TITLE PAGE

The influence of pharmaceutical methods of preparation of *Hydrastis canadensis* mother tincture, on concentrations of Hydrastine and Berberine, using High-Performance Liquid Chromatography

By

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I*, Aaisha Mahmood Vawda*, do declare that this dissertation is representative of my own work, both in conception and execution

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DEDICATION

This work is dedicated to my parents, my husband and my siblings. Thank you for all that you are.

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All Praise and thanks are due to the Almighty for allowing me to reach this point in my journey. The Prophet Muhammad (Peace Be Upon Him) said: “One who is ungrateful to people is ungrateful to the Almighty.” (Hadith. Sunan At-Tirmidhi)

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ABSTRACT

This study used High-Performance Liquid Chromatography to determine the quantities of active ingredients, Hydrastine and Berberine, that were present in different pharmaceutical preparations of Homoeopathic *Hydrastis canadensis* mother tincture. Homoeopathic dilutions, namely 1:3 and 1:10 dilutions of *Hydrastis canadensis* were investigated. Commercial samples of 1:3 and 1:10 dilutions of *Hydrastis canadensis* were purchased from a Homoeopharmaceutical company. A 1:10 dilution was prepared in the Homoeopharmaceutical laboratory at the Durban University of Technology, from the commercial 1:3 dilution. This 1:10 dilution of *Hydrastis canadensis* was compared to the commercial 1:10 dilution that was purchased from the Homoeopharmaceutical company, to compare the difference between the quantities of active ingredients in the same dilutions, prepared under slightly different conditions. A 1:10 dilution is more cost effective, especially for use in Homoeopathic community Healthcare Centres. Therefore, the study aimed to determine whether the wide use of a 1:10 dilution may be justified or negated.

This study aimed to evaluate whether the quantities of alkaloids present in different Homoeopathic dilutions, correlated with the current Homoeopathic uses of the remedy. It also intended to establish whether remedies that are purchased from Homoeopharmaceutical companies, and then further diluted in Homoeopathic laboratories are still comparable and effective.

There are various methods of preparing the same Homoeopathic dilution. This study investigated whether the different methods of preparing the same dilution of a remedy, whilst following standard procedures and guidelines, yielded different quantities of the end alkaloids of the remedy. A new angle of Quality Control in the production of Homoeopathic remedies was investigated as well.

The Homoeopathic scope of practise does not permit the Practitioner to dispense a 1:3 dilution to a patient. Therefore, this study aimed to provide a justification for using a 1:3 to prepare a 1:10 which is commonly used in Homoeopathic practice (2019, pers. comm. Dr S Brijnath. January 2019).

The concentrations of Hydrastine and Berberine were depicted in a chromatogram. This data was displayed using Adobe. The data analysis was depicted using graphs, and SigmaPlot was used to calculate the results of the data obtained. The results were tabulated, and all forms of data were attached as appendices in this dissertation. The statistical analysis of the data was conducted using the Analysis of Variants test.

This research project showed that the Researcher's preparations of *Hydrastis canadensis* contained sufficient concentrations of Hydrastine and Berberine when compared to *Hydrastis canadensis* that was purchased from a Homoeopharmaceutical company. Moreover, the Researcher's preparation of *Hydrastis canadensis* dilutions contained significantly higher concentrations of the

aforementioned alkaloids than that purchased from a Homoeopharmaceutical company.

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DEFINITION OF TERMS

Homoeopathy: Homoeopathy is a medical art that is based on natural principles, which were founded by Dr Samuel Hahnemann (Roberts 1997). It is the administration of a minute dose of the patient's similimum remedy, in order to bring about cure.

Succussion: The process of vigorously shaking a homoeopathic liquid remedy and striking the vial onto the palm or a cushioned surface, in order to alter the dynamic nature of the medicine (Kayne 2006).

Potentisation: The process of making a remedy more therapeutic and stronger-acting, by vigorously shaking or succussing it (Kayne 2006). This process also rids the medicine of toxic properties and encourages the curative properties of the medicine to manifest (Banerjee 2002).

Dilution: The process of adding liquid to another liquid or a substance (Banerjee 2002)**.** This is done in order to eliminate toxic effects of the remedy.

Similimum: A remedy that best fits and is most beneficial to the patient's symptoms in totality (Belling 2017).

