again, as with other species of snake, the venom composition differs between snakes from different geographical locations (Mukherjee & Maity 1998).

The common krait (*Bungarus caeruleus*) has a venom that comprises of potent neurotoxins that induce muscle paralysis and possess phospholipase activity and other toxic components (Bon & Changeux 1975; Bon & Changeux 1977; Lee & Chlm 1976; Abe et al. 1977).
Generally, venom from snakes of the family Elapidae tends to contain more neurotoxic components than venom from snakes of the family Viperidae, which contains more haemotoxins.

9.1.3: Current medical treatment

The current treatment for envenomation is with antivenom, a preparation of IgG antibodies that are able to bind to the venom peptides. Antivenom is typically produced by injecting a low dose of snake venom into an animal such as a horse or sheep. The animal will then produce specific antibodies to neutralise the components of the venom. Antivenoms can be monovalent or polyvalent, with monovalent antivenoms being produced for treatment against

one specific species of snake and a polyvalent antivenom being able to work against venoms from a number of different species of snake. This treatment is effective in treating envenomation and has saved many from death and extensive tissue damage. However, antivenom may contain viruses if derived from animal blood (Theakston et al. 2003) and they can produce an allergic reaction which can be severe enough to cause death (de Silva et al. 2011). In rural Asia, where snakebite poses the greatest threat, small medical facilities have difficulty in storing antivenom as it requires refrigeration and electricity may be unavailable, it may also take days to travel to a medical facility that stores antivenom (Alirol et al. 2010). Since it has been shown that within snakes the same species, venom composition can vary depending on age, sex, location and season, this poses a problem for the manufacturing of antivenom (Casewell et al. 2014). Simply using a combination of venoms for the production of polyvalent antivenoms may also be problematic due to the cross-reactivity of venoms (Berger & Bhatti 1989).

Some progress has been made in producing antivenoms that are less likely to produce allergic reactions, such as antivenoms produced from the eggs of chickens injected with low doses of antivenom and with the use of Fab fragments instead of IgG molecules (Clark et al. 2002).

9.1.4: Traditional remedies

In rural areas where snakebite is prevalent, communities have their own traditional remedies for snakebite. The traditional remedies used vary depending on geographical location and between villages in these locations. The traditional use of plants is inevitably strongly influenced by the plants available in the local area.

In India, there are a variety of plants that are traditionally used to treat snakebite, such as *Mimosa pudica* (sensitive plant) and *Mucuna pruriens* (velvet bean) (Kunjam et al. 2013). *Vitex negundo* and *Emblica officinalis* are Indian medicinal plants which have been found to antagonise the activity of *Vipera russellii* and *Naja kaouthia* (Alam & Gomes 2003). *Andrographis paniculata*, *Crateva magna*, *Gloriosa superba* and *Hydrocotyl javanica* reduced haemolysis induced by the venom of *Naja nigricollis* (Kumarapppan et al. 2011). *Withania somnifera* is an important Ayurvedic herb which has also demonstrated inhibitory activity towards the venom toxin, hyaluronidase (Machiah et al. 2006). A wide assortment of plants are used for the treatment of snakebite in other parts of Asia, including Curcuma sp. in

Thailand (Salama et al. 2012), *Gnetum montanum*, *Phyllanthus urinaria* and *Gynura crepidioides* in Vietnam (Sam et al. 2008), and these plants are used against a number of different species of snake.

In South Africa, Mali and the Democratic Republic of the Congo, a large number of plants are used for the treatment of snakebite. Of some of the plants that have been tested for their activity and have exhibited significant activity include *Pupalia lappacea*, *Lanea acida*, *Combretum molle*, *Bahuinia thonningii*, *Strychnos innocua* and *Grewia mollis* (Molander et al. 2014).

In study of some plants traditionally used for the treatment of snakebite in South America reported strong inhibition of haemorrhaging induced by *Bothrops jararaca* venom by *Bellucia dichotoma*, *Plathymenia reticulata* and *Philodendron megalophyllum* and significant activity from a number of other local plants (De Moura et al. 2015). Another study from this region reported a large number of plants which are traditionally used for treating snakebite which included 71 species from 38 families (Vasquez et al. 2015).
Traditional remedies for snakebite come from plants of different families and a vast number of different species across a wide geographical range, making the choice of an effective traditional medicine difficult to correlate with a particular group of plants.

9.1.5: Current research on plant based medicines used for the treatment of snakebite

Since some plants have demonstrated potent activity against snake venom, much work has been done to find which components in these plant extracts are capable of producing these inhibitory and protective activities (Gomes et al. 2010).

Different classes of compounds have shown to be effective in antagonising venom activity, such as piperine (Shenoy et al. 2013), β-sitosterol and stigmasterol (Gomes et al. 2007b), lupeol acetate (Chatterjee et al. 2006), rosmarinic acid (Aung et al. 2010), labdane di- and trialdehyde, labdane lactone, 14-deoxy-11,12-didehydroandrographolide, parthenolide (Salama et al. 2012) and ar-turmerone (Ferreira et al. 1992). These compounds include alkaloids, phenolic and polyphenolic compounds, sterols and terpenes which may act against broad or specific components in venom. Some natural compounds may also potentiate the action of antivenom (Gomes et al. 2007b; Salwe et al. 2011; Ticli et al. 2005).

Based on current findings, antivenom activity is not attributed to any particular plant family or species, nor any particular class of compound, making a wide variety of plants and compounds open for investigation.

9.1.6: Methods for determining anti-venom activity

Antivenom activity can be determined through a number of *in vitro* techniques which aim to determine haemolysis, clotting, enzyme activity, haemorrhaging and cytotoxicity of a venom. *In vivo* tests are able to determine toxicity to specific tissues, lethality and also the extent haemorrhaging caused by venom. This study focuses on the effect that the extract has on the toxicity of venom, which can be tested on cell cultures *in vitro*.

9.2: Materials and methods

9.2.1: Chemicals and reagents

DMSO was purchased from RCI Labscan (Thailand), RPMI 1640, FBS and PBS were purchased from Thermofisher, quercetin, gallic acid and MTT were purchased from Sigma Aldrich (USA), periglaucine A was purchased from ChemFaces (China), palmitic acid, stearic acid, melissic acid and betulinic acid were purchased from Guokang Biotechnology (China).

9.2.2: Culturing of MRC-5

MRC-5 cells were grown in RPMI 1640 with 10% foetal bovine serum (FBS). Freshly trypsinised cells were seeded into a 96 well plate, at 5,000 cells per well and 100 µL of medium per well. The cells were left for 24 h before removing the media and adding samples.

9.2.3: IC_{50} of *C. atrox* venom

After culturing of MRC-5, *C. atrox* venom was dissolved in RPMI 1640 and sterilised by filtering through a 0.2 µm filter. Cells were incubated with different concentrations of the venom and without venom for 72 h after which the media containing venom was removed and the MTT assay was carried out as described in **section 9.2.4**. The experiment was run in triplicate of triplicates (n=9). The IC_{50} was calculated using the equation of the curve

generated by the percentage of growth inhibition against concentration and was the concentration of venom required to completely inhibit cell growth.

9.2.4: Inhibition of *C. atrox* venom by incubation with *P. glaucus* extracts.

The antivenom activity was determined as previously described by Nalbantsoy et al. (2012) with modifications. After culturing MRC-5, plant extracts were prepared by dissolving in DMSO and RPMI 1640 to make a stock solution which had a final concentration of 0.1% DMSO. This stock solution was serially diluted to make different concentrations of the extracts which ranged from 200 to 6.25 µg/mL, each with a final concentration of venom that was equal to the IC_{50}. The extract and venom samples as well as the controls containing the quantity of venom required to induce a 50% reduction in cell proliferation were incubated at 37 °C before being added to the wells containing cells. Cells were incubated with the samples for 72 h after which the extract was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) added. The experiment was run in triplicate of triplicates (n=9).

9.2.5: MTT assay

The MTT was prepared by dissolving the MTT salt in phosphate buffered saline (PBS), making a final concentration of 5 mg/mL. This stock solution was diluted 1:10 into R_0 RPMI 1640. 100 µL of this medium, containing MTT, was added to each well and incubated at 37 °C for 4 h, after which 75 µL of medium was removed from each of the wells. 50 µL of DMSO was added to each well and mixed thoroughly with a pipette before incubating for 10 min. Each sample was mixed again and the absorbance read at 540 nm using a microplate reader. The increase in proliferation of cells compared to the control was calculated using the following formula:

Equation 9.1: % increase in cell proliferation

$$\text{Increase in cell proliferation (\%)} = \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

9.3: Results

The quantity of venom required to inhibit cell proliferation by 50% was found to be 8.6 µg/mL. All samples were found to increase cell proliferation to some degree, as shown in **Figures 9.1 - 9.8**. The fruit extract showed the most significant enhancement of proliferation, increasing the proliferation of cells by up to 54.86% at a concentration of 200 µg/mL. Some of the extracts which enhanced proliferation in the cytotoxicity assays showed some enhancement of proliferation at lower concentrations, but this effect was reduced at higher concentrations as shown by the results of the stem hexane and chloroform extracts and the leaf chloroform extract. The standard, gallic acid, enhanced cell proliferation by 16.57% at its peak.

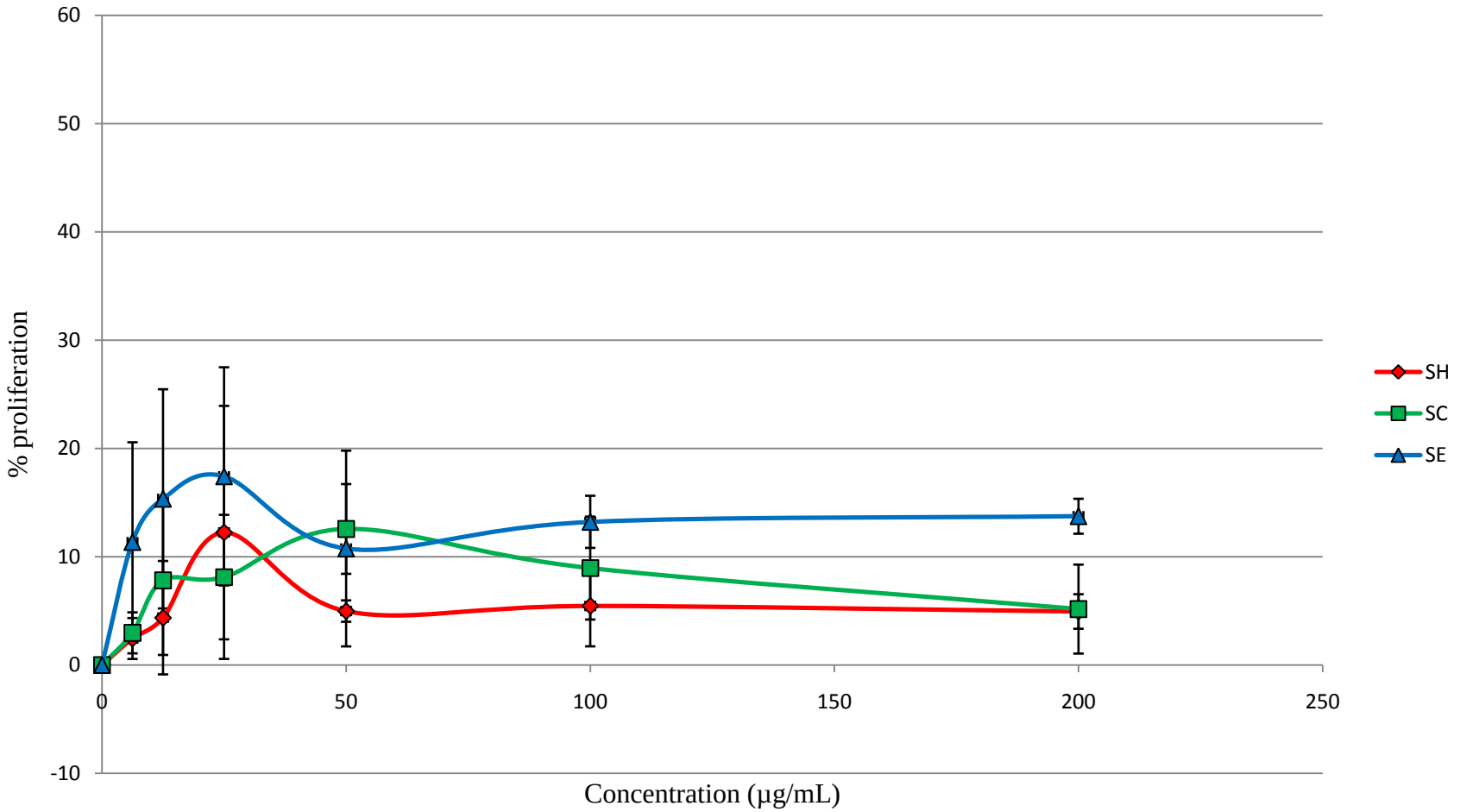


Figure 9.1: Increase in cell proliferation of MRC-5 when incubated with venom and stem extracts.

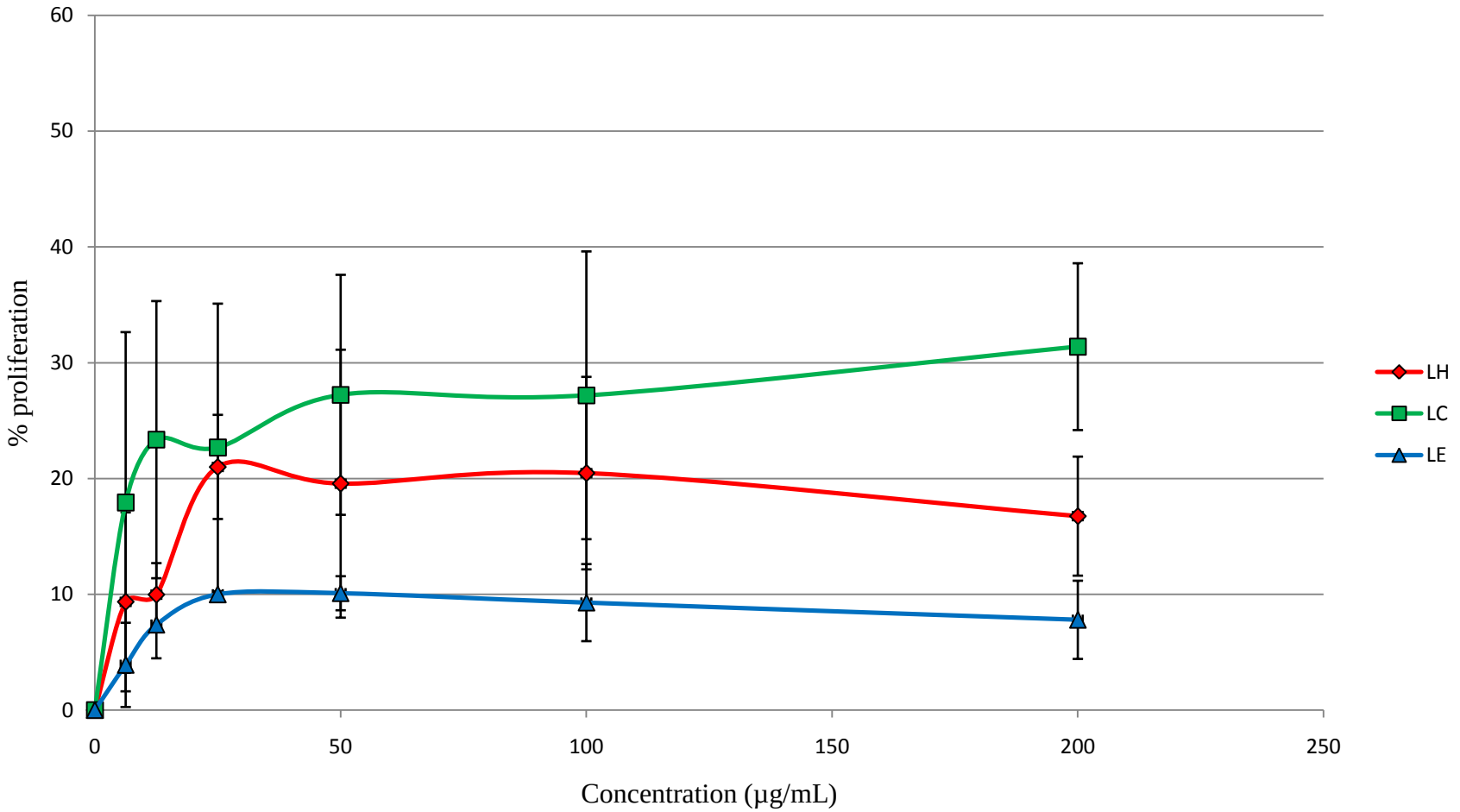


Figure 9.2: Increase in cell proliferation of MRC-5 when incubated with venom and leaf extracts.

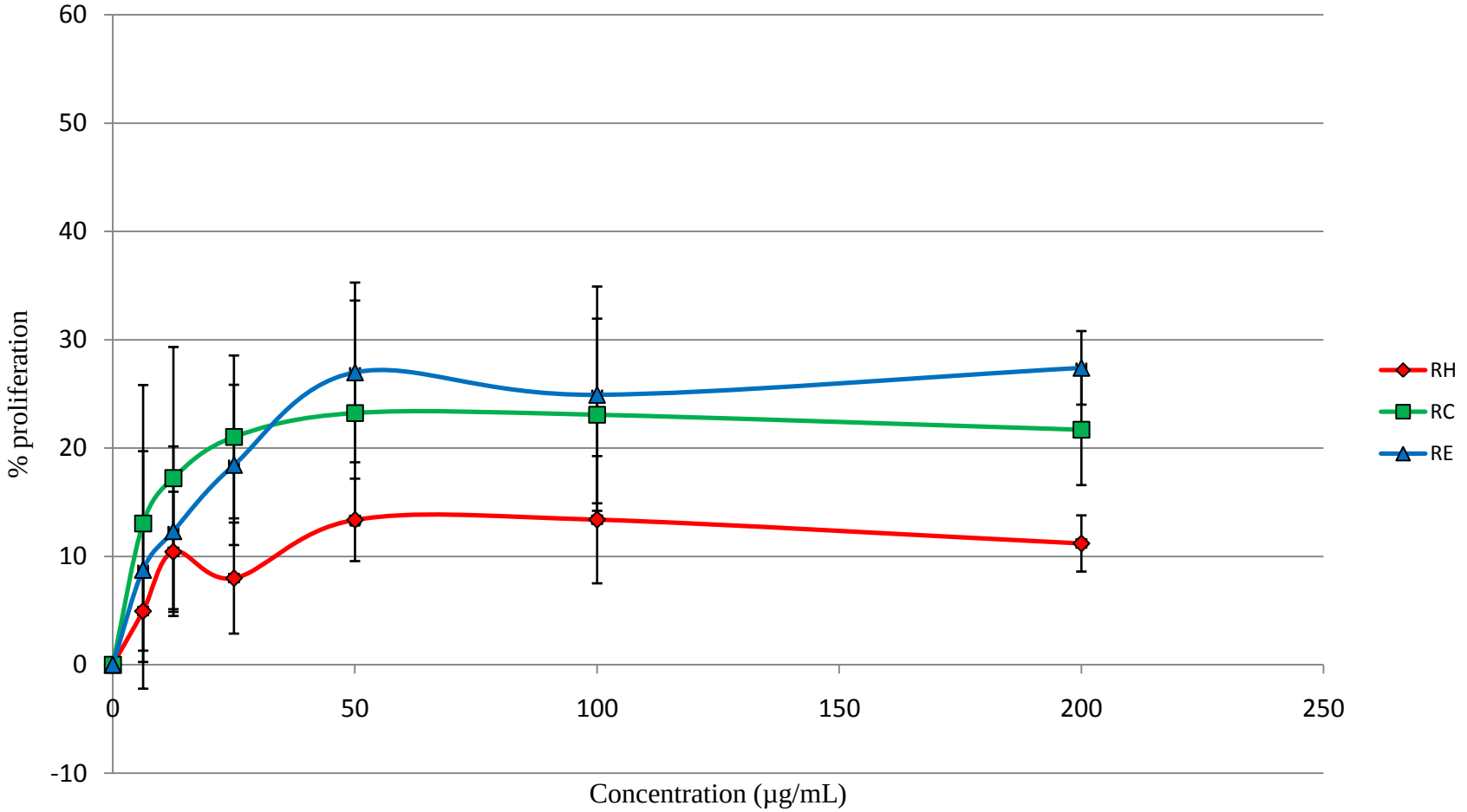


Figure 9.3: Increase in cell proliferation of MRC-5 when incubated with venom and root extracts.

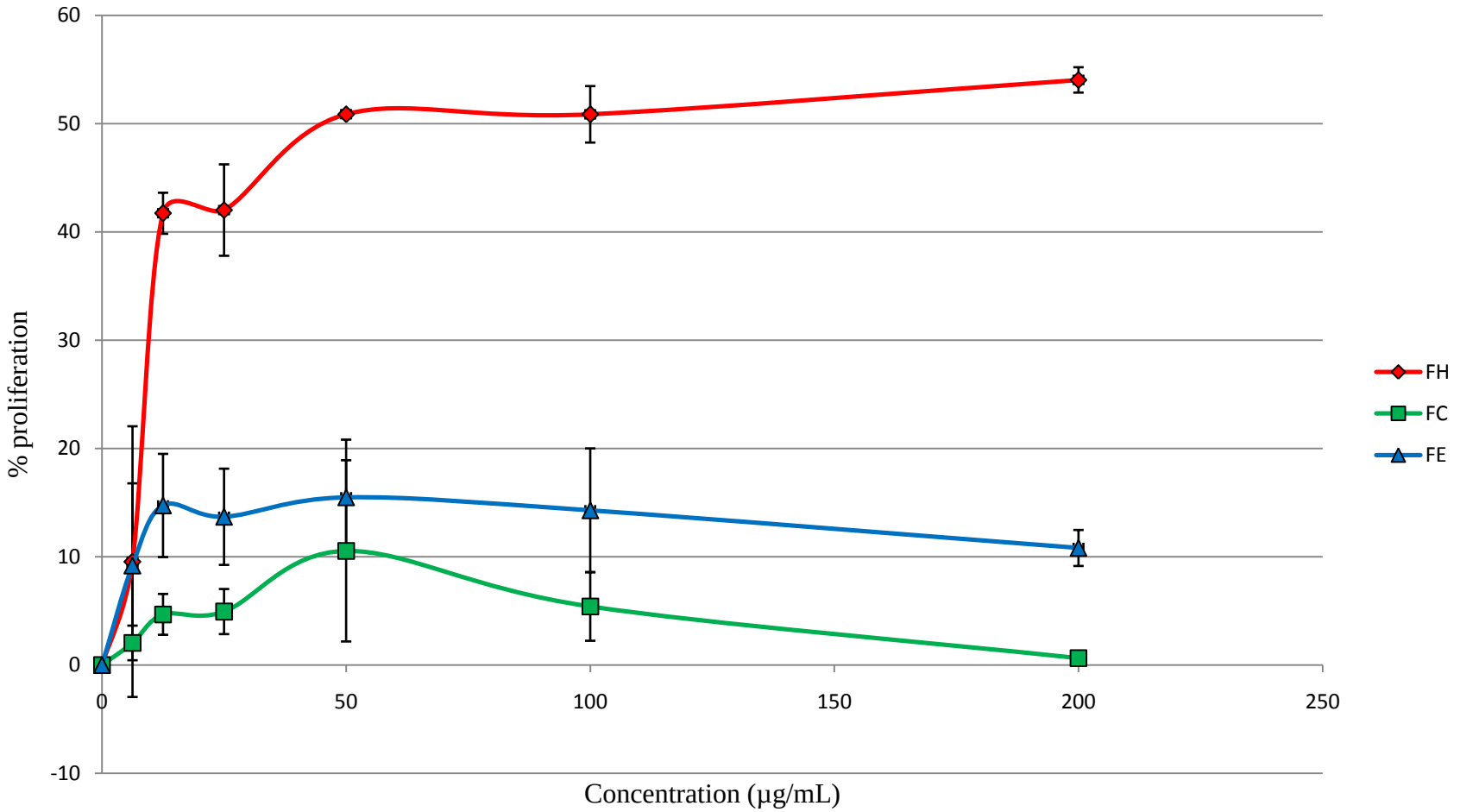


Figure 9.4: Increase in cell proliferation of MRC-5 when incubated with venom and fruit extracts.

