

IT Tralee PhD by Research Programme Details

Title of Project: BioWill – Characterising Willow Bark Bioactives For Skin Therapies
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Research Project Abstract

Willow bark is generally a disposable by-product when growing willow for timber. This project aims to generate *in vitro* data to support the utilisation of willow bark extracts in skin healthcare products. High value natural bioactive extractives can be as effective as synthetic equivalents for analgesic and anti-inflammatory properties, with fewer undesirable side effects.

Secondary metabolites from plant extracts have served as phytotherapeutic antioxidants for centuries. Willow (*Salix*) bark is a known source of salicylates, most notably the anti-inflammatory salicin, which can be metabolised into salicylic acid. Salicylic acid is the precursor for acetylsalicylic acid, more commonly known as aspirin. While the physiological benefits of aspirin are well established, there is growing interest in exploring the bioactivity of unprocessed natural extracts. The antioxidant and anti-inflammatory potential of the salicylate and non-salicylate bioactives (polyphenols, flavonoids and catechols) associated with willow bark will be explored.

As part of the Interreg project BioWILL, this project aims to assess the impact and mode of action of willow bark bioactives, individually and in combination, on human cell culture models of skin injury and disease. Crude willow bark extracts will firstly be examined for antioxidant and anti-inflammatory properties, before undergoing molecular characterisation i.e. size exclusion chromatography (SEC) and liquid chromatography mass spectroscopy (LCMS), to help classify the compounds responsible for these bioactivities. The bioactive fractions with the highest activity will be tested in various models of skin injury and disease (e.g. skin barrier, wound healing, UV insult, melanoma, psoriasis). The mechanisms of their bioactivity will then be assessed at the genetic level using real-time PCR, and at the protein level using ELISAs and western blotting.

Research Context (Technical Merit & Impact)

Aims: This PhD project is part of the Interreg project BioWILL, an integrated "zero waste" biorefinery utilising all fractions of willow feedstock to produce high to medium based bio-chemicals/materials, renewable energy in the form of bio methane production and natural fertilisers. Willow bark is usually considered a disposable by-product when growing willow for timber. The main aim of this PhD project is to generate both *in vitro* and *in vivo* data to support the utilisation of willow bark extracts (WBE) in skin healthcare products. High value natural bioactive extractives can be as effective as synthetic equivalents for analgesic and anti-inflammatory properties, with fewer undesirable side effects. The project will explore the antioxidant and anti-inflammatory potential of both the unprocessed natural salicylates and the non-salicylate bioactives (polyphenols, flavonoids and catechols) associated with willow bark.

Using a variety of human cell culture models, the skin-related bioactivities of WBE (in crude form and subcategorised based on molecular weight and general chemistry) supplied from the BioWILL Interreg project will be characterised.

Objectives:

- i. Measure the anti-inflammatory and antioxidant capabilities of crude WBEs using biochemical assays e.g. cyclooxygenase (COX) and prostaglandin H synthases (PGHS) for anti-inflammatory and oxygen radical absorbance capacity (ORAC) for antioxidant.
- ii. Determine appropriate vehicle/diluent for WBE's application in cell culture models and perform cytotoxicity testing of serial diluted WBE to determine appropriate concentrations for further cell culture assays.
- iii. Measure anti-inflammatory and antioxidant biomarkers in more dynamic *in vitro* skin cell culture models (human dermal fibroblasts and human keratinocytes).
- iv. Fractions collected from the SEC will be characterised for their biochemical antioxidant activity, biological antioxidant activity and biological anti-inflammatory activity. This will identify which fractions contain the highest levels of bioactivity with respect to antioxidant and anti-inflammatory activity. Fractions with the highest levels of activity will be applied a liquid chromatography mass spectroscopy system to achieve a first pass level of molecule identification.
- v. Identify the bioactive fraction(s) with the greatest activity and test their efficacy in human skin models of injury and disease (e.g. skin irritation, skin barrier, UV damage, melanoma, psoriasis).
- vi. Develop a skin cosmetic formulation incorporating the relevant willow bark extracts.

Central Research Questions:

1. Can crude WBE reduce or inhibit free radicals/reactive oxygen species (ROS) biochemically and/or in *in vitro* human skin cell culture models?
2. Can crude WBE reduce inflammation biochemically and/or in *in vitro* human skin cell culture models?
3. Where present, can the bioactivities of the crude WBE be partially identified based on molecular size and general chemistry?
4. Is there any synergistic bioactivity of the components of the crude WBE versus sub-categorised fractions?
5. Are these bioactives beneficial when tested in *in vitro* skin injury and disease models?
6. What are the cellular mechanisms of the WBE bioactivities?
7. What are the molecular mechanisms of the WBE bioactivities?
8. Can a formulation using the WBE be created for topical application?