Pharmacopoeia: A Pharmacopoeia is a legal manual that provides the standard preparations, formulae and description of remedies (Das 2016). It provides the description of the remedy or drug substance as accepted in the current medical practice. It contains unique monographs for each remedy, which stipulates the methods of preparation, standardisation and testing of the raw materials and the remedy (Owen 2007).

Tincture: The starting point for a remedy is referred to as a tincture (Owen 2007). It consists of the crude substance of a source material that is dissolved in alcohol and water (Banerjee 2002).

Decimal dilution: A dilution that has been prepared in parts of one to ten (1:10), with one part of medicinal substance to ten parts of liquid solvent, such as water or alcohol.

Centesimal dilution: A dilution that has been prepared in parts of one to one hundred (1:100), with one part of medicinal substance to one hundred parts of liquid solvent, such as water or alcohol.

Chromatography: Chromatography is the process of separating a sample mixture into its individual components, using a separation column (Meyer 2013). Liquid or gas is used to separate components in a mixture, which are produced either as coloured

bands or peaks, respectively (Gault and McClenaghan 2013). These results are displayed on a computer and can be interpreted for qualitative and quantitative analysis, of individual constituents in a mixture.

Retention time: The period in which the autosampler or injector injects the sample until the formation of a peak, produced by the detected analyte, on the chromatogram (Meyer 2013).

1 CHAPTER 1

INTRODUCTION

1.1 Introduction to the Study

In Homoeopathy, there are different pharmaceutical methods used to prepare the mother tincture of all remedies, including *Hydrastis canadensis. Hydrastis canadensis* is widely used for its antimicrobial and anti-bacterial effects, especially in Homoeopathic community Healthcare Centres. The main methods of preparations are 1:2, 1:3, 1:5 and 1:10 diluted preparations (Benyunes 2005). There was a lack of knowledge over the years regarding which method of starting preparation, such as whether using a 1:10 or a 1:3 diluted preparation to produce subsequent dilutions, would have been more potent in terms of its curative effects. Additionally, there was insufficient research regarding the different methods of homoeopathic dilutions, and the concentrations of alkaloids that each diluted preparation yielded.

This study investigated the quantities of active ingredients, Hydrastine and Berberine, present in different pharmaceutical preparations of *Hydrastis canadensis* mother tincture, using High-Performance Liquid Chromatography (HPLC). Once a homoeopathic remedy is prepared, it is impossible to differentiate between potencies. The use of HPLC to perform a qualitative and quantitative analysis of Berberine and Hydrastine present in different pharmaceutical preparations of *Hydrastis canadensis*, provided for a new angle of quality control to be implemented on different remedies that are prepared by Homoeopaths on a daily basis.

Therefore, this study aimed to determine which method of preparation yielded a higher quantity of Berberine and Hydrastine; and whether the current Homoeopathic use of this remedy, according to the end quantity of these alkaloids, correlated with the Homoeopathic remedy's current uses.

This also allowed the researcher to establish whether there was a difference in the amount of Hydrastine and Berberine present in the end products of the different pharmaceutical preparations of *Hydrastis canadensis.* Furthermore, there is insufficient research about whether remedies that are purchased from Homoeopharmaceutical companies, and then further diluted in Homoeopathic laboratories, are as effective in treating patients. Additionally, this allowed the researcher to determine whether different dilutions of homoeopathic remedies are comparable and effective. Hence, quantifying the alkaloids Hydrastine and Berberine that were present in diluted preparations of *Hydrastis canadensis*, allowed the researcher to determine whether these diluted preparations are still viable as this would reduce the cost of medications.

1.2 Rationale of the Study

The purpose of this study is to determine the concentrations of Hydrastine and Berberine present in the end products of different Homoeopathic dilutions of *Hydrastis canadensis*. This study investigated which diluted preparation of *Hydrastis*

canadensis, the 1:3 or the 1:10 dilution, yielded a higher quantity of Berberine and Hydrastine. It also intended to establish whether Homoeopathic remedies that are purchased from Homoeopharmaceutical companies and then further diluted in Homoeopathic laboratories, were comparable to that of the same dilution that could be purchased.

1.3 Aim of the Study

This study aimed to determine the quantities of active ingredients Hydrastine and Berberine that were present in different pharmaceutical preparations of *Hydrastis canadensis* mother tincture, using High-Performance Liquid Chromatography. It aimed to investigate which method of preparation yielded a higher quantity of the active ingredients.