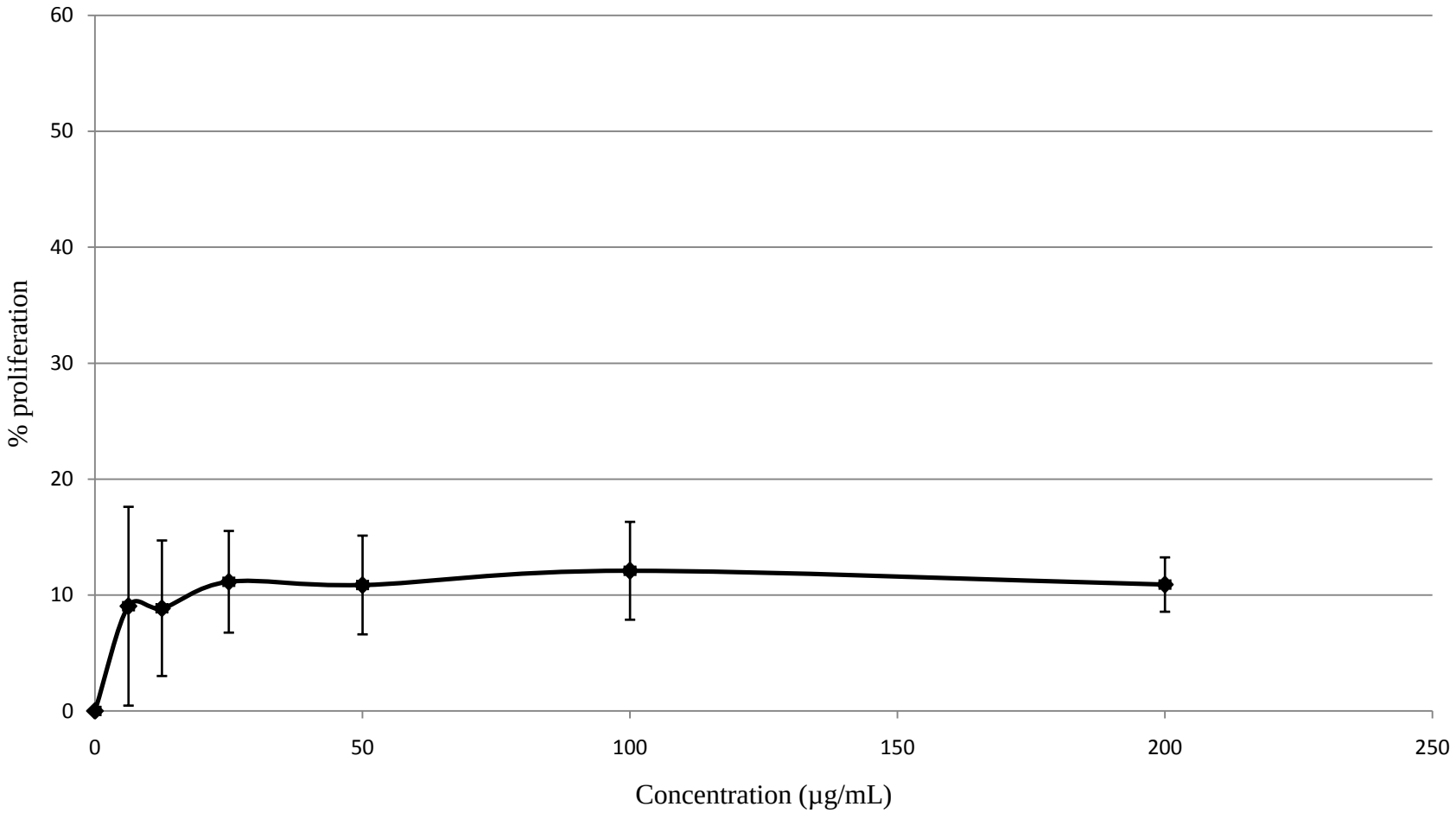


Figure 9.5: Increase in cell proliferation of MRC-5 when incubated with venom and the stem alkaloid extract.

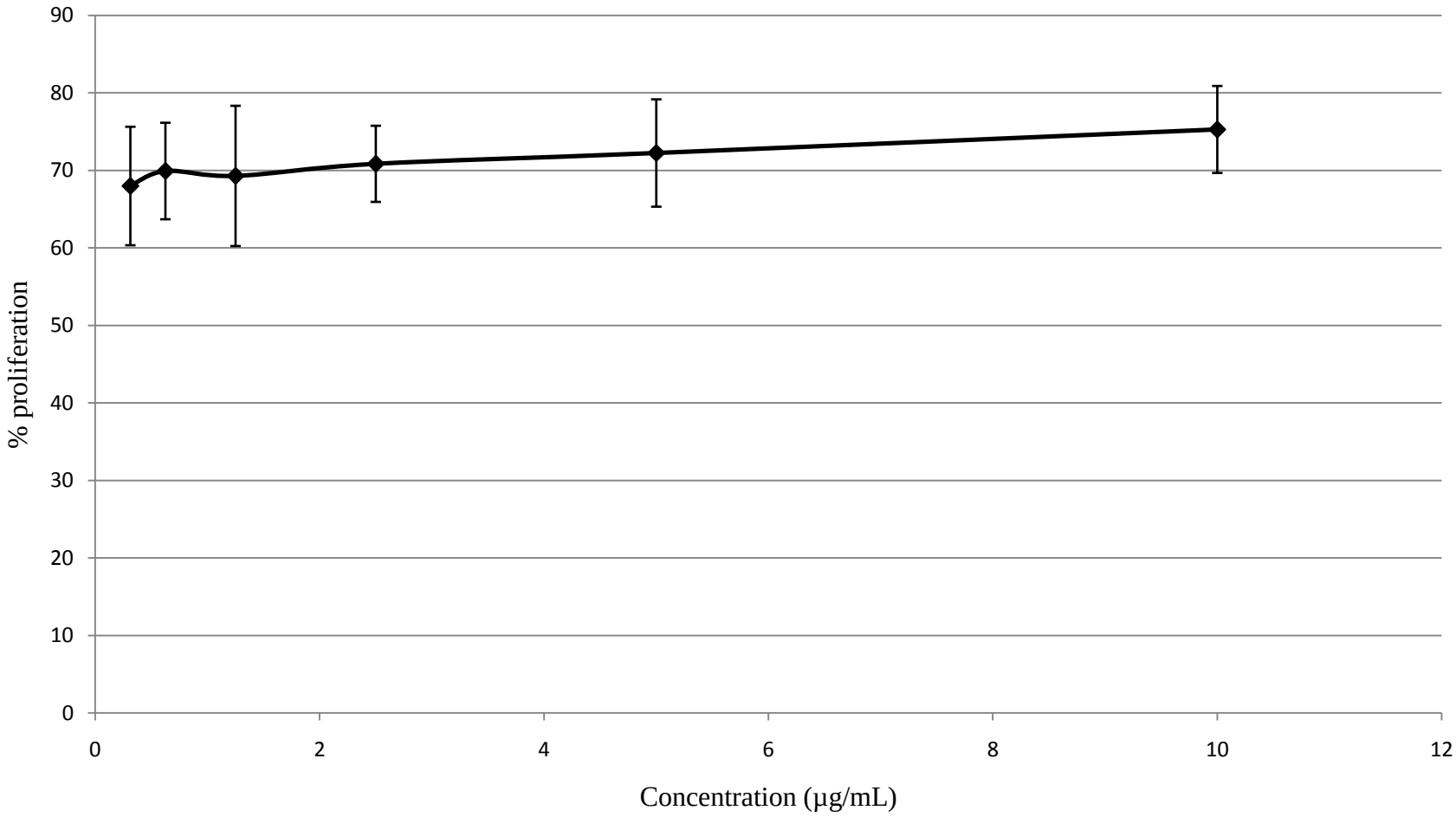


Figure 9.6: Increase in cell proliferation of MRC-5 when incubated with venom and periglaucine A.

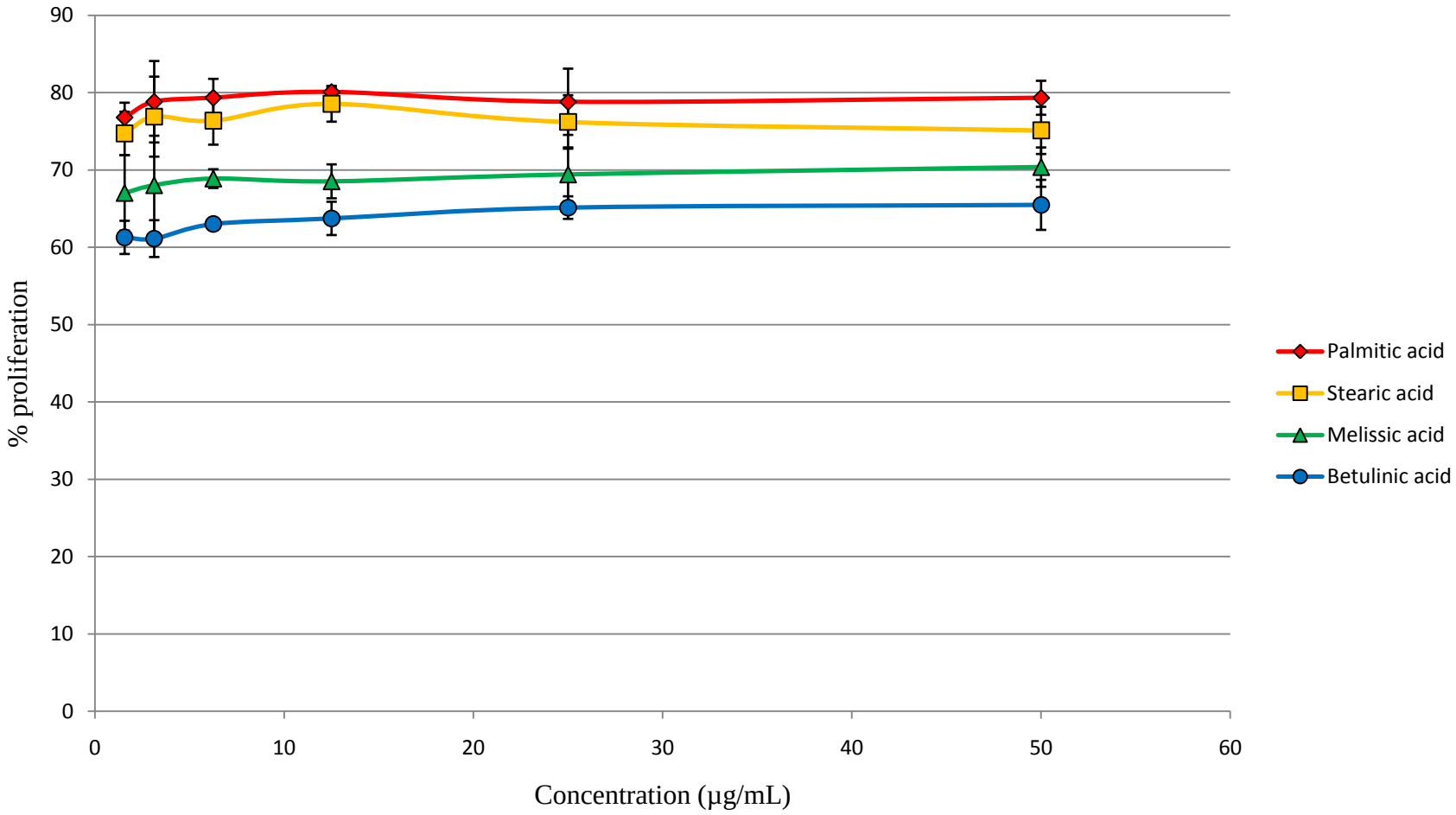


Figure 9.7: Increase in cell proliferation of MRC-5 when incubated with venom and fatty acids and betulinic acid.

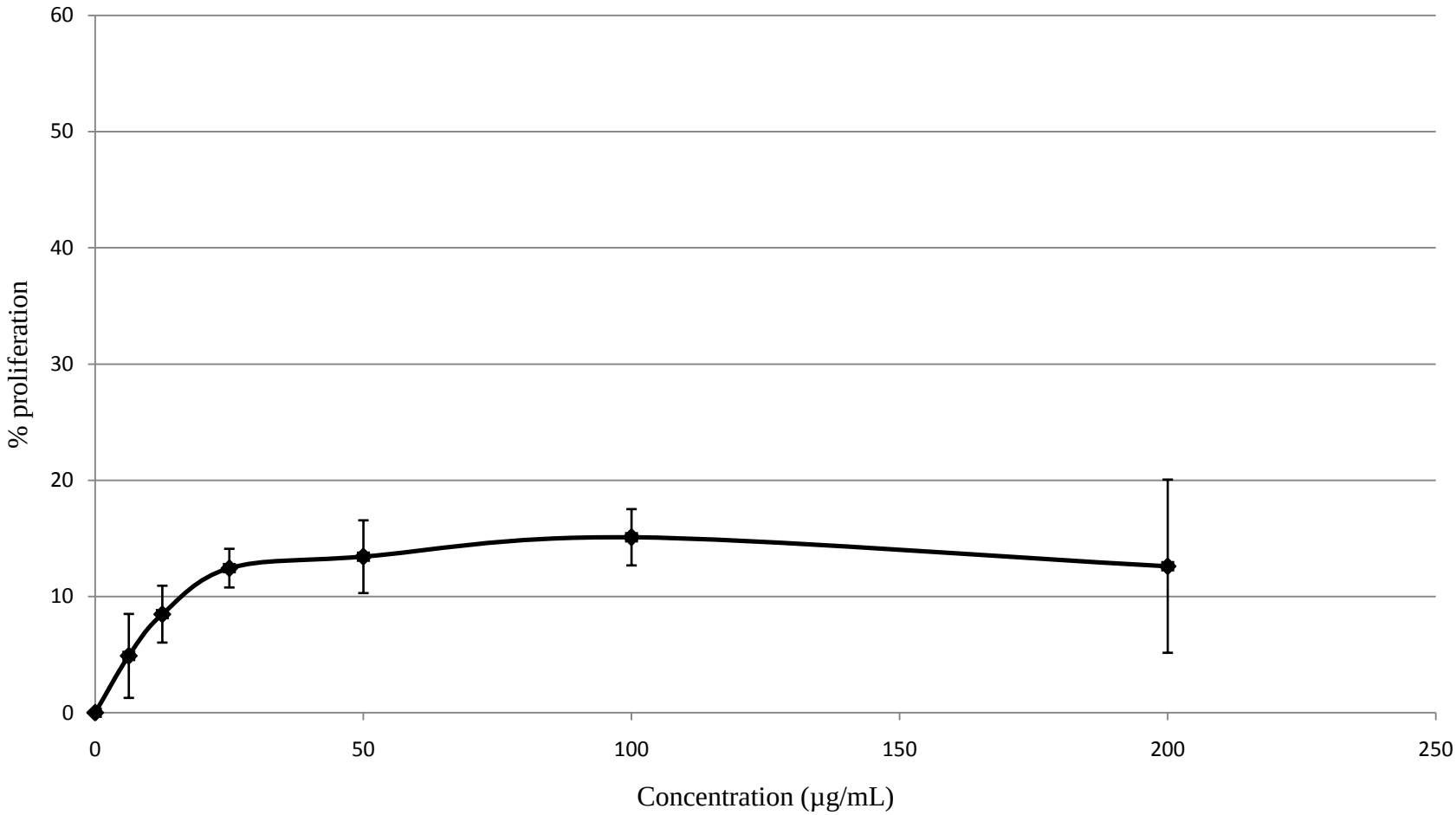


Figure 9.8: Increase in cell proliferation of MRC-5 when incubated with venom and gallic acid.

9.4: Conclusion

The results of the assay indicate that the fruit extract, given its ability to greatly enhance the proliferation of cells in the presence of *C. atrox* venom, may have venom neutralising or protective properties. Further studies should be done on the direct affect of the extract on the venom in order to determine what effect the extract may have on the venom as some natural compounds inhibit the components of venom with varying degrees of efficacy (Janardhan et al. 2014). Since venoms may act in a number of different ways to induce toxicity, a number of other assays could be performed to further explore the effect of the extracts on venom. The ability of the extracts to inhibit haemolysis could be tested as well as the ability of the extracts to directly inhibit specific enzymes found in venom.

Considering the fact that *P. glaucus* is traditionally used to treat venomous snakebites Vietnam (Sam et al. 2008), it is possible that *P. glaucus* is used to treat envenomation by a variety of snakes such as cobras, kraits and vipers. This study tested *P. glaucus* extracts against *C. atrox* venom, which has a different venom composition to snakes found in Vietnam. Although many snake venoms have certain similarities, the results of the antivenom activity of *P. glaucus* from this study may not accurately reflect the degree of activity that is seen with traditional use.

Other studies have found that the haemorrhagic activity of *C. atrox* venom is susceptible to inhibition by the aqueous extract of *Pentaclethra macroloba* and the degree of inhibition is similar to that of other viperids such as *Bothrops alternatus*, *Bothrops jararaca*, *Calloselasma rhodostoma* and *Lachesis muta* (Da Silva et al. 2005). As such, *P. glaucus* may also be active against other snake venoms although this would have to be tested in further studies.

Some of the antivenom activity observed may have been due to photooxidation as the samples were not protected from atmospheric oxygen or light during preparation and it has been observed that photooxidation of venom may occur when irradiated and incubated with trace amounts of methylene blue which leads to detoxification of the venom (Kocholaty 1965). Other compounds in the extracts may also be capable of providing conditions that favour photooxidation of venom peptides and the results may not represent the conditions under which the plant would be traditionally used. The assay should ideally be repeated under

more carefully controlled conditions to prevent any possible photooxidation. The test should also be repeated with a wider range of concentrations so that a clearer relationship between the concentration and venom neutralising activity can be determined, particularly in the case of periglaucine A, the fatty acids and betulinic acid.

Chapter 10: Discussion future perspectives

10.1: Phytochemical, and antioxidant activity results

Much similarity was seen between the stem and root extracts from the phytochemical study. The stem and leaf extracts also appeared to be similar in composition based on the TLC analysis with the fruit extracts showing a distinctly different chemical composition. The HPLC analysis also showed strong similarities between the stem and root extracts and the antioxidant activity and trends seen by the stem and root extracts in **Figures 4.1 and 4.3** were also very similar.

Despite the similarities between some of the extracts, the antibacterial activity demonstrated by extracts from the stem and root were different, with the stem extracts showing antibacterial activity against a number of different bacteria, while no antibacterial activity was observed from the root extracts.

The hexane extracts appeared to provide the best protection to proteins from heat induced denaturation, as such, the non-polar compounds are mostly responsible for this activity. The fatty acids were also able to prevent denaturation, with palmitic and stearic acid providing the greatest protection. Long chain fatty acids are relatively non-polar compounds due to their hydrocarbon tail, the majority of the fatty acids in the plant would have been extracted by hexane and therefore be present in the hexane extract. Although fatty acids are also soluble in the other solvents used in the extraction process, the hexane extraction was performed first so it is likely that the majority of fatty acids were extracted with this solvent.

It has been noted that thermophilic Clostridia have a greater saturated fatty acid composition in comparison to mesophilic or psychrophilic Clostridia, with the latter having a greater unsaturated fatty acid composition (Chan et al. 1971). The high level of saturated fatty acids in the lipid membranes of a thermophilic organism help to maintain the integrity of this membrane during heat exposure. The mechanism that protects ovine albumin from denaturation when mixed with the fatty acid involves no lipid membrane, so in the protein denaturation assay, it is possible that the saturated fatty acids exerted their protective activity by acting as chaperones, stabilising the albumin during exposure to heat by interacting with

the hydrophobic sites of the protein via their hydrocarbon tails and forming hydrogen bonds with the surrounding water via their carboxylic acid group.

During heat exposure, a protein may partially unfold which may expose the hydrophobic portion of the protein to water. The fatty acid may interact with the protein in such a way as to act as an additional protective layer, preventing the exposure of the hydrophobic residues to water when made vulnerable by heat. Binding pockets have been identified in human serum albumin (HSA), which can accommodate a number of different saturated fatty acids including some branched chain fatty acids (Hamilton 2004). Although the function of these binding sites has not been linked to protein stability ligands are able to affect protein stability, even during the process of denaturation (Shrake & Ross 1990). It is possible that the occupation of these binding sites by fatty acids could prevent exposure of heat sensitive portions of the protein to an aqueous environment but further tests would have to be conducted to test this hypothesis. The structures of ovalbumin and HSA are different however, since ovalbumin is able to bind to other molecules, it may also be able to bind to certain fatty acids which may affect the stability the protein.