Research Methodology

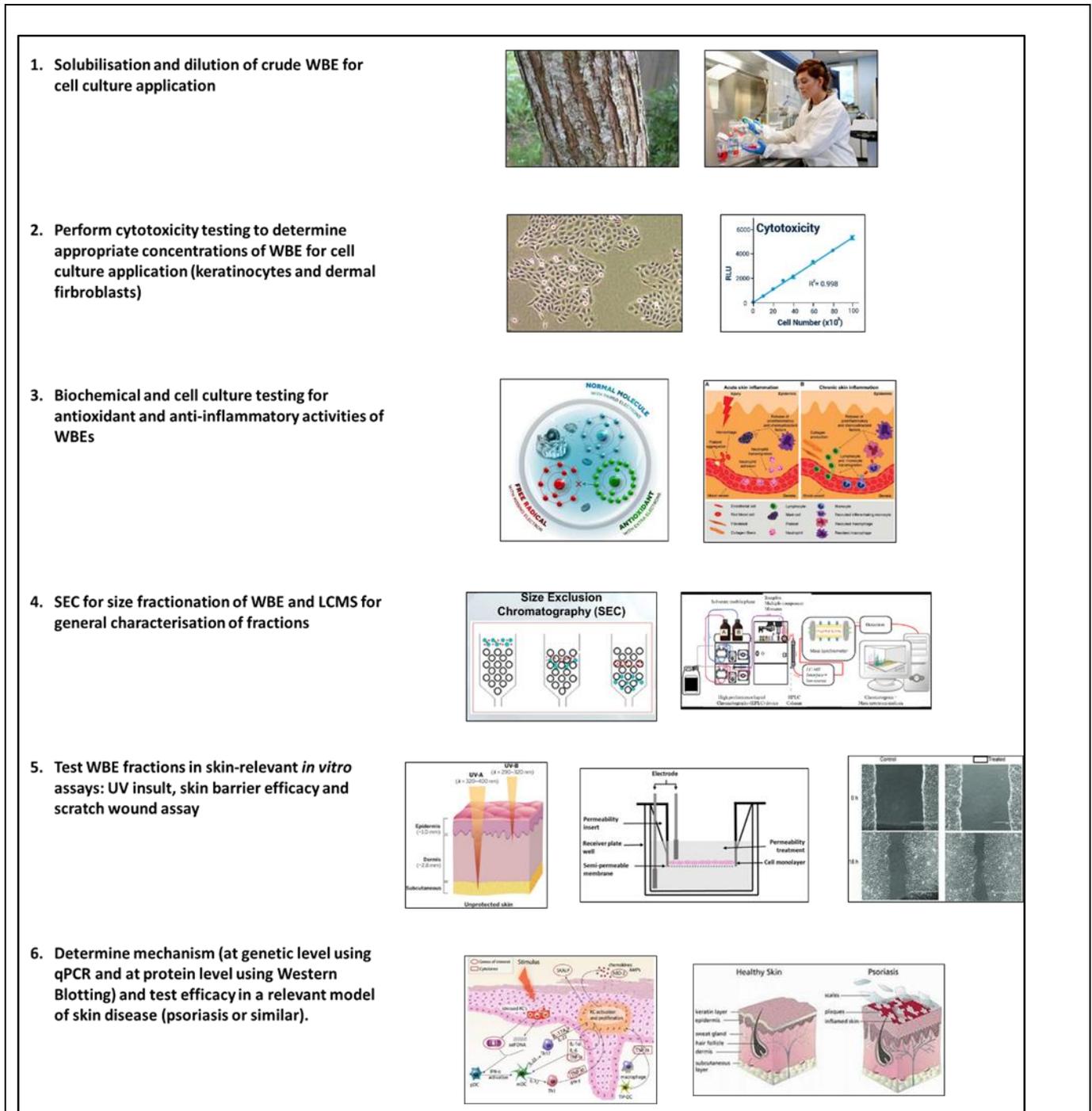


Figure 1. Flow chart of workplan and methodology of project.

The project will require the student to become proficient in cell culture practices such as aseptic technique, cryopreservation and thawing of cells, passaging cells and counting and seeding of cells. These methods are implemented daily by Shannon ABC staff at IT Tralee. The relevant knowledge and expertise in this regard will be available to the student throughout the project.



In order to perform the various bioassays, the learning and implementation of multiple molecular and cellular biology techniques will be required, including (but not limited to) ELISA, qPCR, SEC, western blotting. The Shannon ABC laboratory at IT Tralee has the relevant equipment to perform (as well as experienced staff to teach) these techniques and assays and support and guidance will be provided at any stage where required.