1.4 Statement of the Objectives

- To determine the quantities of Hydrastine and Berberine in a 1:3 dilution of *Hydrastis Canadensis* obtained from a Homoeopharmaceutical company, using High-Performance Liquid Chromatography.

- To determine the quantities of Hydrastine and Berberine in a 1:10 dilution of *Hydrastis canadensis* obtained from a Homoeopharmaceutical company, using High-Performance Liquid Chromatography.

- To determine the quantities of Hydrastine and Berberine in a 1:10 dilution of *Hydrastis canadensis*, prepared in the Homoeopharmaceutical laboratory at the Durban University of Technology, from a Homoeopharmaceutical company 1:3 dilution, using High-Performance Liquid Chromatography.

- To compare the difference between the quantities of Hydrastine and Berberine that are present in both 1:10 dilutions made up in step 2 and 3.

2 CHAPTER 2

LITERATURE REVIEW

2.1 Homoeopathy

Homoeopathy is a system of alternative treatment that was invented by Dr Samuel Hahnemann. There are various principles that Homoeopathy is based on, which is discussed in detail.

2.1.1 The Law of Similars

One of the principles that Homoeopathy is based on is “like cures like”, or The Law of Similars. This principle translates that a substance that is capable of producing harmful or undesirable symptoms when administered to a healthy individual, can be diluted according to standard Homoeopathic preparations, to form a Homoeopathic remedy. If this diluted substance, which is the remedy, is administered to a diseased individual who presents with these same symptoms, the individual’s immune system will remove the cause of these symptoms (Kayne 2006). It does this by strengthening and favourably altering the vital force of the body. After some time, these symptoms will cease (Lessell and Greenwood 1994).

2.1.2 The Law of the Single Dose

Hahnemann states in Aphorism 272 of the Organon of Medicine that it is not necessary to administer more than a single dose of Homoeopathic medicine at a time (Hahnemann 2002). His reasoning for this is that a single dose is potent enough to stimulate the vital force of the body on an energetic level. Moreover, administering a single dose at a time prevents possible unforeseeable interactions between different Homoeopathic remedies (Kayne 2006). In addition, it becomes clear whether or not the dose of a remedy is having any effect on the patient or whether the remedy needs to be changed. If multiple doses are administered, there is also a possibility that one remedy may counteract the other remedy, thereby preventing any medicinal effects (Thomas 2012). Hence, administering a single dose is a safer and more potent option.

2.1.3 The Law of the Infinitesimal Dose

The use of an undesirable substance to produce a Homoeopathic remedy, means that this substance needs to be diluted in order to prevent adverse reactions or an aggravation of symptoms when administered to a patient. Hahnemann observed that although the crude substance or a less diluted dose of a remedy caused an aggravation in the patient, it was often followed by cure. However, this is undesirable to the patient, so Hahnemann discovered another method of treating the patient with the same substance, but in a more diluted form. Thus, the same remedy results in curative effects in the patient but without aggravating their symptoms beforehand (Aphale and Rajgurav 2016). This law states that if the selected remedy fits the patient's symptoms in its totality, then only a minute amount is needed to elicit a

curative reaction (Vithoulkas 2002). A remedy that best fits and is most beneficial to the patient's symptoms in totality, is referred to as the simillimum (Belling 2017). If the simillimum is given to a patient in a high dose, it will produce symptoms in the patient that are characteristic to that remedy; whereas if it is administered in a small dose, it will produce a cure in the individual. Therefore, a small dose of the simillimum is sufficient in producing a cure. This process requires patience and compliance from the patient. The dose may be repeated, depending on the patient's response to the remedy administered.

2.1.4 The Law of Potentisation

According to Hahnemann, potentisation is a process whereby the mechanical action of trituration and succussion of the remedy causes an alteration to the medicinal substance which increases its effectiveness (Ive 2010). Hahnemann noticed that by vigorously shaking or succussing a diluted remedy before administering it to a patient, it not only decreased the toxic effects of the substance, but also increased the curative effects of the remedy that was administered, thereby making it more potent (Kayne 2006) . Therefore, contrary to popular belief, diluting a Homoeopathic remedy does not minimise its curative effects but instead, it minimises the aggressiveness of the remedy (Lessell and Greenwood 1994). This study aimed to compare different diluted preparations of a Homoeopathic remedy by determining the quantities of Berberine and Hydrastine that are present in these dilutions.