The stem and root extracts showed somewhat similar membrane stabilisation activity when lysis of RBCs was induced by heat, except the chloroform extract of the roots did not maintain its protective activity at higher concentrations and enhanced lysis at 200 µg/mL. The hexane extracts tended to enhance RBC lysis, as did palmitic acid, although stearic acid and melissic acid helped to prevent lysis. When RBCs are exposed to excessive heat, this causes deformation of the cell membrane, echinocytosis and rupture (Choi & Pai 2002). Prevention of lysis would involve stabilisation of the membrane proteins and cell membrane. Fatty acids can be incorporated into the phospholipid membrane of erythrocytes in an energy dependent process which is also dependent on cellular metabolism (Shohet et al. 1968) however, it is unknown what quantity of the fatty acids were incorporated into the lipid membranes during incubation period in the HRBC membrane stabilisation assay as this was not tested.

Although the hexane extracts were found to prevent protein denaturation, the enhancement of RBC lysis by these samples under the stress of heat would suggest that stabilisation of membrane proteins did not occur or did not contribute to protection from heat induced lysis and that the direct destabilisation of the phospholipid membrane may have caused the lysis. If

this was due to the fatty acid component of the hexane extracts, Leekumjorn et al. (2010) found that palmitate caused membrane destabilisation, for which the cause was postulated to be due to the more compact arrangement of straight chain phospholipids which allowed the release of water and reduced membrane fluidity, or due to the formation of aggregates with the membrane which allowed pore formation. This would support the results of the present study which found that palmitic acid enhanced lysis under thermal stress.

Palmitic acid, enhanced lysis under the stress of heat while the longer chain fatty acids, stearic and melissic acid, provided protection from heat induced lysis. If the longer chain fatty acids were incorporated into the membrane, they may have been able to provide greater protection from heat due to their lower fluidity and greater stability with increased temperature, as longer chain fatty acids have greater stability at higher temperatures compared to shorter chain fatty acids.

The production of fatty acids with high melting points, such as saturated fatty acids by thermophilic bacteria allows them to survive at high temperatures, as such, the incorporation of fatty acids into the membrane of erythrocytes could be assumed to aid in stabilising the membrane when exposed to heat, however, this hypothesis would contradict the conclusions drawn from the model used in the study conducted by Leekumjorn et al. (2010), which suggested that greater rigidity contributed to greater membrane destabilisation, although this study did not test stability under thermal stress. The application of heat may have allowed the membrane to become more stable. Thermophilic bacteria are known to alter their fatty acid composition rapidly as the temperature of their environment changes as the properties of their membranes will change with variations in temperature (Koga 2012). Although the incorporation of fatty acids with high melting points may be beneficial under thermal stress, without that stress, these fatty acids may cause destabilisation instead, resulting in the differences produced by the different fatty acids in the membrane stabilisation assay.

The erythrocytes may have exhibited toxicity towards palmitic acid, as they can be taken up by cells passively (Abumrad et al. 1998) and certain cell lines are sensitive to certain fatty acids (Martins de Lima et al. 2006). Since fatty acids can induce apoptosis by inducing changes in mitochondrial membrane potential and erythrocytes do not have mitochondria, the possibility of apoptosis being induced by this particular mechanism can be eliminated, instead, lysis may have occurred due to neutral lipid accumulation (Martins de Lima et al.

2006) although it is not known whether accumulation of lipids in the time that the erythrocytes and fatty acids were incubated together in the membrane stabilisation assay would be sufficient to cause toxicity by this mechanism or how hypotonicity and heat would affect this process. Stearic acid, being the shortest chain fatty acid in this study, may have been easier for erythrocytes to take up.

The mechanism by which the fatty acids protected erythrocytes from heat induced lysis or enhanced it is still unclear and would require further investigation to determine the precise mechanism of action. The chemicals in the extracts that were able to enhance membrane stabilisation under heat may have been able to bind to the cell membrane and alter the charge of the membrane, repelling compounds with like charges which could cause lysis of the erythrocyte (Debnath et al. 2013).

The stem, root and fruit ethanol extracts provided protection from lysis, as did the stem chloroform extract. Based on the results, a particular class of compound that could be responsible for this protection from lysis cannot be determined. Some saponins and polyphenols are known to stabilise lysosomes and erythrocytes, however the stabilising activity observed cannot be directly correlated with polyphenol concentration or the presence of saponins since the leaf chloroform, leaf ethanol and stem ethanol extracts contained saponins, yet the trends seen in their responses was different. Also, the highest concentration of polyphenols was found in the chloroform extracts, yet no strong correlation between polyphenol content and membrane stabilisation activity can be made based on the results of this assay.

The leaf chloroform, leaf ethanol extracts, root chloroform, fruit hexane and fruit chloroform extracts all demonstrated membrane stabilising activity at lower concentrations, but reduction in protection and enhancement of lysis at higher concentrations. Based on the difference in the results obtained from the hypotonicity induced lysis, it would appear that different mechanisms are involved in lysis and protection from lysis under these different conditions. Some of the graphs generated displayed biphasic behaviour, which has been observed in erythrocytes exposed to acidic media (Arvinte et al. 1989). Some of the extracts may alter the pH of the media, making it more acidic and contributing to the biphasic effect and also by causing oxyradical formation which could increase lysis (Ivanov 1999), however it may also imply a more complex mechanism behind the stabilisation of membranes.

No particular trend was seen between the polarity of the extracts and the ability of the extracts to inhibit the COX enzymes although the hexane extracts were more likely to show less inhibitory activity which could possibly be due to fatty acid content as explained previously. Based on the classes of compounds in the solvent extracts, the inhibitory activity could not be accurately attributed to any particular class of compounds however, the alkaloid extract and periglaucine A did demonstrate inhibitory activity demonstrating a definite link between the alkaloids and COX inhibition, with COX-2 being more sensitive to the effects of the alkaloids compared to COX-1. While it has been found that many alkaloids do not directly inhibit the COX enzymes (Huss et al. 2002) some alkaloids can inhibit COX in a by downregulating or inhibiting expression (Zhao et al. 2013; Lee et al. 2003). The alkaloid fraction and periglaucine A were found to inhibit COX-1 and COX-2 through direct interaction, it is possible that they may also inhibit the COX enzymes by other means such as reduced expression.

10.2: Antibacterial results

Since bacterial infection can cause inflammation, antibacterial compounds are useful in treating wounds where inflammation is caused by microbial infection. The antibacterial activity of *P. glaucus* was found to be mild, however, it may exert a stronger effect against certain other bacteria. Further studies should include antibacterial testing against a larger number of pathogenic bacteria to better elucidate the antibacterial potential of this plant.

10.3: Anti-inflammatory results

Cytotoxicity and wound healing can cause inflammation. Some of the extracts were found to be cytotoxic, yet inhibit COX-1 and -2, such as the ethanol extracts of the stem, leaf and root. When used traditionally, water is used as the solvent for extraction and delivery of the active components of *P. glaucus*, so it is likely that the polar compounds, which are present in the ethanol extracts, will be in the final preparation of the traditional medicine. Although it is unknown whether or not the cytotoxic compounds in the extract are also responsible for the COX inhibitory activity, some natural compounds with anti-inflammatory activity (Gupta et al. 1969; Yuan et al. 2006) have also been found to be cytotoxic towards some cell lines (Murata et al. 2013; Tsai et al. 2015; Fabiyi et al. 2012). Cytotoxic compounds are also able

to cause inflammation (Comer et al. 2014), as such, more research would have to be conducted to determine the action of these extracts on cells and in a living organism. The hexane extracts and fatty acids showed significant proteoprotective activity, which may be due to the action of fatty acids or other non-polar compounds as chemical chaperones, which have demonstrated therapeutic potential in treating inflammatory diseases (Cortez & Sim 2014; Cao et al. 2013).

The non-cytotoxic extracts were chosen to be tested for their wound healing activity, but it is possible that at low concentrations, the extracts that did show strong cytotoxicity towards MRC-5 could benefit wound healing through eustress or hormesis. Although many natural products that promote wound healing possess anti-inflammatory activity, it is important to note that inflammation is a normal and necessary part of wound healing that prevents the spread of any damaging agents to the surrounding tissues, is required for the removal of dead cells and bacteria and helps wound healing to progress.

Inflammation is a major symptom of many snakebites with a few exceptions, such as envenomation by the krait which initially produces minimal pain and inflammation yet can prove deadly. Triggering inflammation helps to prevent the spread of venom throughout the body, however, can become detrimental to health in extreme cases, contributing to compartment syndrome and tissue necrosis. A study on scorpion venom concluded that the action of the venom could possibly be treated with COX inhibitors (Zoccal et al. 2016) and another study, also on scorpion venom, found that a number of anti-inflammatory drugs reduced mortality rates and increased survival times of mice injected with *Leiurus quinquestriatus* venom (Abdoon et al. 2006). The venom of many venomous animals contain inflammatory components, such as inflammasomes, including the venoms of a number of snakes (Palm & Medzhitov 2013). It is possible that certain anti-inflammatory drugs may also be beneficial in the treatment of snakebites from snakes whose venom contains pro-inflammatory agents, as some plants which are known for their anti-inflammatory activity are also used for the treatment of snakebite (Mensah et al. 2011) and this has been attributed to the inhibitory action on PLA_2 in some studies (Samy et al. 2012). Since many of the extracts tested as part of this study were found to inhibit COX-1 and COX-2, including the alkaloid, periglaucine A, the traditional use of *P. glaucus* for the treatment of snakebite may be partly due to its anti-inflammatory action, further study could be undertaken to assess the inhibitory

action of *P. glaucus* extracts and components of the extracts towards other toxic enzymes such as PLA_2 as inhibition of this enzyme may also be beneficial in treating snakebite.
The use of some anti-inflammatory drugs, in particular, those which thin blood, may be contraindicated for the treatment of bites from snakes whose venom is particularly haemorrhagic as it may cause excessive bleeding.

10.4: Cytotoxicity results

The ethanol extracts tended to exhibit greater toxicity towards HK1 and MRC-5. A similar link between polarity and cytotoxicity has been observed in other studies (Guha et al. 2011; Irjayanti et al. 2015), however, this is not always the case and the type of activity observed by different extracts varies considerably between plants (Talib & Mahasneh 2010). The ethanol extracts of the leaves and stems both tested positive for saponins and terpenes. There are examples of compounds from these two classes which exhibit anticancer activity (Man et al. 2010; Vuong et al. 2015; Kanchana & Balakrishnan 2011; Yan et al. 2009; Salminen et al. 2008; Aiello et al. 2003), but since the leaf chloroform extract was also found to contain saponins and terpenes but enhanced growth, the effect cannot be attributed simply to the class of compounds present in the extracts, especially since compounds in one class can be responsible for a wide variety of activities. Saponins from Ginseng have been found to enhance proliferation (Fang et al. 2007), so to find the compounds specifically responsible for the cytotoxic activity, the ethanolic extracts would have to be fractioned and compounds isolated from these fractions to be tested individually.

The hexane and chloroform extracts were more likely to enhance growth and, in the phytochemical test, were found to contain sterols. Sterols are similar in structure to hormones and may have been acting as growth promoting hormones. A number of studies have identified sterols which cause apoptosis in some cell lines, such as beta-sitosterol treated U937 cells (Park et al. 2007), campesterol treated HUVECs (Choi et al. 2007) and stigmasterol treated A7r5 cells, but sterols can also enhance proliferation. Beta-sitosterol, which causes apoptosis in U937 cells, enhances proliferation of T-cells (Bouic et al. 1996) and cholesterol is required for the proliferation of some cells (Chen 1984). Ergosterol and daucosterol have been previously isolated from *P. glaucus* and the former has been found to inhibit cancer cell growth and induce cell death as well as prevent cell cycle progression in the presence of cholesterol (X. Li et al. 2015; Subbiah & Abplanalp 2003; Suárez et al. 2002;

Li et al. 2016). Daucosterol has been found to enhance proliferation of neural stem cells (Jiang et al. 2014) and inhibit cancer proliferation (Zhao et al. 2015). A decrease in proliferation was only observed when HK1 was treated with the leaf hexane extract at higher concentrations while all other leaf and stem extracts which tested positive for sterols enhanced cell proliferation. It is also possible that the sterols are able to interact with the membranes of the cells and alter membrane enzyme activity which may alter cell signaling and growth (Hartmann 1998).

Interestingly, the stem alkaloid extract promoted HK1 growth while periglaucine A reduced proliferation. In the case of MRC-5, the stem alkaloid extract reduced proliferation while periglaucine A enhanced proliferation. Based on the HPLC analysis of the stem alkaloid extract and periglaucine A, it would appear that many compounds are present in the stem alkaloid extract and periglaucine is not a major component of that extract. As such, the differences in the affect of the stem alkaloids on the cell lines would be mostly due to a combination of the other major components of the stem alkaloid extract which exert a different activity to periglaucine A. Based on these results, periglaucine A would be a better candidate for an anticancer agent than the crude alkaloid extract due to its toxicity towards HK1 and lack of toxicity towards MRC-5. Since different cell lines can respond in different ways to chemicals, with some being more toxic towards some cell lines than others, from the results collected in this study, it can only be deduced that periglaucine A exhibits toxicity towards at least one cell line *in vitro*, HK1.

Palmitic, stearic and melissic acid decreased proliferation of HK1, while only melissic acid reduced proliferation of MRC-5. Fatty acid induced apoptosis may have been due to suppression of autophagy and caspase mediated cleavage of Beclin 1 (Mei et al. 2011), although the reason for the difference in toxicity between cell lines by fatty acids of different lengths is unknown.

Betulinic acid has been widely studied as an anticancer agent and was found to exert greater cytotoxicity towards HK1 than MRC-5 in this study, although it reduced proliferation of both cell lines. The mechanism of action that betulinic acid has on these cell lines is not clear although some studies have found that betulinic acid downregulates lamin B1 in pancreatic cancer cells (Li et al. 2013), alters mitochondrial morphology via inhibition of steroyl-CoA-desaturase and cardiolipin modification (Potze et al. 2016), upregulates pro-apoptotic genes

and downregulates antiapoptotic genes as well as affecting the expression of numerous other genes to promote apoptosis (Xu et al. 2014) and inhibits NF-κB transcription and the oncogene Meq (An et al. 2015). Betulinic acid is non-toxic to some normal cell lines (Heiss et al. 2014; Zuco et al. 2002), while more toxic to some normal cells in comparison to their cancerous counterparts (Surowiak et al. 2009). HK-1 appeared to be more sensitive to betulinic acid compared to MRC-5, although the precise mechanism by which this happens was not determined and could involve any or none of the previously mentioned mechanisms. A proteomic study would have to be conducted to investigate any upregulation or downregulation of gene expression.

Some antibiotics are able to negatively affect mitochondrial function in cells and, given the similarity that mitochondria bear to bacteria and their likely ancestry, this is not surprising. Certain cell lines are more susceptible to toxicity by antibiotics, suggesting that toxicity is not due simply to mitochondrial apoptosis pathways, but involves other factors (Duewelhenke et al. 2007; Ayaki et al. 2012). Antiseptics have also been found to show some toxicity towards cells (Damour et al. 1992), demonstrating that a certain amount of toxicity is to be expected from certain antibiotics and antiseptics. Despite this association between cytotoxicity and antibacterial activity, no particularly strong antibacterial activity was seen by the most cytotoxic extracts, such as the stem, leaf and root ethanol extracts.

The less polar extracts were more likely to be proteoprotective and were also less toxic. The ethanol extracts of the stem, leaf and root were significantly cytotoxic and all ethanol extracts showed relatively less proteoprotective activity compared to other extracts of the same plant part with the exception of the root ethanol extract, which showed little to no proteoprotective activity, but did not enhance protein denaturation as much as the root chloroform extract. The non-polar extracts may contain chemicals that are able to act as chemical chaperones which have been shown to protect cells from certain types of damage (Woltjer et al. 2007) which may partly explain why the non-polar extracts were less cytotoxic than the more polar extracts.

Although some of the ethanol extracts were cytotoxic, when combined with *C. atrox* venom, these same extracts helped to reduce cytotoxicity induced by the venom and did not enhance cytotoxicity. These results indicate that the cytotoxic components of the extract may be responsible for the venom neutralising activity. Aristolochic acid is one example of a

compound that has been shown to be toxic to cells (Nitzsche et al. 2013), yet is able to bind to components of snake venom (Vishwanath et al. 1987) and counter their activity (Girish & Kemparaju 2006). It is possible that components of the extracts are able to work in a similar way to neutralise components of the snake venom, although this would have to be studied further to confirm this.

10.5: Wound healing results

Given the ability of some of the extracts to enhance proliferation, the wound healing activity of all the non-cytotoxic extracts was assessed using the wound healing assay. Numerous other plants have been found to enhance wound healing activity, and often use polar extracts (Divya et al. 2011; Lien et al. 2015; Basanta Kumar Jena, Bhabagrahi Ratha, Subrat Kar, Satyaranjan Mohanta, Monali Tripathy 2012; Amin et al. 2015; Demirci et al. 2014; Tam et al. 2011; Muhammad et al. 2013; Harishkumar et al. 2013; Brusotti et al. 2015; Ruszymah et al. 2012; Wong & Ling 2014). Some of the *in vivo* wound healing activity is attributed to the antimicrobial activity of the plant (Rajendran et al. 2012), however, the disruption of wound healing due to infection was eliminated in this assay as it was done under sterile conditions. Some of the plant derived compounds capable of enhancing wound healing *in vitro* include the quinone, β-lapachone (Kung et al. 2008), sterols (Tanaka et al. 2015), polyphenols (van Hien & Cherry 1997; Amatori et al. 2016; Nirmal et al. 2014), saponins (Sevimli-Gur et al. 2011) and alkaloids (Porras-Reyes et al. 1993). Although corticosteroids slow wound healing (Gerarde & Jones 1953; Jung et al. 2001), anabolic steroids can promote it (Demling 2005; Ahmad 2013). Some plant sterols could possibly enhance wound healing in the same manner as anabolic steroids due to their similar structure and activity, and the wound healing activity observed by the stem and leaf chloroform and hexane extracts, which tested positive for steroids, may partly owe their activity to their steroid composition although this cannot be proven by the results of this study as the sterols would have to be isolated and tested individually.

The chloroform extracts tended to enhance wound healing to a greater extent than other extracts, with the exception of the leaf hexane extract which exhibited greater wound healing activity than the leaf chloroform extract. This indicates a possible link between the quantity of polyphenols in the extracts and their ability to enhance wound healing, stimulate proliferation or promote cell migration. Other studies have also detailed the effect of

polyphenols and antioxidant activity on wound healing (Archit et al. 2014; Geethalakshmi et al. 2013; Tsai et al. 2016), however, oxidants are also important for cell signalling during healing, yet wound healing may still be promoted by compounds that act as antioxidants due to promotion of gene expression associated with wound healing as well as quenching free radicals that are produced during healing (Sen et al. 2002).

FBS showed much greater wound healing activity than any of the extracts, most likely due to the growth factors present. This model is limited in the fact that wounds are not two dimensional and involve more than a single monolayer of cells, as such, a more substantial model involving layers of cells would be a more accurate model for the wound healing process (Gonzalez-Andrades et al. 2016).

10.6: Antivenom results

All extracts were able to increase cell proliferation compared to the control to some degree when incubated with snake venom. The lowered toxicity of the ethanol extracts towards MRC-5 suggests that the cytotoxic components interacted with the components of the *C. atrox* venom to reduce the toxicity of both. Since some compounds, such as polyphenols, are able to aggregate proteins (Haslam 1989), the cytotoxic compounds which have an affinity for the venom peptides may have precipitated the peptides, making them and the compounds themselves unavailable to the cells, thus reducing the cytotoxic effects of both. Further studies would have to be conducted to test this hypothesis. Although the chloroform extracts contained the greatest quantity of phenolics compared to the other solvent extracts, they did not necessarily produce the greatest effect. It is possible that the polyphenols may have had some inhibitory action on the cytotoxicity of the venom peptides, however, the affinity of a polyphenol for a peptide depends to a certain extent on the residues in the peptide and the size of the polyphenol (Charlton et al. 2002). The inhibitory activity could not be directly correlated with the polyphenol content, however, the different molecular weights of the polyphenols present in the extracts may have resulted in the different effects seen by the polyphenol rich extracts, if the antivenom activity was indeed a result of the action of this type of compound (Hemingway & Laks 2012).

Other compounds present in the extracts may also neutralise venom peptides and this was demonstrated by the ability of the stem alkaloid extract to neutralise venom and by

periglaucine A, which significantly reduced the toxic effects of the venom. Other studies have reported interactions between alkaloids and proteins which may involve binding or electrostatic interaction and may interfere with protein stability and function (Hossain et al. 2011; Khan et al. 2013). Polyphenols have been noted to bind with high affinity to proline residues as well as histidine residues (Naurato et al. 1999; Hagerman & Butler 1981) and some of the components of *C. atrox* venom contain a significant proportion of proline residues, such as hypotensin (Bonilla 1978). The carbohydrate recognition domain (CRD) of the rattlesnake lectin (RSL) from *C. atrox*, which is conserved among numerous other viperids, consists of 3 amino acids, one of which is a proline residue (Sartim & Sampaio 2015) which may serve as a target for polyphenol binding. The crude extracts of many plants have been found to act against venom, although the chemicals directly responsible for this activity cannot be deduced with all certainty from many of these studies (De Almeida et al. 2004; Shenoy et al. 2013; Gopi et al. 2014; Fernandes et al. 2011; Dhananjaya et al. 2011; Arts 2009; Ticli et al. 2005; Salwe et al. 2011; Aung et al. 2010; Kumarapppan et al. 2011; Alam & Gomes 2003; Krishnan et al. 2014). Some compounds have been isolated from plants and other sources and have been found to inhibit the activity of snake venom or its components, including caffeic acid (Pereañez et al. 2011), aristolochic acid (Girish & Kemparaju 2006; Vishwanath et al. 1988), vanillic acid, stigmasterol, beta sitosterol (Gomes et al. 2007a), 2-hydroxy-4-methoxy benzoic acid (Alam & Gomes 1998), wedelolactone (Pithayanukul et al. 2007), quercetin (Lindahl & Tagesson 1993), gallic acid (Pereañez et al. 2011), ar-turmerol (Ferreira et al. 1992) and lupeol acetate (Chatterjee et al. 2006).

8.6 µg/mL of venom was used and 0.3125 µg/mL of periglaucine A was able to produce almost a 70% increase in proliferation, this may be because the site that periglaucine A binds to on the venom peptides is able to cause precipitation at low concentrations or it may bind to a component of the venom that is mainly responsible for the cytotoxicity of the venom. The lack of increase in proliferation with increasing periglaucine A concentration may indicate that the peptides were saturated with the alkaloid. Due to the small quantity of periglaucine A available during this study and the possible significance of the results, the test should be repeated to ensure reproducibility.