The focus tissue type of this project is human skin and with that in mind two *in vitro* human cell lines have been chosen, one to represent the epidermis (keratinocytes) and the other the dermis (dermal fibroblasts).

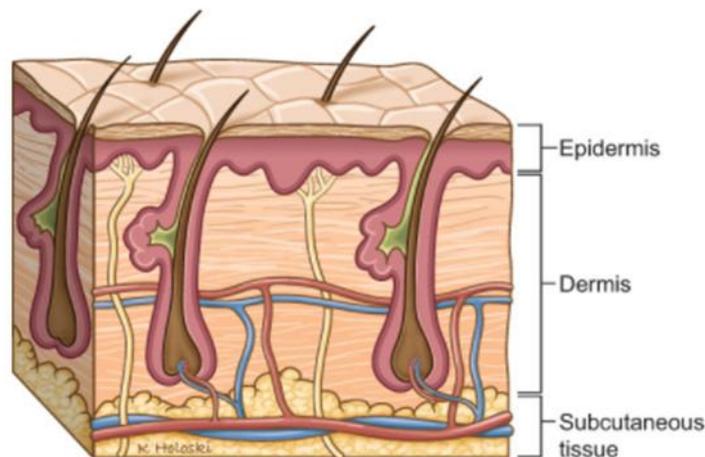


Figure 2. The epidermis is the outermost layer of the skin and is made up of a number of layers, mostly containing keratinocytes. The dermis is generally the thickest layer of the skin, which is still just a few millimeters. In this layer are blood vessels, lymphatic vessels, hair follicles, sweat glands, oil glands, nerve endings and fibrous tissue. The main cell types of the dermis are fibroblasts, as well as macrophages and mast cells (immune cells). Image from <https://headandneckcancerguide.org/adults/introduction-to-head-and-neck-cancer/skin-cancer/anatomy/>

Keratinocytes; are the predominant cell type in the epidermis (outermost skin layer). HaCaT cells are a long-lived, spontaneously immortalized human keratinocyte line which is able to differentiate *in vitro*. They provide an excellent model to study epithelial function and disease, skin biology and toxicology. When grown on inserts and provided a liquid/air interface, they differentiate into a stratified squamous epithelium, forming a physiological 3D tissue.

Human Dermal Fibroblasts (HDF); are responsible for producing the extracellular matrix forming the connective tissue of the skin and play a crucial role during wound healing. They provide an excellent model system to study many aspects of cell physiology, and have been utilized in dozens of research publications, particularly those related to skin biology and reprogramming/induced pluripotency studies.



PROJECT SCHEDULE / GANTT CHART

Milestones:

- i. Biochemically test for relevant bioactivities (antioxidant and anti-inflammatory)
- ii. Test various diluents/vectors for WBE for application in cell culture
- iii. Test a range of concentrations of crude WBE to use in cell culture (cytotoxicity)
- iv. Test bioactivity of WBE in cell culture models
- v. Subcategorise/fractionate crude WBE by molecular size and general chemistry
- vi. Test most promising WBE fractions in various models of skin cell injury
- vii. Test on in vitro human skin disease model
- viii. Incorporate most promising WBE fractions into formulation for application on a 3D skin cell model
- ix. Incorporate most promising extract fraction into a cosmetic formulation

Deliverables:

- i. Determine initial indication of antioxidant and anti-inflammatory potential of crude WBE
- ii. Determine the most appropriate diluent/vector for WBE for application in cell culture
- iii. Determine the most appropriate concentrations of WBE for application in cell culture
- iv. Determine if antioxidant and anti-inflammatory properties can be detected in cell culture models
- v. WBE fractionated by molecular weight
- vi. Determine the bioactivity of WBE fractions in models of skin cell injury
- vii. Determine the bioactivity of WBE fractions in models of skin disease
- viii. Determine if promising results can be translated from 2D model into more physiological 3D model of skin injury
- ix. Provide prototype formulation to be considered for development by collaborating partners in BioWILL



	Month											
	0-4	4-8	8-12	12-16	16-20	20-24	24-28	28-32	32-36	36-40	40-44	44-48
Literary review												
Solubility of crude WBE												
Cytotoxicity												
ORAC assay												
Cell culture antioxidant and anti-inflammatory												
Size fractionation and LCMS												
Skin injury models												
<i>In vitro</i> skin disease model												
3D <i>in vitro</i> models												
Formulation assessment												
Thesis submission												
Viva												

- Funding for this project includes stipend of €850 per month, student registration fees and consumables budget
- Closing date for application – 16th October 2020