2.1.5 Le Chatelier's Principle

Le Chatelier's principle is used to further demonstrate The Law of Similars. Le Chatelier's principle of thermodynamics states: "*If a chemical system at equilibrium experiences a change in concentration, temperature or total pressure, the equilibrium will shift in order to minimize that change*." (Drofenik 2019).

The equation is as follows: [A] ↔ [B]

Where [A] is the concentration of the reactants and [B] is the concentration of the products. Increasing the concentration of reactants [A] will drive the reaction to the right, while increasing the concentration of products [B] will drive the reaction to the left, in order to achieve equilibrium.

K=[B]/[A] where the equilibrium constant (Kc) is the ratio between the concentration of products and reactants in a chemical reaction (Siyavula 2015a).

The human body is viewed as a biochemical system. Each cell in the human body is linked to form a system. Biochemical reactions occur within each cell, thereby ensuring a normal functioning system (Drofenik 2019). This system must also be in a state of equilibrium and this is demonstrated when the body is in a healthy state and the cellular function occurs as it is supposed to. An ill patient shifts from their state of equilibrium into a state of disturbance. According to Homoeopathic principles, the similimum - a remedy whose substance causes the same harmful substance in a healthy individual but elicits cure when introduced to a diseased system in a diluted form - causes the system to shift in the direction appropriate in order to achieve equilibrium (Drofenik 2019). In this case, the vital molecules of the body will be [A] and

the ingested remedy will be [B]. Since [A] ↔ [B], this reversible chemical reaction implies that the ingested remedy causes an increase in [B], the biochemical system will shift in the direction that counteracts that change, thereby resulting in the system decreasing [B] but increasing [A] or the reverse reaction. Hence, an increase in [A] which is an increase in the vital molecules of the body will ultimately result in a healthier individual.

2.2 Mother Tincture

A mother tincture is the initial preparation of a homoeopathic remedy, consisting of the crude substance of a source material that is dissolved in alcohol and water (Banerjee 2002). The mother tincture is a 1:10 diluted preparation of a remedy, which is used to prepare subsequent diluted preparations of the same remedy, namely 1:2, 1:3 and 1:5 diluted preparations.

The source material of Homoeopathic mother tinctures is commonly derived from plants, animals or minerals (Ahmad 2017). With regards to plant-sourced Homoeopathic medication, the entire plant or only certain parts of the plant will be used to prepare the Homoeopathic remedy, depending on what is stated in that plant's monograph for the remedy that is being prepared (World Health Organization 2009). Fresh or dried plant material, depending on the type stipulated in the plant's monograph, is dissolved in water and alcohol to prepare a liquid Homoeopathic remedy (Brighton 2013).

The use of solvents, namely water and alcohol, allows for maximum solubility of the plant material. From the aforementioned solvents, alcohol, particularly ethanol, allows for maximum extraction of the plant material, thereby ensuring that the desired alkaloids are present in the homoeopathic remedy (Kayne 2006). Ethanol is used as a solvent in different strengths, such as 30%, 60% or 90%. According to a study done by Meier (cited in Bone (2003), the optimal strengths of ethanol that are used for extractions of phytochemicals are between 40% and 60%. Additionally, ethanol's denaturing properties allow for it to break through cell walls of plant material, thereby extracting the active ingredients from the plant (Last 2020). Alcohol has bactericidal properties which also preserve the mother tincture, so that it may be used to create other diluted preparations of the same remedy. Moreover, when the medicine is dispensed to a patient, he or she is able to take the medication for a longer period of time without the remedy expiring.

A mother tincture is either prepared through maceration or percolation of the plant material (Bone 2003). Depending on the remedy that is being produced, either the entire plant will be used or only a certain part or parts of the plant, such as the leaves, stems or roots. In the case of *Hydrastis canadensis* mother tincture, high concentrations of the two alkaloids, Hydrastine and Berberine are found in the roots and rhizomes of the plant (Douglas *et al.* 2013). As a result, the roots and rhizomes of *Hydrastis canadensis* are commonly used for their medicinal effects as an antimicrobial, particularly as an antibacterial.