Palmitic, stearic and melissic acid also showed significant inhibition of venom cytotoxicity with betulinic acid showing the least activity, but still over a 60% increase in proliferation. The fatty acids appeared to be able to inhibit the action of the venom at low doses, and

appeared to have reached a saturation point at these low concentrations. This effect has not been described in previous literature and further studies would have to be conducted to determine how the fatty acids are exerting this protective activity.

Many components of snake venom are also produced in bacteria, such as PLA_2, which is produced by *Helicobacter pylori* (Smoot 2014) and collagenases, which are produced by *Clostridium* (Sanjuan-Cerveró 2016). Hyaluronidases are produced by bacteria such as *Staphylococcus aureus* (Ibberson et al. 2014) and *Streptococcus pyogenes* (Starr & Engleberg 2006) and contribute to pathogenesis by degrading tissues and allowing passage of bacteria deeper into tissues. As such, the ability of a chemical to neutralise venom may also be beneficial in slowing the progression of certain bacterial infections.

When considering the practical aspect of administering polyphenols to a snakebite victim for the purpose of aggregating the venom peptides, it is worth noting that polyphenols will bind to various different proteins and peptides with varying affinity depending on available binding sites (Charlton et al. 2002), as such, are able to bind to proteins normally present in an organism, such as serum albumin and cellular proteins. The binding of polyphenols to non-offensive proteins may reduce the quantity of polyphenol available to bind to venom peptides, meaning a large quantity of polyphenol may be required to produce a venom neutralising effect.

It is possible that the hasubanan alkaloids are capable of not only neutralising components of venom, as demonstrated by the results of this study, but reducing some of the symptoms associated with snakebite. Hasubanan alkaloids have been found to bind to some opioid receptors (Carroll et al. 2010) which is not surprising considering the resemblance that hasubanan alkaloids bear to morphinan alkaloids which are known to bind to opioid receptors (Benyhe et al. 2015). Since the traditional use requires the plant extract to be injected into the site of the wound, this would allow the alkaloids to reach the brain where they can bind to opioid receptors, providing some relief from the pain of the bite. Opioids that are able to bind to κ-opioid receptors have been found to also act as anti-inflammatories (Walker 2003), however, no hasubanan alkaloids have been found to act on κ-opioid receptors to date and they have only been found to act on δ- and μ-opioid receptors (Carroll et al. 2010).

In a number of assays, unusual trends were observed which showed that the samples did not exert a dose dependant effect. This has been explained as being the result of biphasic behaviour or a hormetic response in some cases, however, it may also be due to contamination by trace amounts of solvent. When the extract is produced, it is dried by rotary evaporation to remove the solvents used for extraction, however, if trace amounts of solvent remain in the extract this can cause discrepancies in the results. A study by Kardile et al. 2016 found that trace amounts of ethanol and methanol in a sample can yield false positive results in the HRBC membrane stabilisation assay. Traces of solvent could also potentially corrupt other assays in a similar way. The samples in this study were not tested for traces of solvent after rotary evaporation, as such, it is possible that the results were affected by solvent contamination. In the future, this can be tested for using HPLC to detect trace quantities of solvent in samples before using them in assays to prevent this type of problem from occurring.

10.7: Traditional uses

To conclude, the use of traditional plants for the treatment of certain ailments may or may not be a reliable indicator of its practical use, it is possible that some traditional remedies may work, at least in part, due to the placebo effect. This study aimed to determine whether the use of *P. glaucus* for the treatment of a selection of ailments, guided by traditional use, could be established scientifically. A summary of the results in relation to traditional use is shown in **Table 10.1**.

Table 10.1: Traditional uses of *P. glaucus* and their relation to assay results.

Traditional use	**Relation to results**
Cure a swollen spleen and associated fever Plant part used and relevance: Leaves, pounded and taken orally. Since only the leaves are used for this purpose and they are	*P. glaucus* leaves have been found to have some anti-inflammatory activity which may work via proteoprotection, membrane stabilisation or COX-1 and -2 inhibition which may help to alleviate swelling. A swollen spleen can be caused by infection or certain cancers and this study has found that the leaves possess some antibacterial activity towards certain Gram negative and Gram positive bacteria. Another study has found that some compounds in the plant inhibit

taken orally, compounds that are not rendered inactive by digestion or that can be broken down into biologically active compounds will be responsible for this activity.	antibiotic resistant bacteria (Zhao 2008).Other studies have found that components of the plant have antiviral activity (Yan et al. 2008), anti-tumour activity (Zhao & Cui 2009; Zhao 2008; Zhao et al. 2005) and that the extracts have some antiparasitic activity (Khaw, Leerach, Yap, Jaturas, Mahboob, Tan, Dungca, LosBanos, et al. 2015). Betulinic acid has been found to have some antibacterial activity, although this activity is only towards certain bacteria and is mild (Fontanay et al. 2008; C. M. Wang et al. 2016), however, betulinic does have significant anti-inflammatory and immunoregulatory activity which may be beneficial in treating an enlarged spleen (Yi et al. 2010; Tsai et al. 2011; Yun et al. n.d.). Depending on the cause of the swollen spleen, it is possible that *P. glaucus* could be effective in treating this condition.
Cure high fever Plant part used and relevance: Leaves, infused and taken orally Only the leaves are used for this preparation and an infusion is made, meaning that the more polar, heat stable compounds are extracted for consumption.	Fever is commonly caused by an infection, as such, the antibacterial, antiparasitic and antiviral activity demonstrated by *P. glaucus* in this and other studies (Yan et al. 2008; Khaw, Leerach, Yap, Jaturas, Mahboob, Tan, Dungca, LosBanos, et al. 2015; Zhao & Cui 2009; Zhao 2008) supports the use of the plant for this purpose.
Treat cough, laryngitis, pulmonary disease and asthma Plant part used and relevance:	Due to the presence of hasubanan alkaloids that are similar in structure to morphinan alkaloids, it is possible that these alkaloids may suppress a cough in the same manner as morphine, although, due to the very low alkaloid content of the leaves, the treatment of cough

Leaves, infused and taken orally	may be due more to the antibacterial activity. Also, the mechanism of action of antitussives is not fully understood and although *P. glaucus* has been found to have sedative effects (Kifayatullah, Mustafa, et al. 2015), this is not necessarily linked to the antitussive properties of a compound (Dickinson et al. 2014) and some coughs may not be suppressed by compounds which act on opiate receptors while others are (Takahama & Shirasaki 2007), as such, further work would have to be conducted to determine whether or not the hasubanan alkaloids in *P. glaucus* are able to suppress cough. Although *P. glaucus* also has antiviral activity, it is unknown whether or not it will exhibit antiviral activity towards a virus commonly associated with cough, such as a rhinovirus, as this has not been tested. The anti-inflammatory activity via COX-1 and COX-2 may induce asthma (Kowalski 2007), however, inhibition of COX-2 without inhibition of COX-1 does not (Harrington et al. 2008). The leaf hexane extract showed greater, but not complete, selectivity towards COX-2, so would be less likely to aggravate asthma. The ability of the extracts to inhibit the COX enzymes may not be beneficial to treating inflammation in asthma. It is unknown whether *P. glaucus* contains components that are able to inhibit TGF-beta-mediated conversion of pulmonary fibroblasts into myofibroblasts, activate HDAC2 or that possess anti-histaminic activity, however, it is possible that the antibacterial and antiviral components could treat asthma which has been induced by infection.
Soothe headache Plant part used and relevance:	The anti-inflammatory activity, antibacterial (Zhao 2008), antiviral (Yan et al. 2008) and anticancer (Zhao & Cui 2009; Zhao 2008; Zhao et al. 2005) activity of *P.*

Leaves, pounded and applied topically Since this preparation is applied topically, the compounds responsible for this activity will be limited to those which can be transported effectively through the skin.	*glaucus* could treat a headache depending on the cause. Since the treatment is applied topically, it can be assumed that the plant is applied to the site of the pain. If the headache is caused by muscle tension or by a toothache, topical application may be effective in relieving the symptoms in that area, however, it is unlikely that a topical application would be effective in treating a brain tumour or viral infection. Many factors can affect how effectively compounds can penetrate the skin and this will affect which compounds are able to exert their activity to treat headaches.
Remedy for hair loss Plant part used and relevance: Leaves, pounded and soaked before mucilage applied to the scalp	Hair re-growth can be stimulated by angiogenesis or sensitization to UV light, however this was not tested in the present study or by any other studies on *P. glaucus*. Some studies have reported that betulinic acid, a component of *P. glaucus*, inhibits angiogenesis (Kwon et al. 2002; Mukherjee et al. 2004) and betulinic acid can protect cells from UV-C damage (Salti et al. 2001). It is unknown whether other compounds would promote angiogenesis despite the presence of anti-angiogenic and UV protective compounds. It is also unknown whether the plant could stimulate hair growth by inhibiting dihydrotestosterone production, causing an immune response or sensitising skin to UV damage. Other plants have been found to successfully promote hair growth (Begum et al. 2015; Shin et al. 2015), similar tests could be conducted to determine whether *P. glaucus* also promotes hair growth and by what mechanism.
Treatment for conjunctivitis, boar bites and boils Plant part used and relevance:	Conjunctivitis can be caused by a bacterial or viral infection or by allergens or irritants, resulting in inflammation. *P. glaucus* stem extracts showed inhibitory activity towards COX-1 and -2, with the

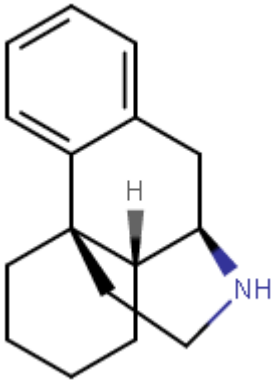
Stem, juiced, leaves and roots respectively.	hexane extract showing the least inhibitory activity and since the juice of the stem is traditionally used, the polar compounds would be used for this treatment. COX inhibitors, particularly inhibitors of COX-2, may be beneficial in the treatment of conjunctivitis (Oka et al. 2004). The anti-inflammatory activity combined with the mild antibacterial activity of the stem may contribute to its use as a treatment for conjunctivitis in traditional medicine. Boar bites can cause bleeding and lead to infection, while boils are caused by a bacterial infection. It is unknown whether a preparation of *P. glaucus* would help a wound to clot, however, the current study has found that the plant possesses mild antibacterial activity, anti-inflammatory activity and some wound healing activity which supports the use of the plant for the treatment of inflammation due to infection and trauma.
Treatment for snakebites Plant part used and relevance: Roots, fluid extract is injected at the site of the snakebite	Since an aqueous extract is used, the ethanol extract used in this study relates best to the traditional use of *P. glaucus* for the purpose of treating snakebite, which correlates well with the results of the antivenom assays, since the ethanol extract showed the greatest venom neutralising activity when compared with the other root extracts. The root did not exhibit any antibacterial activity, however, the stem alkaloid extract did have very mild antibacterial activity. It is unlikely that the traditional use of the root for the treatment of snakebite will prevent infection, however, further research should be conducted to learn more about the possible antibacterial activity of the root extract against other types of pathogenic bacteria since the sensitivity of bacteria to antibiotics can be very specific. The alkaloid extract demonstrated some anti-venom activity, while

	periglaucine A significantly neutralised *C. atrox* venom. Periglaucine A was not clearly detected in the HPLC analysis due to the large number of peaks at the same elution time, however, if it is present in the stem alkaloid extract, it is only present in very small quantities. These results demonstrate that periglaucine A is more active than the other major alkaloids present in the stem, and that the stem does have some venom neutralising activity. *C. atrox* is not found in the areas where *P. glaucus* is traditionally used to treat snakebite, however, many snake venoms contain similar components so the results of this assay indicate that *P. glaucus* may also be active against venoms of other snakes. Further work would have to be done on venoms from snakes native to Southeast Asia, particularly Vietnam, to accurately determine whether *P. glaucus* is effective against the venoms of these snakes.
Fractures Plant part used and relevance: Leaves	It is unknown whether *P. glaucus* would aid in the healing of a fracture, however, it may be able to relieve some discomfort caused by the inflammation associated with a fracture. Since *P. glaucus* has demonstrated inhibitory activity towards the COX enzymes, and inhibition of these enzymes is considered the main mechanism by which NSAIDs exert their beneficial effect, it is possible that *P. glaucus* could hamper bone repair as commonly used NSAIDs have been found to impair the healing of bones (Giannoudis et al. 2015).
Narcotic properties Plant part used and relevance: Root	Toxicity testing has found that the ethanolic extract of the leaves of *P. glaucus* has sedative properties (Kifayatullah, Mustafa, et al. 2015) and the compounds responsible for this activity are unknown. Some hasubanan alkaloids are able to bind to δ-opioid receptors (Carroll et al. 2010) and the roots were found

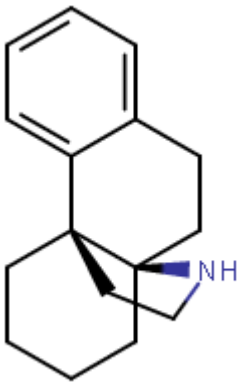
	to contain alkaloids, more so than the leaves, although it is not known what alkaloids are contained in the root as all alkaloids isolated from this plant were isolated from the aerial parts (Yan et al. 2008). It is possible that some of the same alkaloids in the aerial parts of the plant are also found in the roots and may even be present in higher concentrations. These may be able to produce narcotic effects via opioid receptor binding but further studies would have to be conducted to confirm this.
Treatment of tumours Plant part used and relevance: Leaves	The ethanol extract of the leaves of *P. glaucus* did exhibit toxicity towards HK1, although the root and fruit extracts were significantly more toxic. The leaf ethanol extract was also more toxic towards MRC-5 than HK1, making it unattractive as a chemotherapeutic medicine.
Treatment of tetanus Plant part used and relevance: Leaves	The ethanol extract of the leaves possess mild antibacterial activity and sedative activity (Kifayatullah, Mustafa, et al. 2015). The mild antibacterial activity could possibly help to control infection while the sedative effects could help control spasms (Rodrigo et al. 2014). The peptide toxins that are responsible for the spasms and pathogenicity may also be neutralised by compounds that are able to bind peptides.
Rheumatic pains Plant part used and relevance: Leaves	The symptoms of rheumatism include pain and inflammation of the joints, which *P. glaucus* may be useful in treating based on current literature and the results of the present study. Rheumatism is caused by a poorly understood autoimmune disorder, as such immunoregulatory compounds could help to treat this illness. Daucosterol has been found to have immunoregulatory properties and is a component of *P. glaucus*, although it has not been tested as a possible treatment for rheumatism.
Treating itches	A combination of the anti-inflammatory, antibacterial

Plant part used and relevance: Leaves	and sedative activity of the leaves may contribute to the use of *P. glaucus* for treating itches and would enable the treatment to work on itches with a variety of causes.
Eclampsia Plant part used and relevance: Leaves	The sedative effects of the leaves may help treat seizures, reduce inflammation and possibly reduce pain.

It has been reported that the roots of *P. glaucus* possess narcotic properties. When comparing the hasubanan and morphinan core structures, there is little difference between the two (**Figure 10.1**). Since structure and activity of chemicals can quite often be linked, it is possible that the morphinan and hasubanan alkaloids exhibit similar activities due to their similar structure. The hasubanan alkaloids, periglaucines A-D in *P. glaucus* may therefore be responsible for the reported narcotic properties of the plant. Since the root of the plant is reported to possess this property, this suggests that the alkaloids are concentrated mainly in the roots of the plant.



Morphinan core structure



Hasubanan core structure

Figure 10.1: A comparison of the hasubanan and morphinan core structures

Morphine, a morphinan alkaloid, has cough suppressant activity and, most notably, analgesic and sedative activity. It is a narcotic and works by binding the μ-opioid receptors in the central nervous system. Upon binding, the analgesic, sedative and respiratory depression activity are experienced, as well as euphoria and possibly physical dependence.

A number of hasubanan alkaloids were found to have δ-opioid binding affinity (Carroll et al. 2010) which would support the structure-activity relationship between hasubanan and morphinan alkaloids.

The leaf ethanol extract of *P. glaucus* has been found to exhibit no toxicity in mice, only inducing sedation at higher doses (Kifayatullah, Mustafa, et al. 2015). Some of the unique compounds in this plant may offer useful biological activities which should be investigated further to better establish the extent of their activity and their efficacy. It is very possible that *P. glaucus* contains different proportions of active phytochemicals during different times of the year and when grown in different locations, as such, there may be variation in results obtained by researchers who study *P. glaucus* or communities who use it for traditional medicine. Traditional medicines often use water as the solvent for extraction, it also uses plant parts that can be harvested in adequate quantities for use as medicine. Although the use of water as a solvent is most practical, it may not necessarily contain the most active components of a plant extract. Due to the hormetic and possible biphasic responses seen by many of the extracts, the dosing of this plant may be particularly important so as not to induce a toxic effect. The hexane extracts demonstrated good anti-inflammatory activity, yet the compounds in the hexane extract are likely to be poorly soluble in water. Although the fruit extract demonstrated better venom neutralising activity than the root, it is not traditionally used to treat snakebite. It was personally noted by the present researcher that the fruits of *P. glaucus* were particularly difficult to find and that the plant was rarely observed flowering throughout the year, the lack of availability of this particular plant part may contribute to its lack of use as traditional medicine, despite the promising results that it displayed in tests. Traditional medicines vary from place to place as the plants that are used are largely determined by what is available, therefore the availability of a specific part of a plant will also determine what can be practically used as traditional medicine. Preclinical tests are not always an accurate indicator of the effect you might expect in a clinical trial, as such, results obtained by researchers may not accurately reflect the practical results observed by communities who use traditional medicines.

Although the results of the *in vitro* experiments conducted may support the traditional use to a certain extent, the action demonstrated in these experiments should not be expected in an *in vivo* model and it is unknown how the plant extract would exert its activity, if any, in a

controlled clinical trial. The results of the studies would be of much greater value if accompanied by results of *in vivo* experiments which also supported the traditional use.

10.8: Final remarks and future perspectives

Traditional medicines may serve as good starting points for the discovery or development of practical new drugs, however, due to the regulations and financial aspects involved in the development of new drugs, the use of natural products as drugs is currently limited and they serve more as templates for new synthetic molecules which are more marketable.

The use of antioxidants as therapeutic compounds to prevent oxidative damage is subject to some debate as, although they may hold significant value as preventers of diseases linked to oxidative damage, this is not necessarily true for all antioxidants and may not be due directly to antioxidant activity. In the preparation of traditional medicines, antioxidants may possess other useful biological activities, such as antimicrobial activity, and help to preserve other sensitive compounds in the medicine. Often, the traditional medicine requires that the plant be taken orally, which may alter the activity of certain compounds. When digested, the bioavailability of polyphenols is reduced as they are hydrolysed into other compounds and these products may not possess the same antioxidant or biological activity (Scheepens et al. 2010; Krook & Hagerman 2012). Antioxidants may also be useful in synthesis of certain nanoparticles, as has been demonstrated by the use of reishi extract in the production of graphene (Muthoosamy et al. 2015). Given the potential to cause harm due to excessive intake of antioxidants and the uncertainty of whether or not antioxidants to provide any significant health benefit in the management or prevention of disease, a healthy diet should eliminate the need for supplementation and is recommended by many health professionals.

NSAIDs that effectively target the COX enzymes impact such a large number of systems that inhibiting COX produces potentially serious negative side effects such as thrombosis and gastrointestinal bleeding. The mechanism of action of popular NSAIDs may involve more than just COX inhibition, the popular NSAID, paracetamol, has been shown to act in a variety of ways which may contribute to its pain killing effects (Bertolini et al. 2006; Högestätt et al. 2005). Ideally, researchers should focus on a different target than COX, that will provide relief from inflammation with fewer side effects, such as PLA_2 (Singh et al. 2004) or cannabinoid receptor agonists (Malan et al. 2002). Chemicals that target PLA_2 have been the target of more recent research into anti-inflammatory drugs, but they may also be effective in treating some snakebites since PLA_2 is found in many snake venoms.

Although antibacterial resistance is a growing problem, there are many natural compounds that are known to be effective in treating infections by antibiotic resistant bacteria and are widely available. Research into novel antibiotics is unnecessary given the abundance of antimicrobials that are known. Instead, focus should be on conducting further clinical trials, making these compounds commercially available and approved for the use of treating bacterial infections, however, this problem has arisen due to our reliance on profit driven companies for the production of pharmaceuticals. Until regulations change and the attitude of drug developers and manufacturers change to benefit the production of effective medicines rather than profitable medicines, antibiotics should be used responsibly by humans and farmers so that we can reduce the incidence of the emergence of antibiotic resistant strains. The development of resistance in microbes is an inevitable process, as such, simply finding new drugs to treat infections is not enough to solve a constantly developing problem and the solution to such a problem is beyond the scope of this current project.

Snakebite is a problem which affects mostly poor populaces in developing countries. It is a neglected disease and receives little attention as far as development of practical treatments go. Since the main market for snakebite drugs is poverty stricken, developing countries, it is unlikely that they will be able to afford such drugs, making the development of new treatments for snakebite and the manufacturing of antivenom unappealing to drug companies.

Traditional medicines can provide researchers with useful starting points for the development of new drugs since they can be effective in treating ailments and they have already been tested on humans, however, the reliability of diagnosis, variability between chemical composition of the same species of plant, variation in dosing, route of administration, possible misidentification of plants and whether the plant manages symptoms or cures the disease are all factors which need to be taken into consideration when conducting pre-clinical studies and determining the mechanism of action of a plant and its active constituents. Given the results of this study and other studies on *P. glaucus*, There is good reason to believe that it would be effective in treating a number of diseases or ailments which it is traditionally used for. Although some activities of the extracts were determines by the results of the present study, further tests should be carried out to determine the mechanism of action of the extracts and their active components.

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Appendix A

LSBE-1337
The antibacterial activity of *Pericampylus glaucus* (Lam.) Merr

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Abstract

Objectives: *Pericampylus glaucus* can be found in many parts of Asia, where it has been traditionally used as a medicinal plant. The traditional uses of this plant suggest that it may possess a variety of activities, including antibacterial activity. The aim of this study is to identify whether extracts of *P. glaucus* exert antibacterial activity against a selection of gram positive and gram negative bacteria.