Maceration is the process whereby the plant material is cut into small pieces or ground using a mortar and pestle, so that it can be easily dissolved in a mixture of water and alcohol (Banerjee 2002). This plant and liquid solution is placed into a glass jar which is sealed and left to stand for a period of time, so that the active ingredients from the plant may be extracted. This process is conducted at room temperature. The glass jar is shaken daily to allow for the contents to be dispersed within the jar. After five days, this plant material is strained and the resulting liquid from the extraction process is known as the mother tincture (Shane 2017). Marc is the residue remaining from the extraction process. The marc is pressed further to ensure all remaining liquid is extracted and this is mixed with the previously strained liquid. This is left to rest until the new solution becomes clear.

Percolation is the process whereby the herb is ground to a fine material and then moistened with ethanol (Shane 2017). It is then left to sit for a period of time, to absorb the solvent and to allow the moistened, ground herb to expand. It is then left to stand for a few days. The moistened herb is further sifted and thereafter, it is packed into a cone or a percolator. Ethanol is added to the herb that is in the percolator and the herb is pressed to extract more liquid which is then mixed with the extracted liquid and this allows for more of the solution to be extracted from the herb (Bone 2003). This process is conducted at room temperature.

Homoeopathic mother tinctures are prepared according to different methods of preparation, as set out in the different Pharmacopoeias. A Homoeopathic Pharmacopoeia is a legal manual that provides the standard preparations, formulae and description of remedies (Das 2016). It provides the description of the remedy or drug substance as accepted in the current medical practice. Within a Pharmacopoeia, each remedy has its own unique monograph which stipulates its method of preparation. A monograph is a description of the formula for preparing a herbal or homoeopathic medicine (World Health Organization 2019). Monographs may include the active constituents in the remedy, the method of relating the purity and the main chemicals that may be used to identify the remedy or drug substance (World Health Organization 2019). Although Pharmacopoeias contain monographs for the preparation of medicines, some monographs may also appear independently.

The two main preparation methods of preparing Homoeopathic mother tinctures are according to the German Homoeopathic Pharmacopoeia and the French Homoeopathic Pharmacopoeia. The German method of Homoeopathic remedy preparation utilises dried plant material (Benyunes 2005), whereas the French method utilises fresh or dried plant material. The French method of preparation is more time consuming as the plant material must be left to sit for three weeks, whereas the German method of preparation only requires the plant and alcohol mixture to sit for approximately ten days before proceeding to prepare the remedy (Kayne 2006). Depending on which Homoeopharmaceutical company is preparing the remedy, either the French of German method may be used.

2.2.1 Hahnemanian Preparation of a Mother Tincture

According to Aphorism 271 of the Organon of Medicine, Hahnemann prepared plant-sourced Mother tinctures from fresh plant material as they were more superior to dry plants (Hahnemann 2002). This was because the medicinal properties remained intact in the fresh plant. Hahnemann classified plants into different groups, depending on the water content of the plant. Class one contains the highest content of water in the plants, class two includes medium juicy plants, class three includes the least amount of water content in plants and class four includes dry plants, herbs and dried or fresh animal substances (Hahnemann 2002).

Hahnemann prepared the Mother tinctures of plant sourced remedies according to their classification. Mother tinctures using class one plants are prepared with equal parts of plant juice and alcohol, calculated by weight (Banerjee 2002: 183). The fresh plant material is chopped up and covered in a piece of cloth, which is used to squeeze out the extra water content or juice from the plant material. This juice is quantified and mixed with an equal part of alcohol and bottled. This mixture is left to stand in a cool and dark place for eight days, and then filtered thereafter. For class two drugs, Hahnemann weighed the finely chopped fresh plant and added two parts of alcohol to every three parts of weighed plant material (Banerjee 2002). The chopped plant is moistened with alcohol until the plant material forms a thick pulp and stirred. The remaining alcohol is added to the pulp, which is then strained, bottled and left to stand in a cool, dark place for eight days. For class three plants, tinctures are prepared

using one part of plant to two parts of alcohol by weight (Kumar 2014). The plant is chopped up into a fine pulp and weighed. From the two parts of alcohol that were weighed, one sixth of that is used to mix and moisten the pulp and thereafter, the balance of the alcohol is added to the pulp. The mixture is stirred and bottled and left to stand in a cool and dark place for eight days. After this time, the mixture or tincture is strained and filtered. Class four plant-sourced mother tinctures are prepared using one part of plant material to five parts of alcohol, calculated by weight. One part of the crushed plant material is weighed, and five parts of alcohol is measured and poured onto the plant material. Thereafter, this plant and alcohol mixture is left to stand in a glass bottle, in a dark room of ambient temperature, for eight days. During this period, the mixture is shaken twice daily. At the end of the eight-day period, the mixture is strained and filtered.

2.2.2 Standardisation of Homoeopathic Remedies

According to the World Health Organisation, approximately eighty percent of the world relies on herbal medication, with Homoeopathy being the most commonly used form of it (Das 2016). The global market is a concept that has allowed for different Homoeopathic remedies to be produced in different parts of the world, regardless of where the raw materials for the remedy are found, be it plant or animal sources (World Health Organization 2009). This is advantageous to Homoeopathic practitioners worldwide as they are not limited to remedies whose source materials are only present in their region. An example is this research study which uses *Hydrastis canadensis*, a plant that is native to North America, which was needed to be produced in South Africa

and was therefore ordered by a Pharmaceutical company in South Africa and transported under optimal conditions. Therefore, raw materials for remedies are sourced from different parts of the world. Various different companies prepare medicines, and this increases the need for systematic methods of preparation. It is also important to ensure that the raw materials obtained are safe and free of contaminants and toxic substances.

Although high potencies of Homoeopathic medicines are diluted beyond Avogadro's constant, thereby eliminating the presence of the raw materials in the remedy (World Health Organization 2009), it is still crucial to ensure that these materials are pure, non-contaminated and the remedies prepared are according to Good Manufacturing Practice (GMP) guidelines. GMP guidelines ensure that the entire process of remedy production, including obtaining the raw materials, cleanliness of staff that are preparing the remedies, sanitation of the premises and utensils with which remedies are produced, meticulous and clear labelling of remedies, safe packaging and efficient transportation of remedies under good environmental conditions (Kunle, Egharevba and Ahmadu 2012). These factors are crucial in preserving the integrity of the remedy so that it is safe and effective when it is consumed by patients (World Health Organization 2009). Furthermore, there is the element of accountability where the batch description of the remedy should include the toxic content of the plant material where applicable.

Quality Control (QC) is an important aspect in the preparation of Homoeopathic medication. Homoeopathic remedies are consumed by the public on a daily basis, for acute and chronic illnesses, and consumers deserve to know the quality of the product or medication that they are purchasing (World Health Organization 2009). Regulatory authorities set out guidelines for medication which ensure that consumers are purchasing medications that are safe, pure, potent and effective. QC ensures that the standardisation of a remedy is achieved. Standardisation is the process of laying down uniform guidelines with quantitative and qualitative specifications for the production of a Homoeopathic remedy, as well as clear specifications of what the remedy contains. This method of uniformity ensures that the product or remedy produced is of high quality and that the method of preparation that is set out is safe, effective, practical and most importantly reproducible (Kunle, Egharevba and Ahmadu 2012). Therefore, this research supports the notion that following standardised procedures and guidelines for remedy preparation, produces products of acceptable quality (Kunle, Egharevba and Ahmadu 2012).

The process of Quality Control begins from the time the raw material is obtained until the medication is packaged and transported for consumption. Raw materials undergo tests and inspections to ensure that they are free of impurities or foreign matter, as well as identification tests to ensure that the correct type and part of the source material are used (Kayne 2006). Plant materials are the most common source of Homoeopathic medication. The entire plant, or only certain parts of the plant will be used to prepare the Homoeopathic remedy, depending on what is stated in that plant's monograph for the remedy that is being prepared.

Another integral part of Quality Control for the preparation of plant-sourced or Herbal preparations is Good Agricultural Practices (GAP). This includes the quality of seeds that will be used, sewing them into fertile soil of an optimal pH, the growth conditions in which the seeds are grown, the possible use of a Green House to control temperature and environmental conditions, water availability for the soil, the time of day and time of year at which plants are harvested and the manner in which the plant is stored thereafter (Kunle, Egharevba and Ahmadu 2012). The purity of the plant is also crucial to the preparation of these remedies. Plant materials are often sprayed with pesticides or may affected by the presence of heavy metals, such as lead and mercury which is caused by pollution. This could contaminate the starting material and compromise the safety of the remedy which it is used to make (Malik *et al.* 2013). There are various methods that are used for identifying the presence of heavy metal contamination in plants, one of which is atomic absorption spectrophotometry (Watson 1999, cited in Kunle, Egharevba and Ahmadu (2012).