Methods: Extracts of the leaves and stems of *P. glaucus* were made, using hexane, chloroform and ethanol, then tested for their antibacterial activity against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter freundii*, *Kocuria rhizophila* and *Pseudomonas aeruginosa* using the disc diffusion test. A total of 6 extracts from *P. glaucus* were made and tested in this study.

Results: In the disc diffusion tests, the hexane and ethanol extracts showed antibacterial activity against some of the bacteria, but the chloroform extracts showed no significant activity. The greatest activity was seen by the stem hexane extract against *S. aureus*, the stem ethanol extract against *C. freundii* and *K. rhizophila*, the leaf hexane extract against *B. cereus* and *S. aureus* and the leaf ethanol extract against *C. freundii* and *K. rhizophila*. The antibacterial activity of these extracts was compared to the standard chloramphenicol.

The MIC is to be determined using the broth dilution test and will be presented at a later date.

Conclusion: The initial results showed that *P. glaucus* contains antibacterial compounds. Work is being conducted to isolate and identify the compounds responsible for this antibacterial activity.

Keyword: Pericampylus glaucus, antibacterial, extract, disc diffusion.

1.0 Introduction

Pericampylus glaucus is a climber in the Menispermaceae family. There are 12 species in the genus *Pericampylus*. *P. glaucus* is found in numerous countries in Asia, including China, India, Indonesia, Laos, Malaysia, Philippines, Taiwan, Thailand and Vietnam. The studies conducted to date have pointed out that *P. glaucus* contains pifriedelinol, melissic acid, palmatic acid, stearic acid, bututic acid and daucosterol [1] as well as numerous alkaloids and

has anti-viral activity. The roots have been used to treat laryngitis, cough, pulmonary disease and boils, and the leaves are used on fractures and boar bites. The plant is also used as a sedative, for treating tetanus and for relieving rheumatic pains [2-4]. It has also been found to have anti-cancer activity against a tsFT210 cell line [5]. These results suggest that there may be a number of interesting compounds present in the plant.

There has not been much advancement in the discovery and development of new antibiotics in recent years, even though new antibiotics are more in demand due to the increasing number of reported cases where bacteria have become resistant to antibiotics [6, 7]. One notable example is with MRSA. This bacteria has caused a great number of deaths due to its resistance to methicillin [8]. The approval of a new antibiotic would provide more options for the treatment of bacteria that have become resistant to commonly used antibiotics.

Although the antibacterial activity of *P. glaucus* has not been tested, some of the traditional uses of this plant include uses that imply that it may have antibacterial activity. The aim of this study is to determine whether or not extracts of this plant do indeed have any antibacterial activity.

2.0 Materials and methods

2.1 Collection of Plant Material: Fresh Pericampylus glaucus was collected. The leaves and stems were air dried for 3 weeks, then ground separately. The total dry weight of the leaves and stems came to 119 g and 438 g respectively. The plant material was then macerated for 3 days at 60°C in hexane, chloroform and ethanol respectively and maceration in each solvent was performed 3 times. The extracts obtained from each of the solvents were concentrated using a rotary evaporator and stored at -20°C until further use. Samples were labeled PGSH, PGSC, PGSE, PGLH, PGLC and PGLE, the letters representing the genus, species, plant part (stem or leaf) and solvent (hexane, chloroform or ethanol) respectively.

2.2 Antimicrobial assays: The disc diffusion method was employed [9] to determine the antimicrobial activity of the extracts. This method measures the ability of the sample to inhibit the growth of bacteria around a disc that contains a measured concentration of the sample. If the sample has antimicrobial activity, the bacteria surrounding the disc will not grow and a transparent ring will be seen around the disc, which can be measured to give a quantitative measure of antimicrobial activity. The greater the area of inhibition, the greater the antimicrobial activity is understood to be.

For this assay, the extract was dissolved in a solvent and applied to sterile paper discs, 6 mm in diameter, made from No. 1 filter paper. Each disc contained 1 mg of extract and was allowed to dry overnight before being used. Three gram positive bacteria, Staphylococcus aureus, Bacillus cereus and Kocuria rhizophila, and three gram negative bacteria, Pseudomonas aeruginosa, Citrobacter freundii, Escherichia coli, were grown overnight in tryptic soy broth. The optical density of the bacteria was adjusted to 0.5 before being streaked onto plates of Mueller Hinton agar. The paper discs containing the extracts were applied to the surface of the plates. Negative controls were a paper disc with no extract and the positive control was a disc impregnated with 30 µg of chloramphenicol.

3.0 Results

The results of the disc diffusion test, shown in Table 1, show that none of the extracts had any activity against *E.coli*. The chloroform extracts showed no zone of inhibition against any of the bacteria. The hexane and ethanol demonstrated varying antibacterial activity and only the leaf ethanol extract was able to inhibit the growth of *P. aeruginosa*.

Table 1: Disc diffusion test results

	B. cereus	E. coli	S. aureus	C. freundii	K. Rhizophila	P. aeruginosa
PGSH	1	0	4	0	0	0
PGSC	0	0	0	0	0	0
PGSE	0.5	0	0	4	4	0
PGLH	4.5	0	5	0	0	0
PGLC	0	0	0	0	0	0
PGLE	0	0	0	4	6	2
Chloramphenicol	12.5	10	15	24	14	5

The values in the table correspond to the diameter of the zone of inhibition (mm), but not including the diameter of the disc (6 mm).
Both of the hexane extracts inhibited *B. cereus* and *S. aureus* and both ethanol extracts inhibited *C. freundii* and *K. rhizophila*. The zones of inhibition were much smaller than those seen by chloramphenicol.

4.0 Discussion and conclusion

The antibacterial activity of the extracts was much lower than that of chloramphenicol, but this was expected as the plant extracts contain a mixture of compounds and the concentration of the compounds responsible for the antibacterial activity in the extracts is unknown. The hexane and ethanol extracts showed antibacterial activity towards certain bacteria, this may be because of similarities in chemical composition.
The broth dilution test should also be employed to determine the antibacterial activity of the extracts. Since plant extracts contain a large number of compounds with different properties, the disc diffusion test may not determine the full potential of the extract as an antibiotic. The plant extracts were found to be poorly soluble in water, so may not have been able to diffuse through the agar medium very effectively. As such, the broth dilution will be used to determine the MIC and MBC of the extracts in the future.
The results of the disc diffusion test have shown that *P. glaucus* has some antibacterial activity and further study into this may yield interesting results.

References

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Appendix B

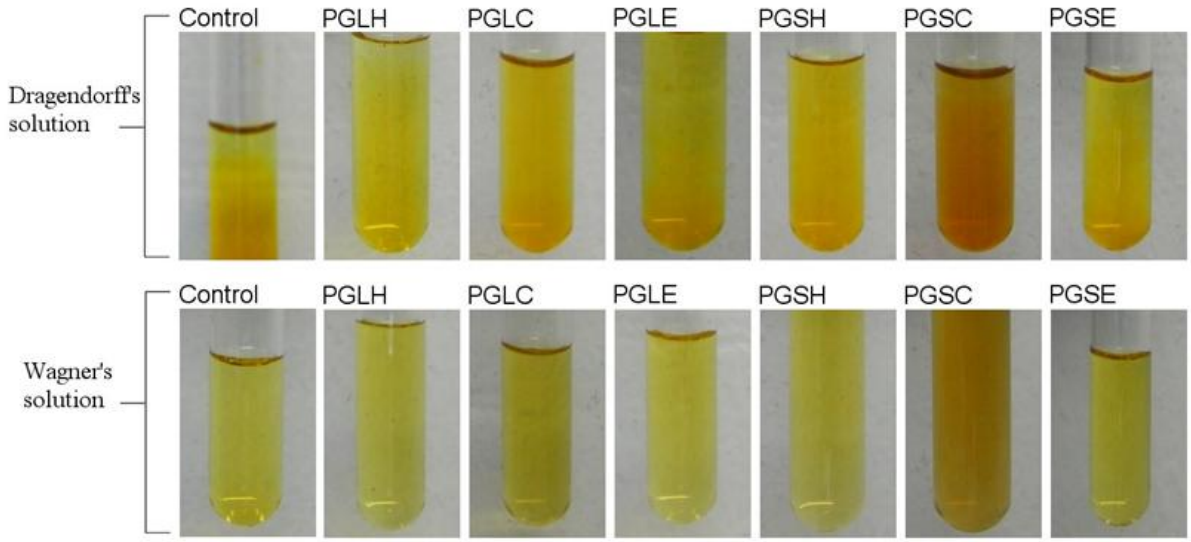


Figure B1: Test for alkaloids.

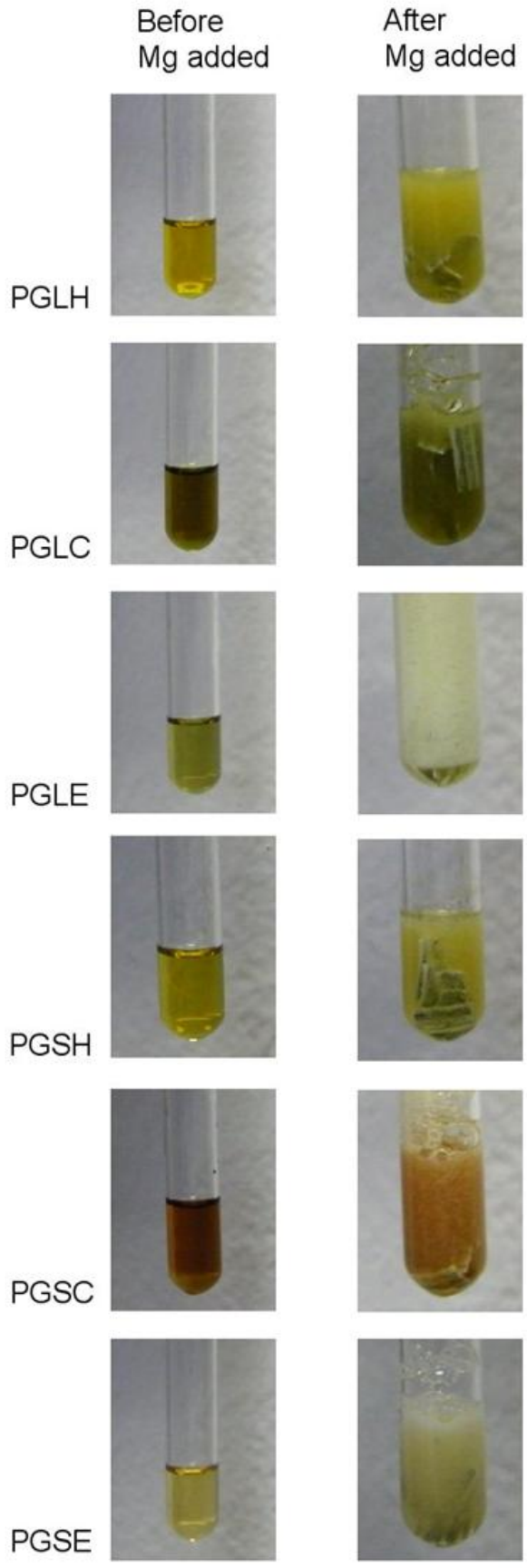


Figure B2: Test for flavonoids.

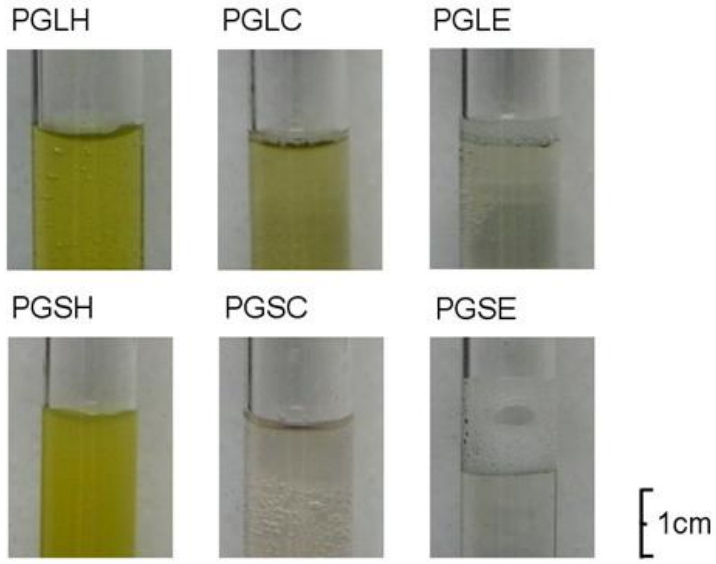


Figure B3: Test for saponins.

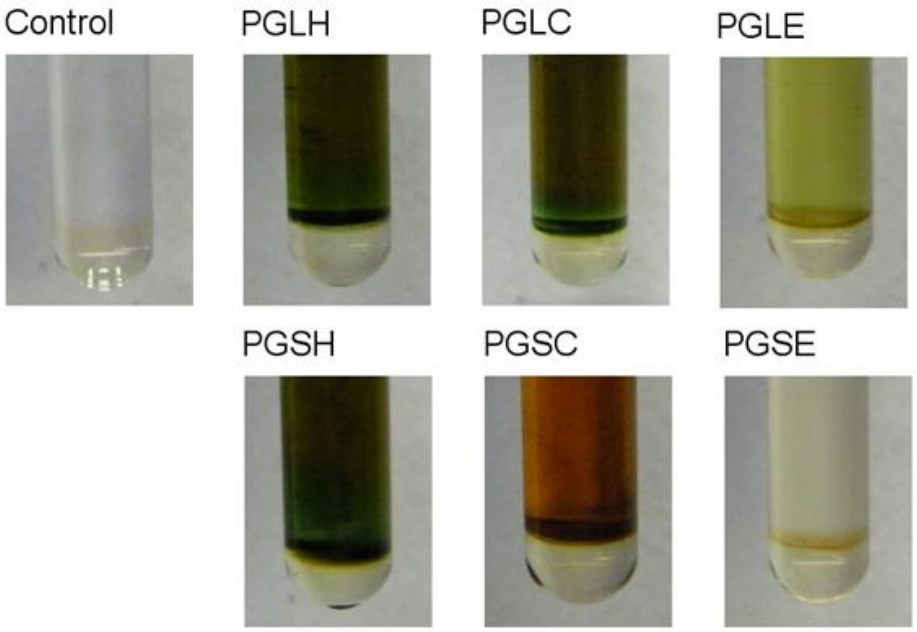


Figure B4: Test for terpenes and sterols.

Appendix C

Table C1: Table showing the TPC, relative TPC ratios for each plant part relative to the extract of the plant part with the lowest TCP, ratio of radical scavenging activity at 800, 200, 25 µg/mL and the DPPH radical scavenging potential at each concentration.

Sample (at 10mg /ml)	TPC (mg GAE/10mg extract)	TCP ratio (lowest value = 1, excluding 0)	Radical scavenging ratio for 800, 200 and 25 µg/mL (lowest value = 1)	Relative potency (radical scavenging activity ratio divided by TCP ratio)
SH	0.01		1:2.6:1.8	1:0.2:0.3
SC	0.11	1:11:6	1:12.9:5.9	1:1.2:1
SE	0.06		1:133:35	1:12.1:5.8
LH	0.01		1:1.2:1.2	1:0.1:0.2
LC	0.09	1:9:6	1:1.7:1.8	1:0.2:0.3
LE	0.06		1:1.6:2.7	1:0.2:0.5
RH	0.01		1:3.7:2.3	1:0.6:0.8
RC	0.06	1:6:3	1:5.7:1.9	1:1:0.6
RE	0.03		1:3.6:0.7	1:0.6:0.2
FH	0		1:1.2*	1:0.9*
FC	0.17	1:1.42*	1:1.1*	1:0.8*
FE	0.12		1:1.1*	1:0.8*

* Ratio includes only samples for which a value greater than 0 was recorded as the TPC

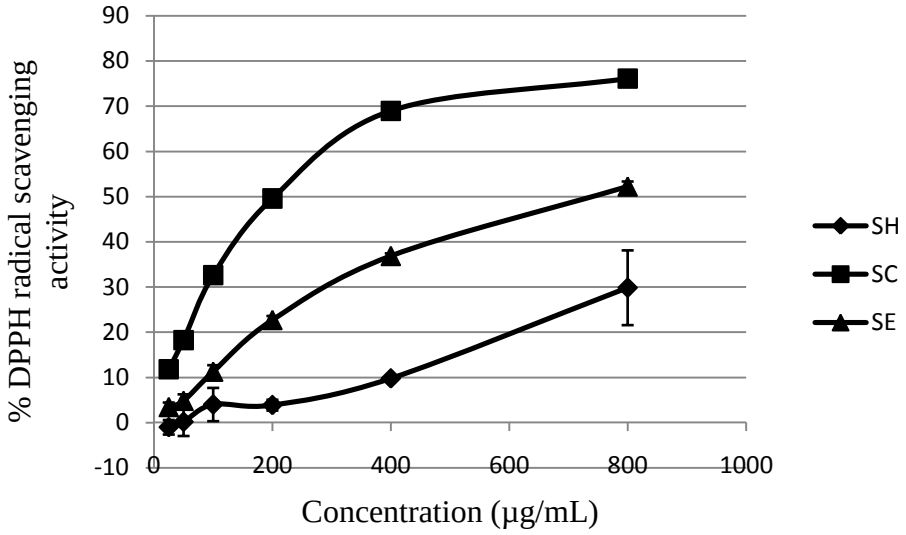


Figure C1: Radical scavenging activity of *P. glaucus* stem extracts.

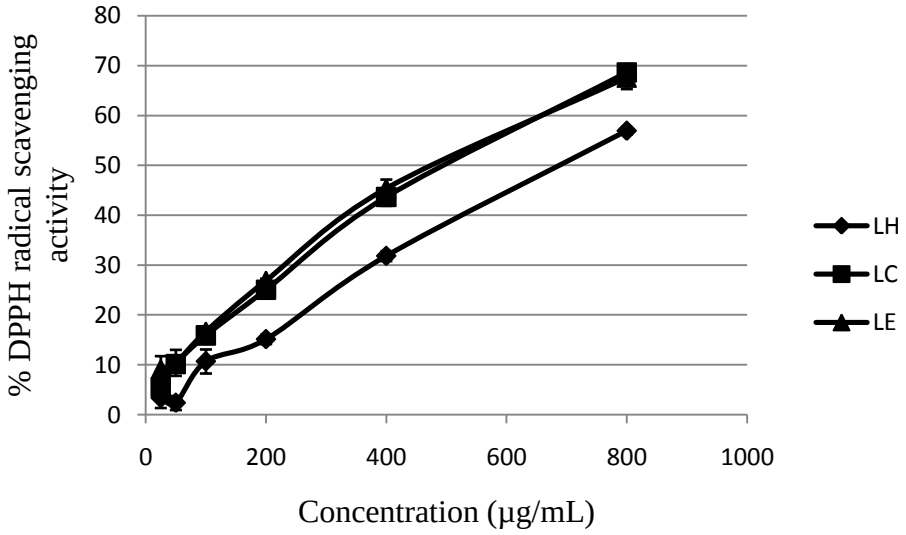


Figure C2: Radical scavenging activity of *P. glaucus* leaf extracts.

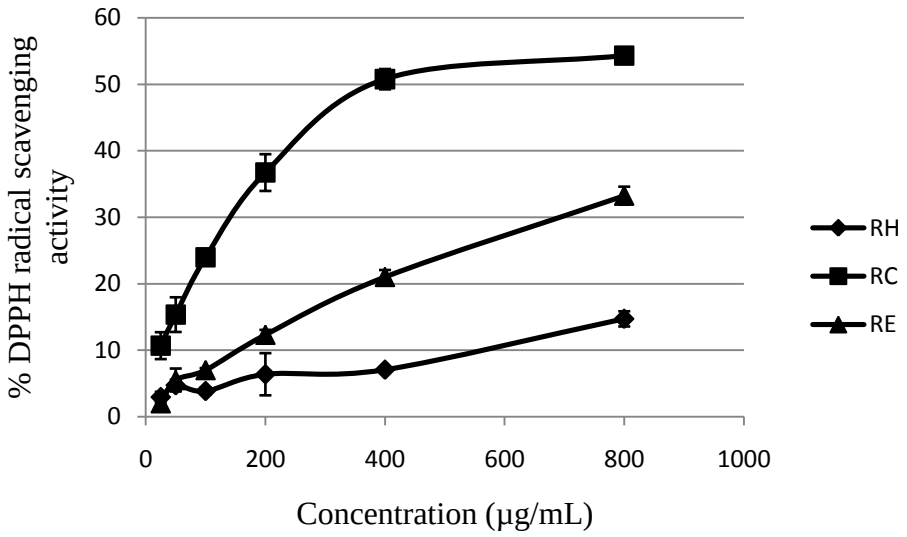


Figure C3: Radical scavenging activity of *P. glaucus* root extracts.

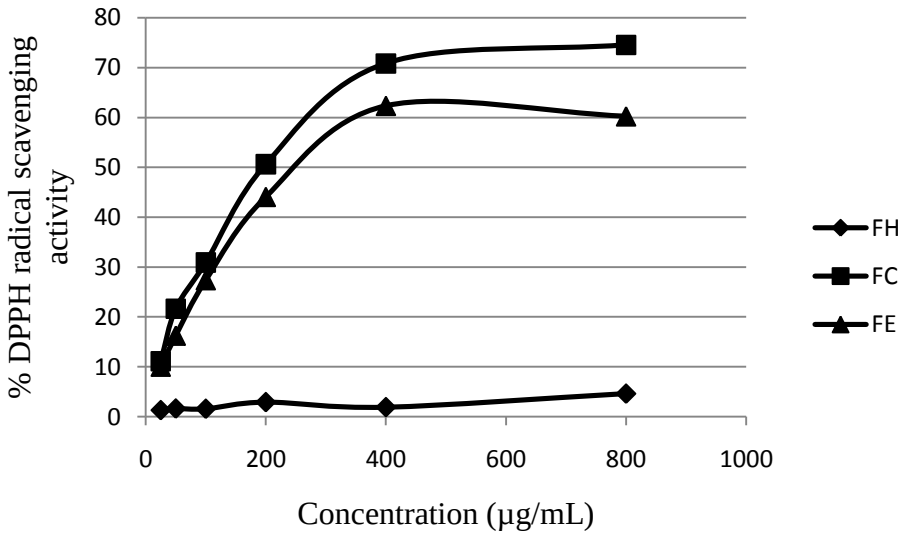


Figure C4: Radical scavenging activity of *P. glaucus* fruit extracts.