Furthermore, correct guidelines must be adhered to when preparing the remedy. The water content of the plant is also taken into account as this could affect the preparation, depending on which Pharmacopoeia is followed for the preparation. The preparation of each Homoeopathic remedy is specified in monographs of different Pharmacopoeias that are available. By following this uniform procedure when preparing Homoeopathic remedies, it ensures standardisation of remedies regardless of where in the world it is being prepared (World Health Organization 2019). This

research aimed to investigate this aspect of Quality Control and standardisation by comparing 1:3 and 1:10 diluted preparations of the same remedy, *Hydrastis canadensis*, as well comparing the same dilution, that is 1:10 dilutions, that were prepared by the Researcher and by a Homoeopharmaceutical company, both of whom followed standard procedures.

The handling of source material, in the case of this research plant-sources, is another important factor that could affect the end product or the remedy. Materials require gentleness and care when handled. Additionally, storage and transportation of raw materials require optimal and cool temperatures, to prevent damaging or denaturing active components in the source material (MediHerb 2020). These are often expensive methods that must be implemented in order to avoid compromising the quality of the remedies that are produced from the source materials.

2.3 *Hydrastis canadensis*

2.3.1 Description and Taxonomy

Hydrastis canadensis, also known as Goldenseal, is a herbaceous perennial plant that is between 20-50cm tall (USDA Forest Service 2003). *Hydrastis canadensis* is known by various other names including Indian dye, jaundice root, ground raspberry, yellow root, eye balm, orange root and yellow puccoon (Asmi and Lakshmi 2013).The plant grows in moist soil, along riverbanks and in open woodlands (Pengelly *et al.* 2012). The natural habitat of *Hydrastis canadensis* is the United States and Canada. This

plant is part of the *Ranunculaceae* family (Pengelly *et al.* 2012). The plant species blooms between April and May and its fruit matures between July and August.

Herbaceous perennial plants have soft, green stems instead of woody stems and they die each winter and regrow during spring and summer. *Hydrastis canadensis* has yellow rhizomes that are 0.5-0.75 inches in thickness, which give rise to hairy stems. The rhizomes are covered with thin, yellow roots to which the term Goldenseal owes its name (Asmi and Lakshmi 2013). The roots and rhizomes of *Hydrastis canadensis* are of particular importance since they contain the major alkaloids of the plant, which is discussed later on in this study (USDA Forest Service 2003). Additionally, the roots and rhizomes produce a yellow-orange juice which contains the active ingredients of this plant (Mahady *et al.* 2003). The yellow, cylindrical stems of the plant give rise to dark green, palmate leaves. The flowers of the plant consist of five sepals, stamens and five yellow sepals (Asmi and Lakshmi 2013). A shiny, berry-like fruit containing 10-20 small seeds develops from the flower head. The fruit ripens in Summer (July-August) and is red in colour when it has matured (Zuiderveen 2019). The plant has an acidic to bitter taste, the latter of which causes salivation when the plant is ingested.

There are twelve known alkaloids in *Hydrastis canadensis* but Hydrastine, Berberine and to some extent Canadine were shown to exhibit the highest alkaloid concentrations in this plant (Chadwick, Wu and Kinghorn 2001). However, the purpose of this research focused on the two more common alkaloids, Hydrastine and Berberine.

Figure 2-1 *Hydrastis canadensis*

Green palm-like leaves, yellow rhizomes, thin roots, plants, red berry fruit and seeds. *Koehler's Medicinal-Plants (1887)* (cited in International Agency for Research on Cancer (IARC) (2018).

Table 2-1 Isoquinoline alkaloids from *Hydrastis canadensis*

Table depicting the Isoquinoline alkaloids (cited in Pengelly *et al.* (2012).

Major Alkaloids	**Minor Alkaloids**
Berberine	Hydrastinine
(—)-(β)-hydrastine	Canadaline
(—)-canadine	Isohydrastidine
	1-β-hydrastine
	5-hydroxytetrahydroberberine
	(S)-corypalmine

	(S)-isocorypalmine
	(S)-tetrahydropalmatine
	Berberastine
	8-oxotetrahydrothalifendine
	Canadinic acid