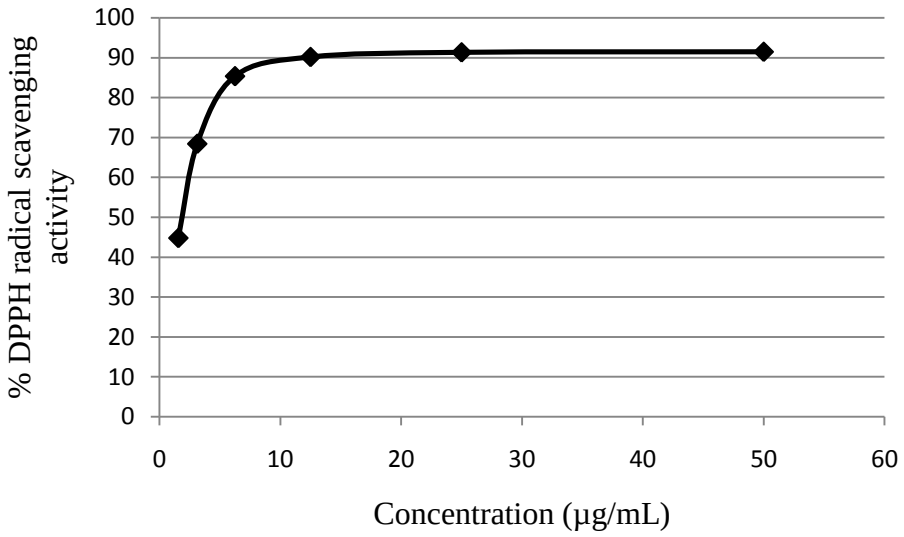


Figure C5: Radical scavenging activity gallic acid.

Appendix D

Appendix D consists of supplementary images for the antibacterial disc diffusion test. Samples 1 – 13 were stem hexane, stem chloroform, stem ethanol, leaf hexane, leaf chloroform, leaf ethanol, root hexane, root chloroform, root ethanol, fruit hexane, fruit chloroform, fruit ethanol and the stem alkaloid extract respectively. 100µg of each sample was loaded onto the discs.

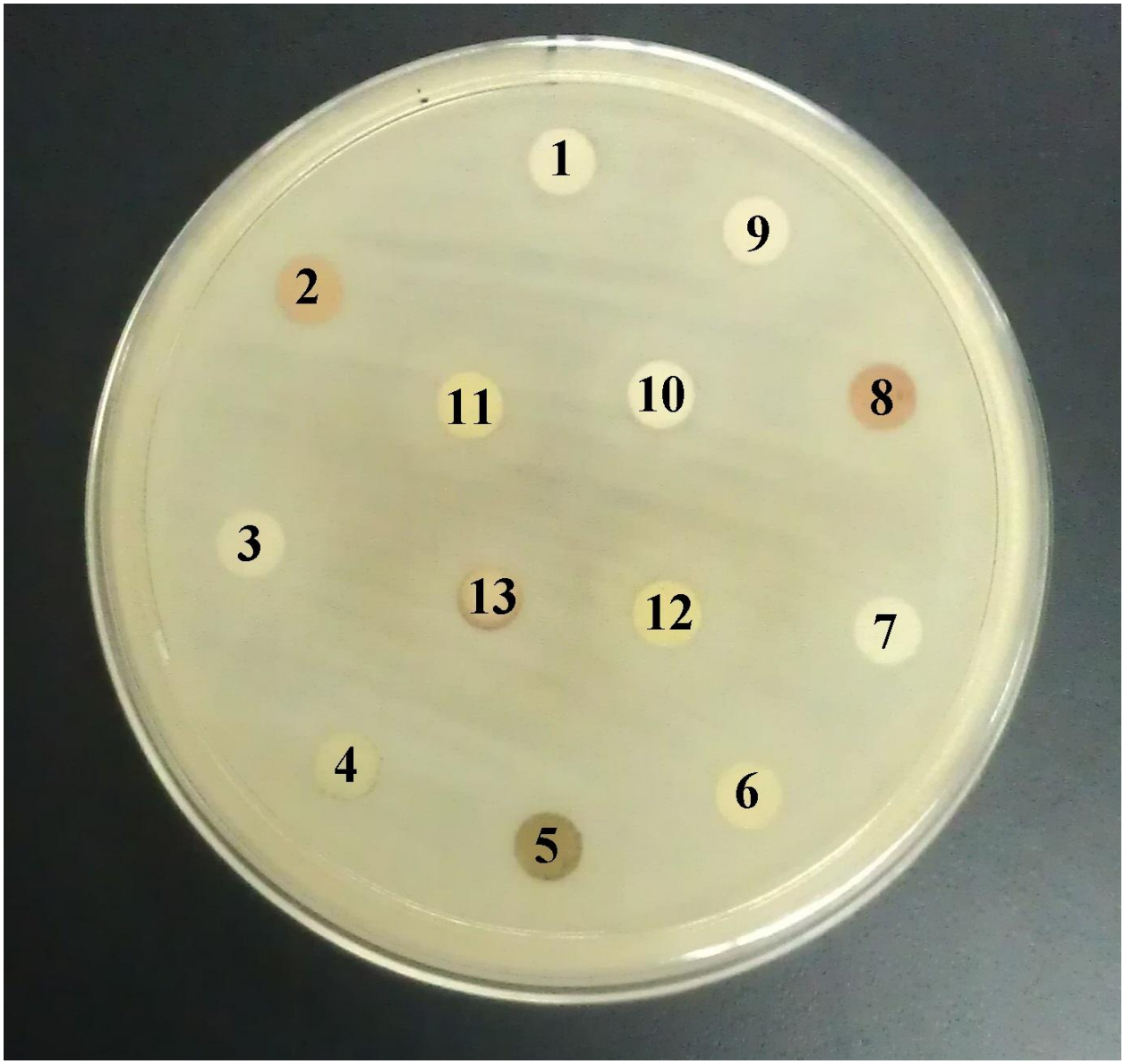


Figure D1: Results of the disc diffusion test showing the activity of extracts against *B. cereus*.

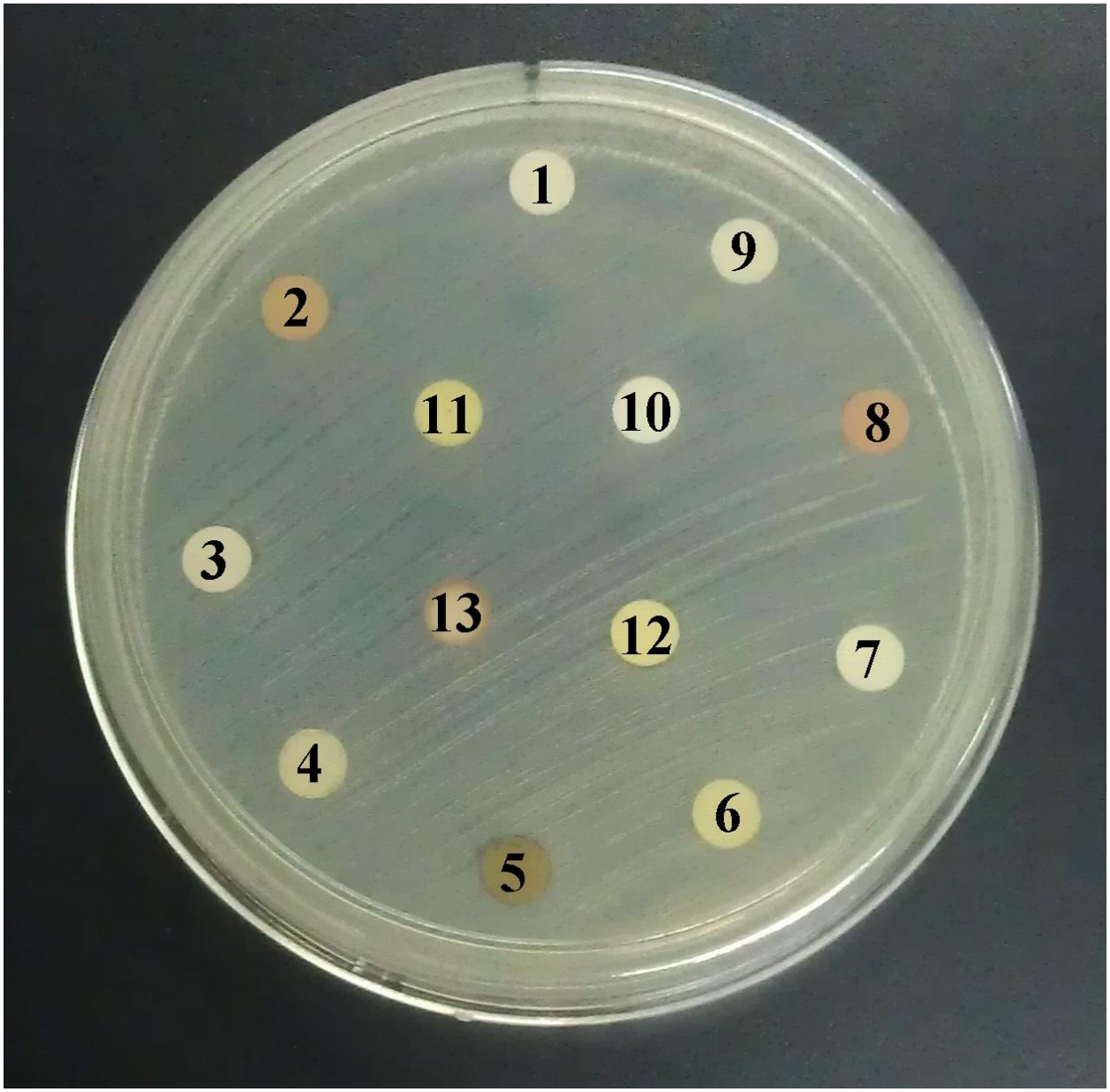


Figure D2: Results of the disc diffusion test showing the activity of extracts against *C. freundii*.

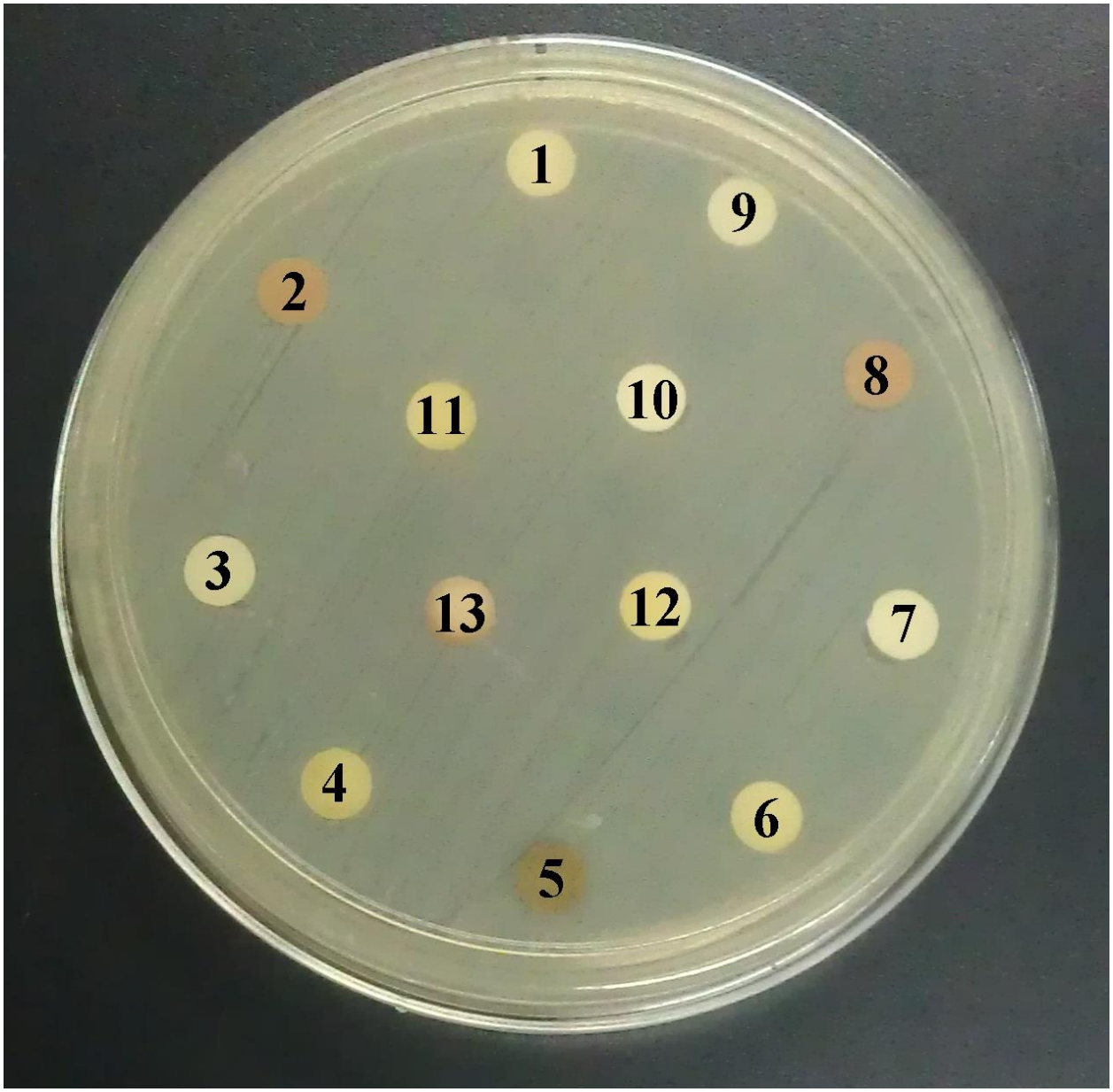


Figure D3: Results of the disc diffusion test showing the activity of extracts against *E. coli*.

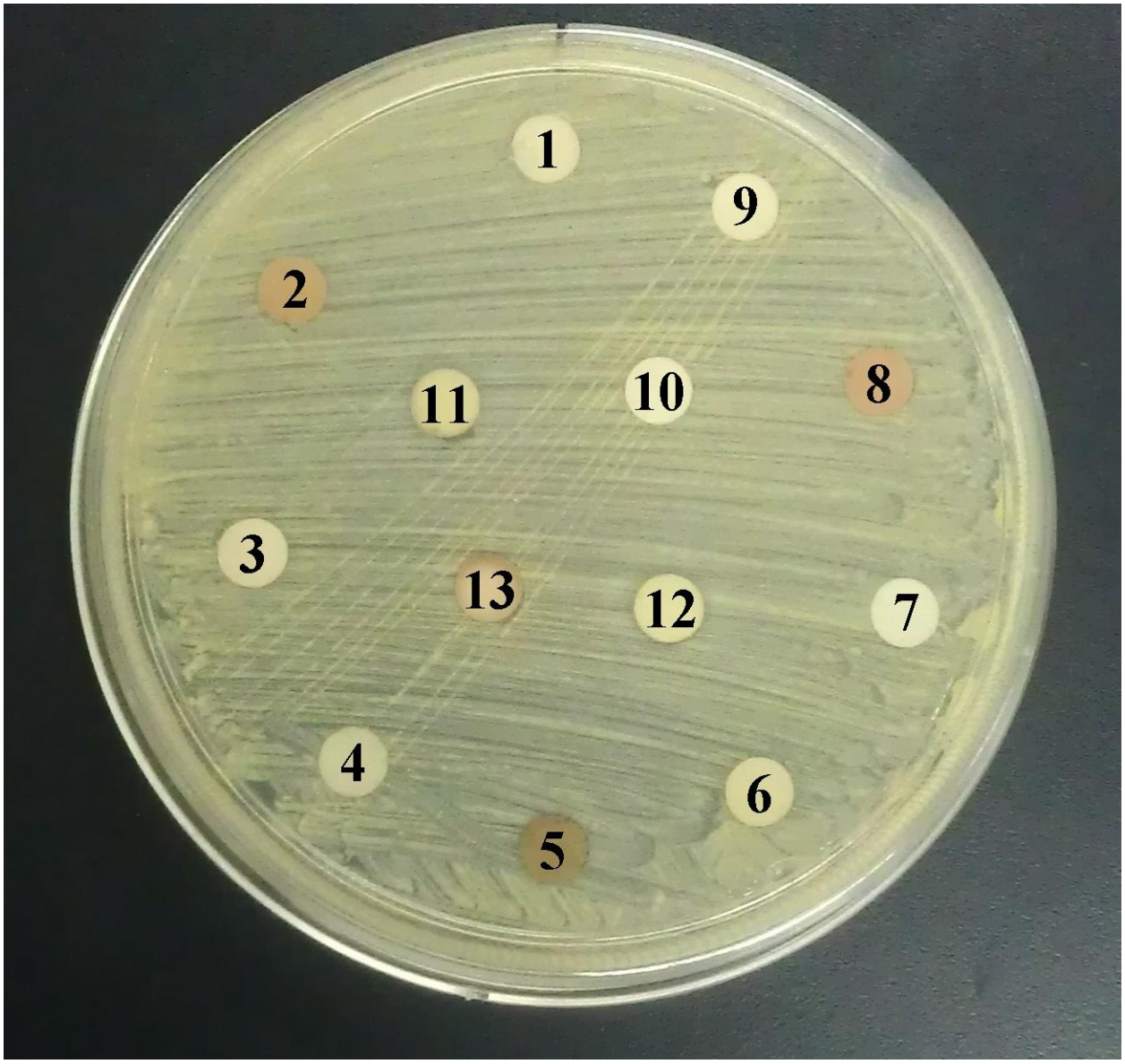


Figure D4: Results of the disc diffusion test showing the activity of extracts against *K. rhizophila*.

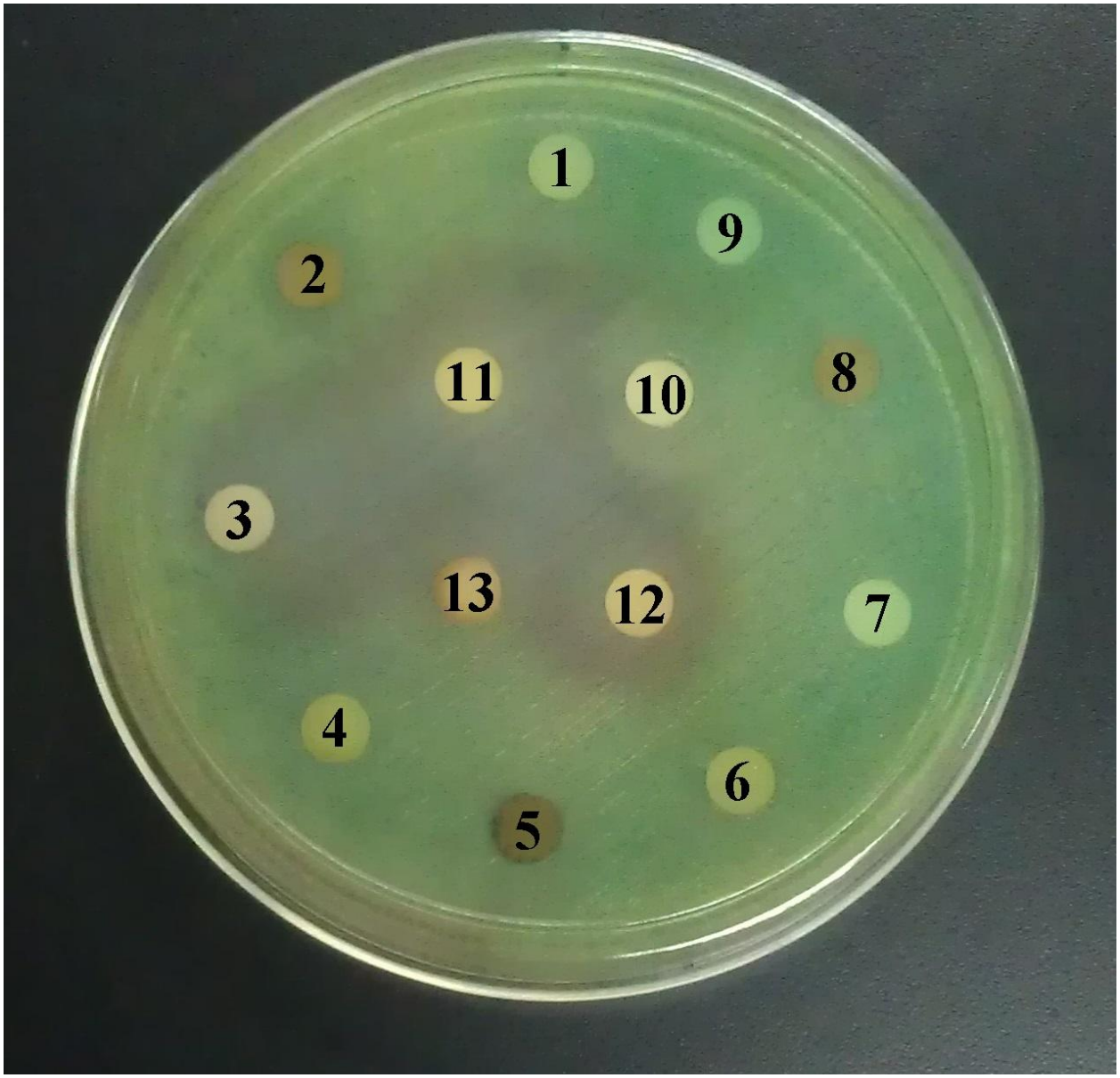


Figure D5: Results of the disc diffusion test showing the activity of extracts against *P. aeruginosa*.

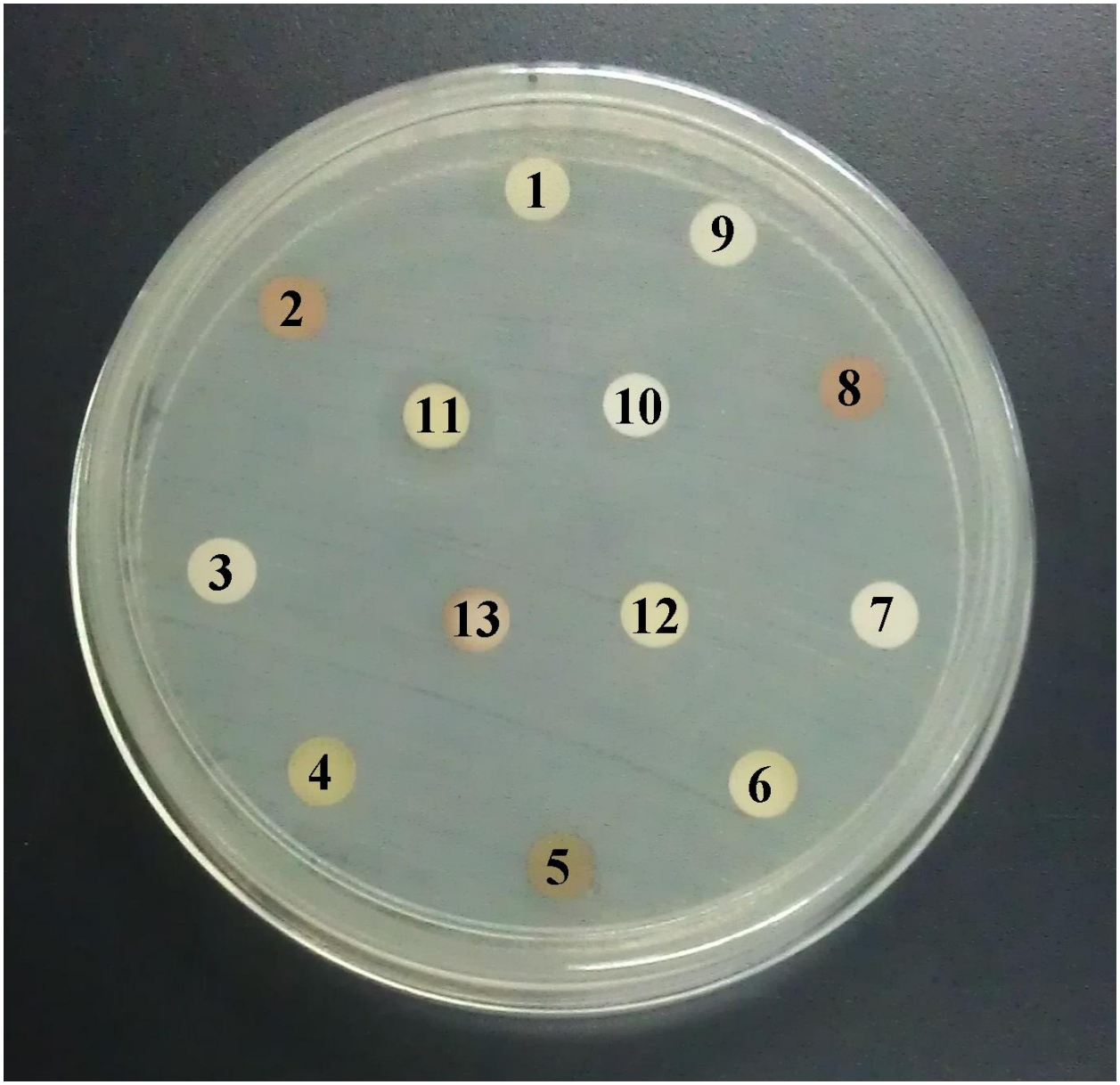


Figure D6: Results of the disc diffusion test showing the activity of extracts against *S. aureus*.

Appendix E

Version 1 (2014)

University of Nottingham Malaysia Campus

Participant Information Sheet

Investigation of the Anti-inflammatory Activity of Potentially Medicinal Plants

You have been invited to participate in the research on the potential anti-inflammatory activity of Malaysian rainforest plants. Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully.

1. **What is the purpose of this study?** The purpose of this study is to discover whether or not extracts of selected Malaysian plants could be used to treat inflammation.
2. **Why have I been invited to participate in this study?** In order to investigate the anti-inflammatory activity of a plant, we need some blood. This means that we need you to give a small quantity of your blood (less than 30ml) for our tests. If you decide to participate you must not have taken any anti-inflammatory drugs for at least 2 weeks before donating your blood, you must not be a hemophiliac and you must be over 18.
3. **What if I do not want to take part in this study, or if I want to withdraw later?** Participation in this study is completely voluntary and it is entirely up to you whether or not you want to participate after being given a brief explanation of the study. You are free to withdraw at any time of the study without the need to give any reason.
4. **What does this study involve?** If you agree to participate, to will be taken to a nearby medical facility, where medically trained staff will take a 30ml blood sample from your arm by venipuncture. This will be done only once and should take less than 10 minutes. The risks involved with this procedure are hematoma, persistent bleeding, vasovagal response (dizziness, sweating, coldness of skin, numbness and tingling of hands and feet, nausea, vomiting, possible visual disturbance, syncope and injury fall from fainting) and there may be swelling, tenderness and inflammation at the site where blood was drawn. In very rare cases thrombosis of the vein may occur due to trauma or infection may occur, resulting in thrombophlebitis. Since this procedure will be carried out by medically trained staff, the risks are low. Generally, there are very minimal risks associated with this procedure and there will be medically trained staff on hand. You will not be provided with transport to or from the medical facility.
5. **Will I benefit from this study?** This study will not benefit you directly, but will help to further research and development of anti-inflammatory drugs.
6. **Will taking part in this study cost me anything, and will I be paid?** Participation in this study will not cost you anything and you will not be paid.
7. **How will my confidentiality be protected?** Any identifiable information that is collected about you in connection with this study will remain confidential and will be disclosed only with your permission, or except as required by law. All documents will be stored in a locked cabinet on

campus. Only researchers and project supervisors involved in this study will have access to any information disclosed.

8. **What happens with the results?** If you give us your permission by signing the consent form, we plan to publish the results. However, in the publication, information will be provided in such a way that you cannot be identified.

Should you have any query please contact Fiona Shipton at khyx3fnp@nottingham.edu.my

Thank you for taking the time to consider participating in this study.

If you wish to take part, please request and sign the consent form.

Version 1 (2013)

Consent Form

Investigation of the Anti-inflammatory Activity of Potentially Medicinal Plants

1. I, FIONA NATALIA SHIPTON agree to participate in this study described in the participant information sheet.
2. I acknowledge that I have read the participant information sheet, which explains why I have been selected, the aims of the study and the information sheet has been explained to me, to my satisfaction.
3. Before signing this form, I have been given the opportunity to ask questions and have received satisfactory answers.
4. I understand that I can withdraw from this study at any time and at any stage without any obligation or under any binding agreement.
5. I agree that the research data gathered from the results of the study may be published provided that I cannot be identified.

(Signature of the participant)

Name: FIONA SHIPTON Date: 10/8/14

(Signature of the investigator)

Name: FIONA SHIPTON Date: 10/8/14

Investigation of the Anti-inflammatory Activity of Potentially Medicinal Plants

Rationale for the research

The purpose of this research is to determine whether or not a particular plant is able to exert an anti-inflammatory activity, as its traditional uses would suggest. The plant being studied has been used traditionally in local areas for the treatment of ailments such as sore throats, cough, bites, fractures and asthma, among other things. These uses would suggest that the plant could possess a number of interesting activities, including anti-inflammatory activity.

Inflammation can cause a number of diseases. In particular, chronic inflammation can lead to conditions such as atherosclerosis, rheumatoid arthritis and cancer. Current anti-inflammatory drugs are classed as being either steroidal or non-steroidal. Steroids are commonly used to treat conditions such as arthritis and hayfever, non-steroidal anti-inflammatory drugs (NSAIDs) have a similar activity and are also used to treat things such as pain from arthritis as well as back pain, menstrual pain and headache.

For this study, plant extracts have been produced, by solvent extraction, from different parts of the plant and it is these extracts that will be used for tests in the study. It is hoped that the extract with the most significant anti-inflammatory activity can be identified from these tests and fractioned by column chromatography. This would separate the compounds in the extract according to polarity. The fractions will then be tested and the fraction with the greatest activity will be focused on for isolation work. Any pure compounds isolated from the most active fraction will be tested using the same tests to determine its anti-inflammatory activity. The anti-inflammatory activity of all the samples will be compared to that of diclofenac sodium, a common NSAID. The identification of a compound, novel or yet to have been tested, with anti-inflammatory activity that is comparable or greater to that of drugs currently available, would be an ideal outcome to this study. This would hopefully aid in the development of new alternatives or better anti-inflammatory drugs. In order to study this activity, a series of test will be conducted, including a protein denaturation test, HRBC membrane stabilization test and COX and LOX inhibitory activity tests.

The protein denaturation test is carried out as inflammation often causes proteins in the site of inflammation to become denatured, so a sample that is able to prevent this type of damage could possibly be used to treat inflammation.

The HRBC (Human Red Blood Corpuscle) membrane stabilization test uses red blood cells (RBCs) to simulate the membrane of a lysosome. When lysosomes release their enzymes, this can damage and cause inflammation of the surrounding tissues. Since the membranes of lysosomes are similar to the membranes of RBCs, RBCs can be used instead of lysosomes in this test as they are easily obtained and lysis can be measured spectrophotometrically. The RBCs can be treated with the sample and subjected to either heat or hyposaline conditions to induce lysis. The ability of the sample to inhibit lysis implies that it would also be able to inhibit the release of lysosomal enzymes and prevent inflammation. Since

the anti-inflammatory activity will be investigated, the blood donor will have to refrain from taking any anti-inflammatory drugs for at least two weeks prior to donating blood.

The enzyme inhibitory assays are used to further determine the mechanism of action of the sample. The ability of the sample to inhibit a particular enzyme that is involved in inflammation, such as COX-1, COX-2 and LOX, will help to further elucidate the mode of action of the sample.

The HRBC membrane stabilization is one part of this project and is the only part of the project that requires ethical approval for the taking of blood, but would help to give us a more complete understanding of how the samples work to treat inflammation.

Number and type of participants

Only one healthy participant, over 18 years of age, will be required for this study. The participant can be male or female and of any ethnicity. The participant must not have taken any anti-inflammatory drugs for at least two weeks prior to donating their blood.

Number and duration of activities participants will be involved in

One participant will be taken to the nearest medical facility and required to give a small quantity of blood (<30ml), only once, and the blood taking procedure should take less than 10 minutes.

Equipment and procedures to be applied

The participant will attend a local medical facility, where medically trained staff, such as a nurse, will take blood from the arm of the participant. The blood will be stored in a leak-proof container marked as biohazardous.

Information about how participants will be recruited

The participant will be a volunteer. The participant will be informed about the purpose of the study, what is required of them (to be over 18 years of age and to not have taken any anti-inflammatory drugs for two weeks prior to giving blood). If the participant agrees to participate, they will sign a consent form.

Whether participants will be compensated

The participant will not be compensated.

How participants will be informed that they may withdraw at any time

Participants will be given an information sheet to read before they begin, informing them of their freedom to withdraw.

Plans to ensure participant confidentiality and anonymity

The name of the participant will not be disclosed to any third party, will be known only to the researcher and supervisor, and is not significant to the results, should the results of the test be published.

Plans for storage and handling of data

The participant's name or other personal details will not be recorded on the blood sample and no personal details will be needed in the presentation of the data collected. The consent form will be kept in an opaque folder, in a locked cabinet and marked as confidential.

Information about what will happen to the data after the study

After 3 years, the consent form will be destroyed by shredding. The data collected from the use of the blood sample may be published, but no information about the participant will be revealed.

Information about how any data and images may be used

The data collected from the use of the blood sample may be published. The participant will be informed, by means of an informational sheet, that the data collected from the use of the blood sample may be published but that no personal details about the participant will be published or mentioned anywhere in the work.

Statistical significance of the number of participants used

Only one participant is required as the response of the RBCs to the samples will be studied and compared relatively. RBCs are expected to behave in the same general manner from person to person. As such, there is no need for more than one participant.

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Science & Engineering Research Ethics Committee (SEREC)
Application form: research studies involving human participants

PART A

APPLICANT DETAILS:

The applicant must be the person who will conduct the investigations; each application must be made by ONE applicant. The applicant may be a member of staff or a student (undergraduate or postgraduate) registered with the University of Nottingham.

The application form must be submitted from a University of Nottingham email account.

Applicant name:	Fiona Shipton
Email address:	khyx3fnp@nottingham.edu.my

If the applicant is an undergraduate OR postgraduate student please also complete the section below.

Student ID number:	012546
Course:	PhD School of Pharmacy
Supervisor:	Dr. Christophe Wiart

For student applicants this form must be submitted via their supervisor's University of Nottingham email account. By doing this the supervisor is indicating that they support the application.

PROJECT DETAILS:

Title of project:	Investigation of the Anti-inflammatory Activity of Potentially Medicinal Plants
Abstract: Include a brief summary of the project (maximum of 300 words)	Three plants have been selected to be screened for their potential biological activity. Based on the traditional uses of the plants, there is reason to believe that one of the plants may possess significant anti-inflammatory activity. In order to investigate this claim, a series of assays will be carried out. The HRBC membrane stabilization test, tests the ability of the plant extract to inhibit lysis of red blood cells. Lysosomes contain enzymes which, when released, can cause inflammation. Since the membranes of red blood cells are similar to lysosomal membranes, this can be used to determine whether or not the plant extracts are able to prevent inflammation that is caused by release of enzymes from lysosomes. As such, this test would provide valuable data to help verify or disprove claims that the plant in question possesses anti-inflammatory properties and the mechanisms behind the action.

PROJECT DETAILS

Application identification number*:	FS090714
Study start date (planned):	1st August 2014
Study completion date (planned):	29th August 2014

**Note: the application identification number is the applicant's initials followed by the date of submission. E.g. If John Wong submitted a proposal on 1st September 2013 then the application identification number would be JW010913.*

Previous applications:

Has a similar study been approved by SEREC? If YES, state the title, application identification number and the submission date in the boxes below. This may speed up the review process.

Title of similar study:	
Submission date of similar study:	
Application identification number:	

PART B

Minimal Risk Checklist

The purpose of this Checklist is to facilitate the review process and to identify projects that can be considered minimal risk.

If you answer "YES" to any of these questions you will need to complete Part C of the application form.

1. Will the population studied include any vulnerable groups, e.g. participants aged under 18, disabled or lacking capacity. (If YES, please give further details)

   YES ☐ NO ☑

2. Will any participants aged under 18 be involved without the consent of their parents, guardians or carers? (If YES, please give further details). SEREC will only approve 'opt-out' consent in very exceptional circumstances.

   YES ☐ NO ☑

3. Excluding consent forms, will it be possible to associate specific information in your records with specific participants on the basis of name, position or other identifying information contained in your records? (If YES, please give further details)

   YES ☐ NO ☑

4. Will persons participating in the study be subjected to physical or psychological discomfort, pain or aversive stimuli, which are more than expected from everyday life? (If YES, please give further details).

   YES ☑ NO ☐

   Blood will be taken from the arm, by trained medical staff, from a nearby medical facility.

5. Will any inducement, other than reasonable expenses / compensation for time, be given to participants? (If YES, please give further details).

   YES ☐ NO ☑

6. Will any participants involved in this study be doing so without their knowledge and consent, e.g. covert observation? (If YES, please give further details).

YES ☐ NO ☑

7. Will the investigation involve any blood or tissue samples being taken from the participant? (If YES, please give further details including an explanation of how these samples will be stored and disposed of).

YES ☑ NO ☐

Stored blood is marked as biohazardous and disposed of in biohazard waste.

8. Will the investigation involve any drugs, placebos or any other substances (supplements, vitamins) being administered to patients? (If YES, please give further details).

YES ☐ NO ☑

9. Will the investigation require the participant to undergo any type of surgical / invasive procedure, physiological / psychophysiological monitoring, TMS or fMRI studies? (If YES, please give further details).

YES ☑ NO ☐

The investigation will require the participant to undergo an invasive procedure. Blood will be taken by venipuncture, by medically trained staff. This procedure has minimal

10. Will the participant be exposed to any unpleasant, loud or prolonged (or multiple) stimuli/testing or deprivation or restriction, e.g. of food or sleep? (If YES, please give further details).

YES ☐ NO ☑

11. Will participants engage in strenuous or unaccustomed physical activity? (If YES, please give further details).

YES ☐ NO ☑

12. Will the investigation use procedures designed to induce participants to act contrary to their wishes or that will affect their ability to give continuing consent, e.g. hypnosis, alcohol use? (If YES, please give further details).

YES ☐ NO ☑

13. Will the investigation involve any procedures which participants might not feel free to withdraw from or that they may regret taking part in? (If YES, please give further details).

YES ☐ NO ☑

14. Is the investigation likely to intentionally or unintentionally induce embarrassment, humiliation, lowered self-esteem, guilt, conflict, anger, discouragement or other emotional reactions? (If YES, please give further details).

YES ☐ NO ☑

15. Will participants be required to recall personal memories or induced to disclose information of an intimate or otherwise sensitive nature, e.g. relationships, feelings of success? (If YES, please give further details).

YES ☐ NO ☑

16. Will participants be deceived or actively misled in any manner or will information be withheld from them that they might reasonably expect to receive? (If YES, please give further details).

YES ☐ NO ☑

17. Are any participants being knowingly investigated for a problem which has received medical, psychiatric, clinical psychological or other similar attention? (If YES, please give further details).

YES ☐ NO ☑

18. Will the research involve potentially sensitive topics, e.g. sexual, racial, religious or political attitudes? (If YES, please give further details).

YES ☐ NO ☑

19. Is there the possibility of the disclosure of confidential information, by participants or researchers, during the course of the study, e.g. to other participants? (If YES, please give further details).

YES	NO
☐	☑

20. Will any of the research activities be taking place outside of Malaysia or the UK? (If YES, please give further details).

YES	NO
☐	☑

21. Are you aware of any other ethical concerns that are not identified in the application? (If YES, please give further details).

YES	NO
☐	☑

If the application relates to an **undergraduate research project** or a research project which forms part of a **postgraduate taught course,** AND you answered "NO" to all of the questions in Part B then the research is considered minimal risk by SEREC.

By submitting this application I confirm that I understand the ethical requirements for my study and have read and complied with the University of Nottingham Code of Research Conduct and Research Ethics.

For "minimal risk" projects the applicant only needs to complete Part A and Part B before submitting the Application Form to their School Research Officer.

PART C

Part C should only be completed if the project is considered to greater than "minimal risk"

This section outlines the supporting information that should be submitted with your application IF it is considered to be greater than minimal risk.

Checklist of information to include with your application (please tick)

☑	A description of the study design (maximum 3000 words) which should cover, but is not limited to: – Rationale for the research – Number and type of participants – Number and duration of activities participants will be involved in – Equipment and procedures to be applied – Information about how participants will be recruited – Whether participants will be compensated (state how this will be done) – How participants will be informed that they may withdraw at any time – Plans to ensure participant confidentiality and anonymity – Plans for storage and handling of data – Information about what will happen to the data after the study – Information about how any data and images may be used – Statistical significance of the number of participants
☑	Copies of any information to be given to participants. (This includes recruitment information e.g. adverts, posters, letters).
☑	A copy of the participant consent form.
☑	Copies of data collection sheets, e.g. questionnaires.
☑	Any other information (please state)

Where possible ALL supporting documents should be submitted as pdf files.

By submitting this application I confirm that I understand the ethical requirements for my study and have read and complied with the University of Nottingham Code of Research Conduct and Research Ethics.

If you answered "YES" to any of the questions in Part B then the completed form must be submitted to the Ethics Administrator.

Click HERE to submit the form.

Appendix F

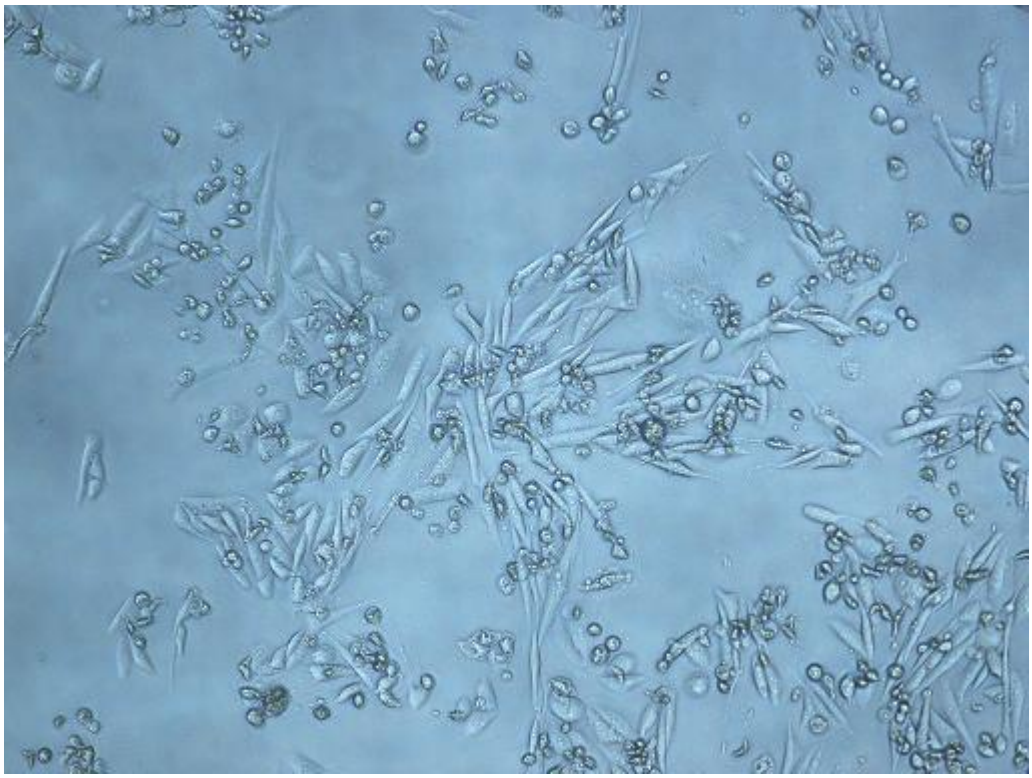


Figure F1: Cell morphology of MRC-5 control after 72 hours of incubation.

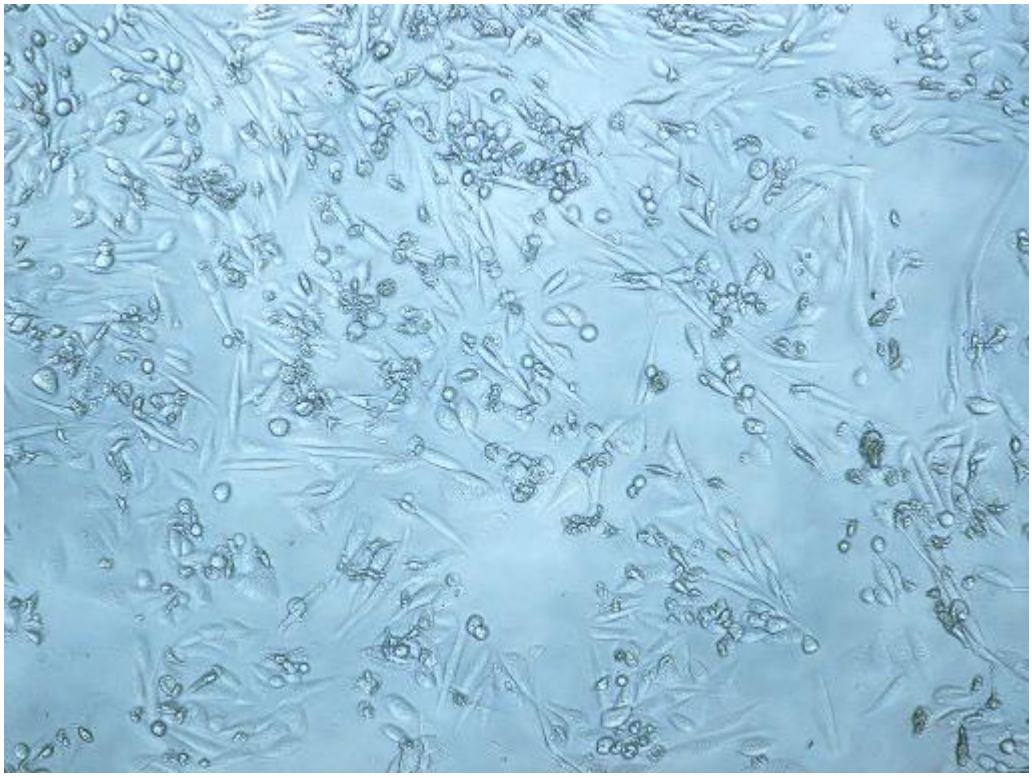


Figure F2: Cell morphology of MRC-5 treated with PGSH at a concentration of 200 µg/ml, after 72 hours of incubation.

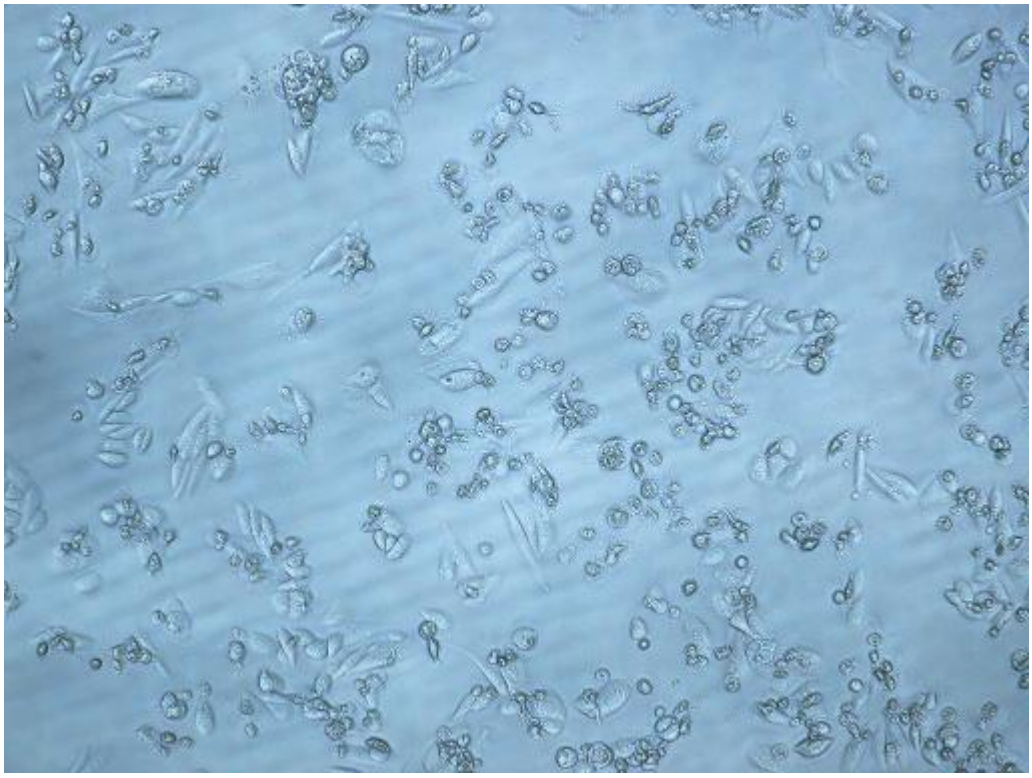


Figure F3: Cell morphology of MRC-5 treated with PGSC at a concentration of 200 µg/ml, after 72 hours of incubation.

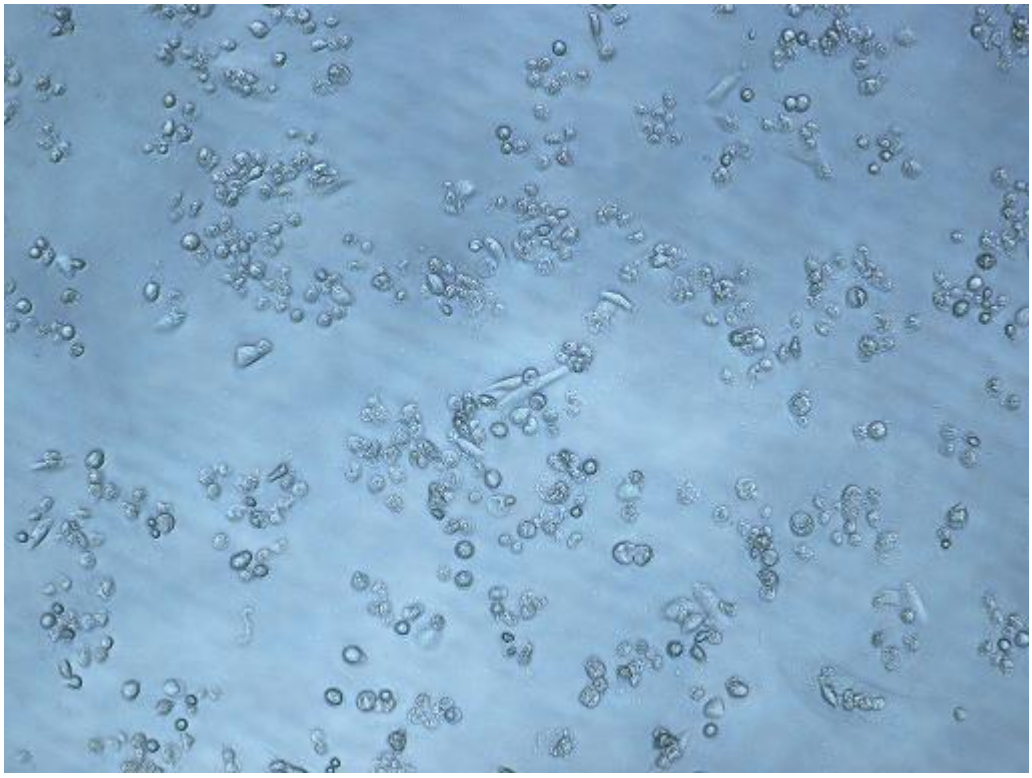


Figure F4: Cell morphology of MRC-5 treated with PGSE at a concentration of 200 µg/ml, after 72 hours of incubation.

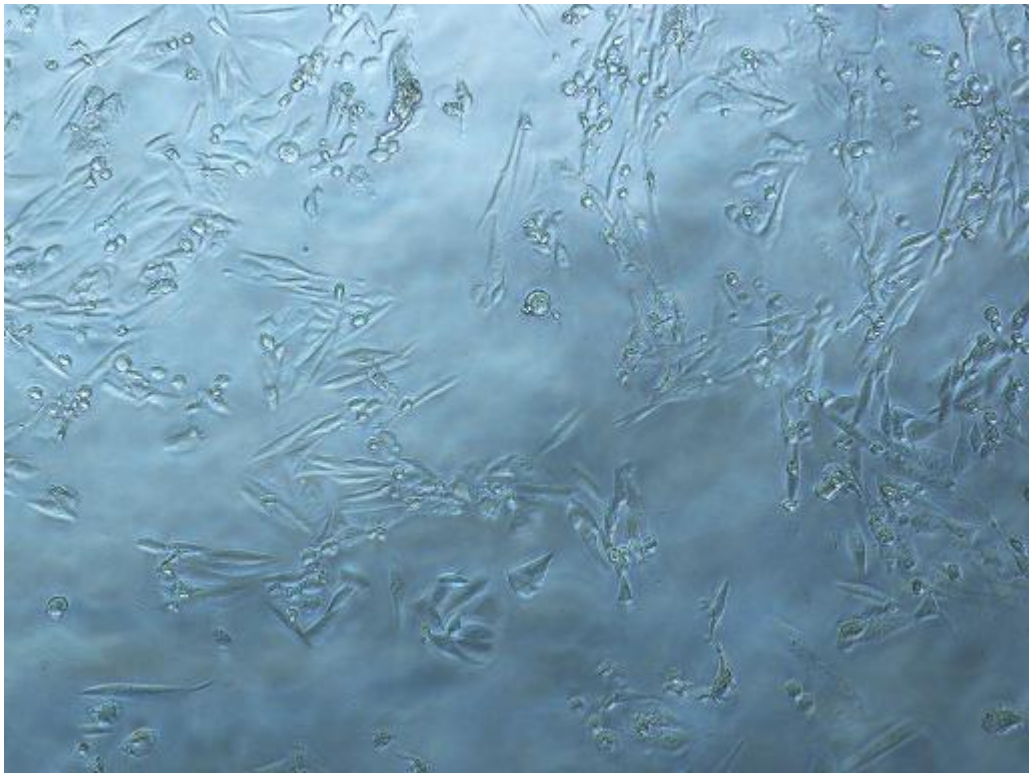


Figure F5: Cell morphology of MRC-5 treated with PGLH at a concentration of 200 µg/ml, after 72 hours of incubation.



Figure F6: Cell morphology of MRC-5 treated with PGLC at a concentration of 200 µg/ml, after 72 hours of incubation.



Figure F7: Cell morphology of MRC-5 treated with PGLE at a concentration of 200 µg/ml, after 72 hours of incubation.

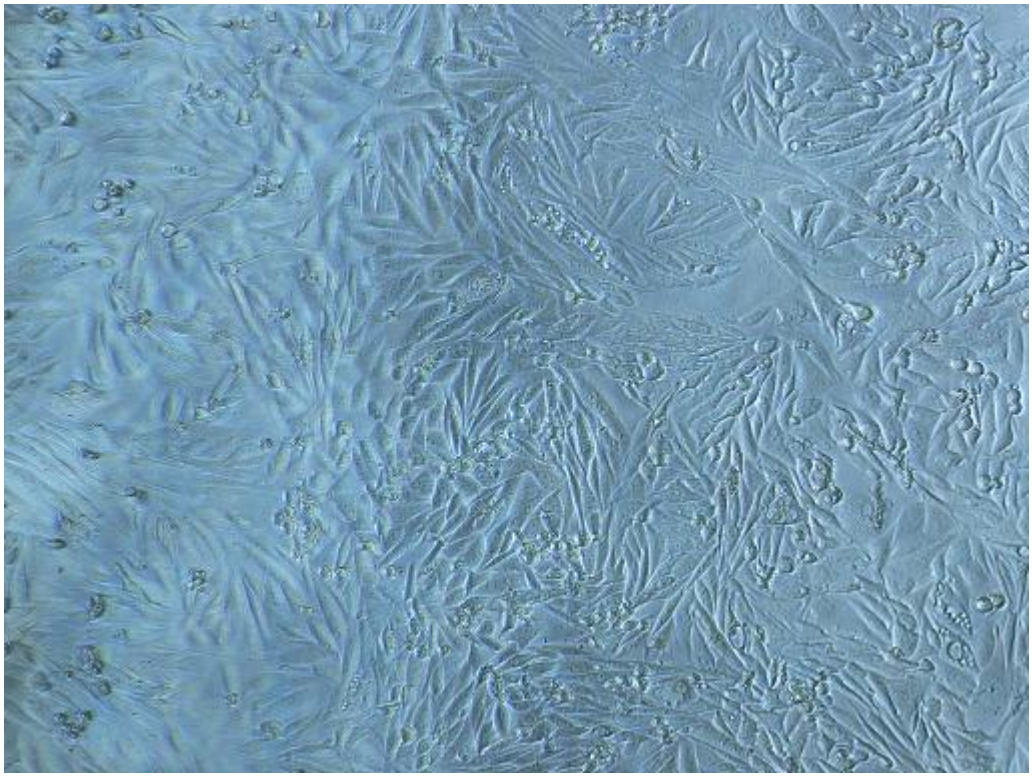


Figure F8: Cell morphology of MRC-5 treated with PGRH at a concentration of 200 µg/ml, after 72 hours of incubation.

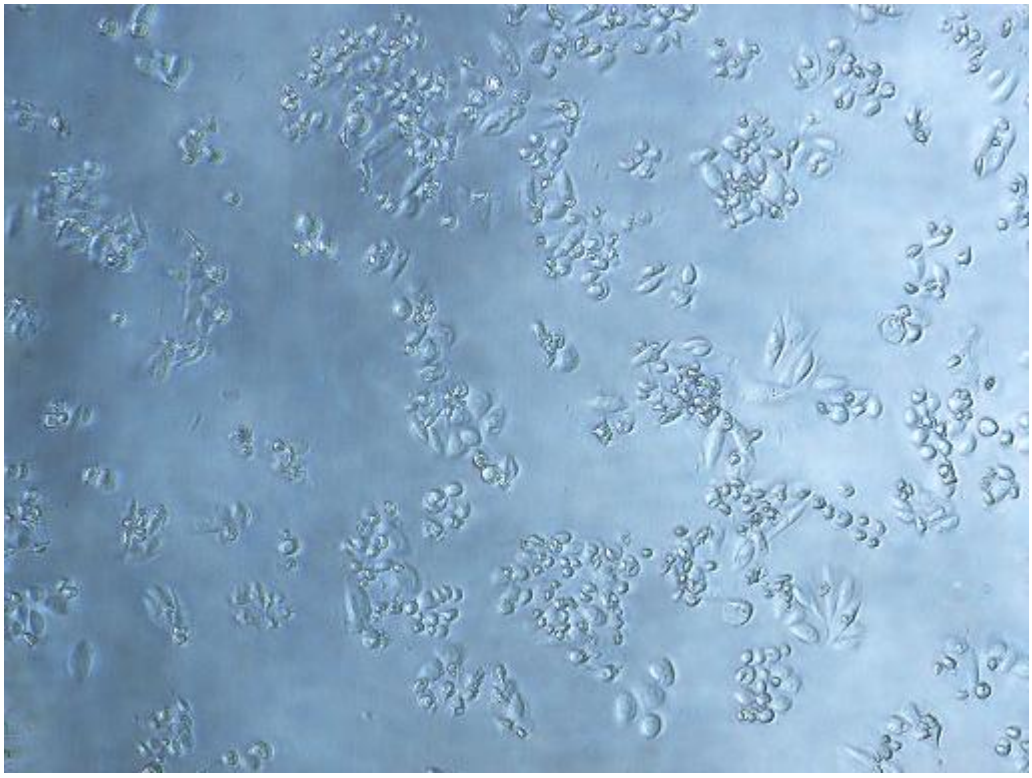


Figure F9: Cell morphology of MRC-5 treated with PGRC at a concentration of 200 µg/ml, after 72 hours of incubation.



Figure F10: Cell morphology of MRC-5 treated with PGRE at a concentration of 200 µg/ml, after 72 hours of incubation.



Figure F11: Cell morphology of MRC-5 treated with PGFH at a concentration of 200 µg/ml, after 72 hours of incubation.



Figure F12: Cell morphology of MRC-5 treated with PGFC at a concentration of 200 µg/ml, after 72 hours of incubation.

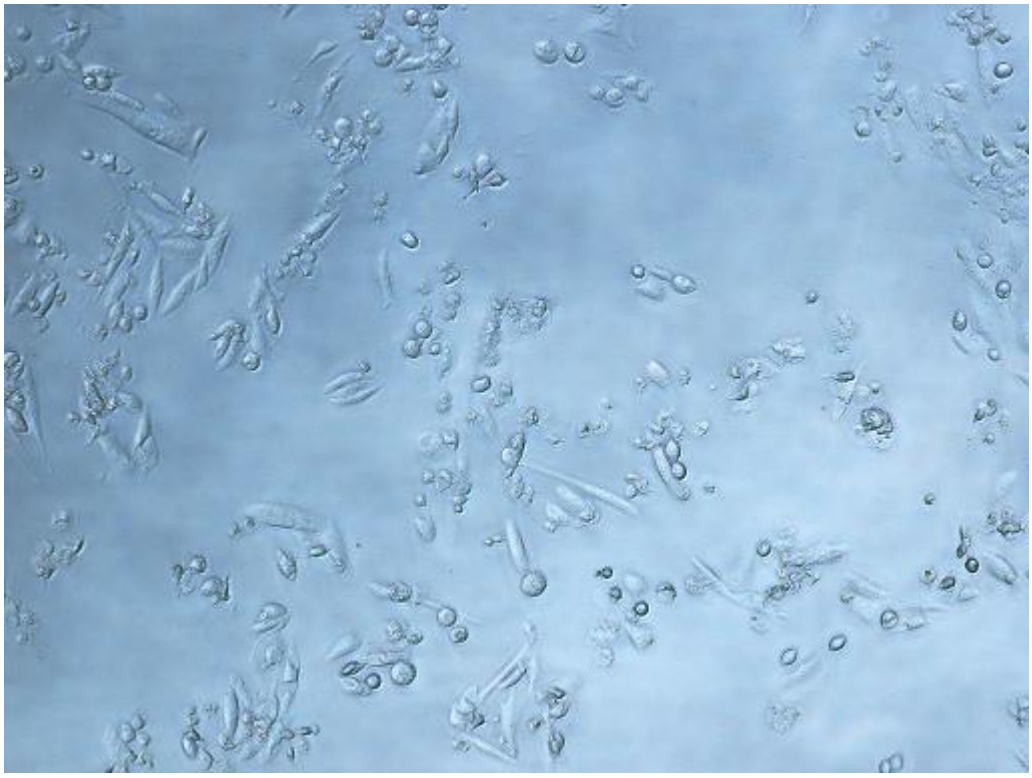


Figure F13: Cell morphology of MRC-5 treated with PGFE at a concentration of 200 µg/ml, after 72 hours of incubation.

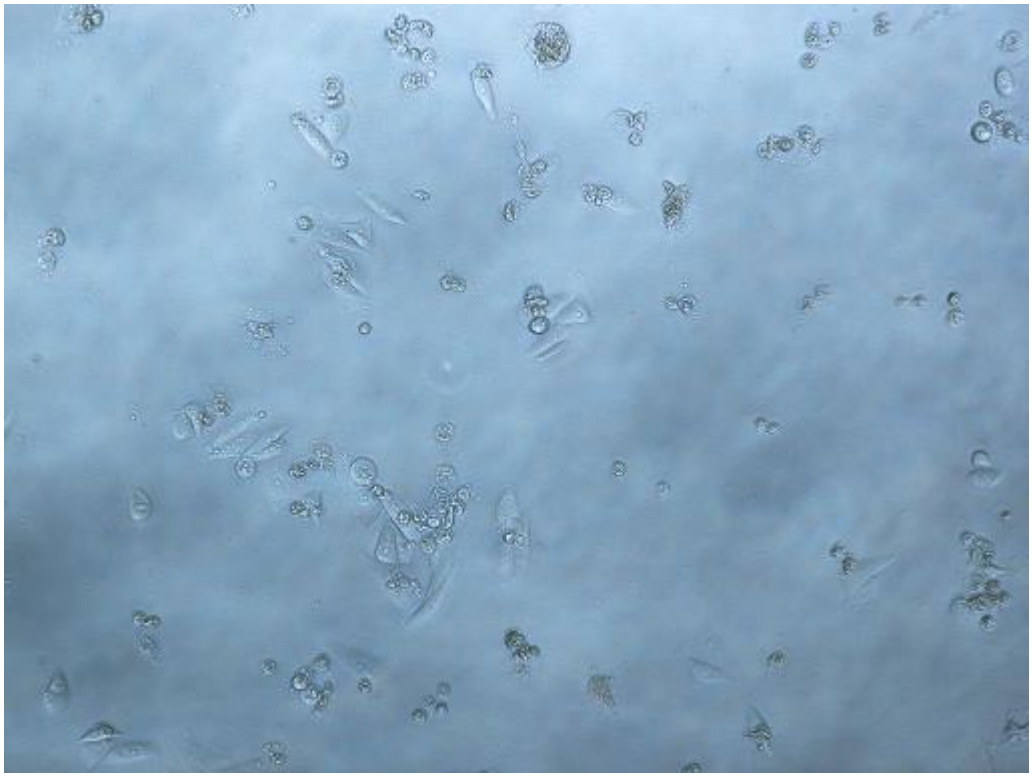
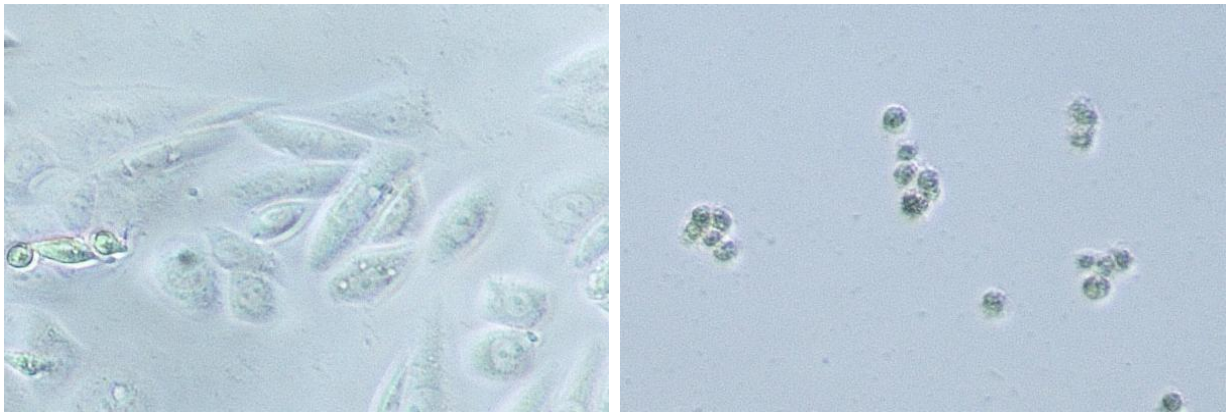


Figure F14: Cell morphology of MRC-5 treated with quercetin at a concentration of 25 µg/ml, after 72 hours of incubation.

Appendix G



(a) (b)

Figure G1: Morphology of cells when (a) untreated and (b) when treated with *C. atrox* venom