2.3.2 Conservational Concerns for *Hydrastis canadensis*

Hydrastis canadensis is becoming increasingly difficult to obtain since it is a slow-growing plant that is at a risk of being over-harvested for its medicinal properties (Gagnon 1999; Lubbe and Verpoorte 2011, cited in Zuiderveen (2019).This remains the main threat since the presence of its alkaloids prevents consumption of the plant by herbivorous creatures. In a study conducted by Sinclair & Catling (2001) (cited in Douglas *et al.* (2013), the optimal growth for *Hydrastis canadensis* is in shady conditions. Additionally, the study cited in Douglas *et al.* (2013) revealed that the plant grew best in moist soil with a pH of between 5.7 and 6.3. The conservational concerns for this plant affect its availability of various potencies. Hence, this research was conducted using the most commonly available and commonly used potencies of *Hydrastis canadensis*, which are 1:3 and 1:10 dilutions.

2.3.3 Toxicology of *Hydrastis canadensis*

The two main alkaloids that are present in *Hydrastis canadensis*, are Hydrastine (1.5% – 4 %) and Berberine (2.5%) (Pengelly *et al.* 2012). If *Hydrastis canadensis* is administered in large doses, these alkaloids can produce toxic effects which include vomiting, digestive disturbances, contractions of the uterus, hallucinations and delirium (van Wyk and Wink 2015). The benefit of Homoeopathic dilutions, is that since it decreases the concentrations of the active ingredients in the remedy, toxicological effects are not very common when administering a Homoeopathic remedy.

A comparative study on the toxicities of *Hydrastis Canadensis L., Berberis aristata DC. and Achillea millefolium L*. against Brine Shrimps depicted that *Hydrastis canadensis* was the most toxic when used in high doses (Karim *et al.* 2015). Despite Berberine's ability to exhibit phototoxic properties, *Berberis aristata* which contains Berberine, was shown to be safer than *Hydrastis canadensis*. Furthermore, this study was in agreement with a previous study conducted by Dunnick *et al.* (2011) (cited in Karim *et al.* (2015) which showed that *Hydrastis canadensis* exhibited tumorigenic properties, or the ability to form tumours in male and female mice that were used as study subjects.

It has been shown by Inbaraj *et al.* (2001) that exposure to Ultraviolet A (UVA) from light sources whilst using topical products containing *Hydrastis canadensis*, particularly the use of Berberine, caused DNA damage and cell death (or a decrease in viability) of HaCaT keratinocytes. HaCaT cells are immortalised cells (Gabbott and

Sun 2018). These cells are genetically engineered from human cells, for the purpose of research. Therefore, patients using topical products containing *Hydrastis canadensis* would be advised against direct sun exposure and encouraged to take precautions when there is a risk of sun exposure.

Furthermore, another study was later conducted by Inbaraj *et al.* (2006), wherein Berberine was shown to be more phototoxic than the other alkaloids present in *Hydrastis canadensis*, when administered at concentrations of 20 *μ*M and below. The wavelength of sunlight is 290 nm (Inbaraj *et al.* 2006). This study also showed that when it was exposed to Ultraviolet A radiation, Berberine which has the highest concentration in *Hydrastis canadensis,* caused DNA damage in keratinocytes (Inbaraj *et al.* 2006). On the contrary, although high concentrations of Hydrastine were present in *Hydrastis canadensis*, this alkaloid did not cause photodamage to keratinocytes. In a study that was conducted by Chignell et al. (2007), it was shown that Berberine has a considerable absorption only above 400 nm. Wavelengths that are greater than 400 nm penetrate the retina, causing phototoxicity to retinal cells. Hence, Berberine has the potential to damage the retina (Chignell *et al.* 2007).

2.3.4 Homoeopathic Uses of *Hydrastis canadensis*

In Homoeopathy, there are different pharmaceutical methods used to prepare the mother tincture of *Hydrastis canadensis,* which is used widely for its antimicrobial and anti-bacterial effects, especially in Homoeopathic community Healthcare Centres in South Africa. Some methods of Homoeopathic preparations include a 1:2 diluted

preparation, a 1:5 diluted preparation and a 1:10 diluted preparation, according to most standard Homoeopathic tincture productions. Homoeopathic Pharmacopoeias utilise 1:2 and a 1:10 dilutions.

There has been much debate over the years about which method of preparation is more potent in terms of its curative effects. Furthermore, there has been insufficient evidence regarding the quantities of active ingredients present in different homoeopathic dilutions of remedies. Therefore, this study aimed to determine which method of preparation yielded a higher quantity of Berberine and Hydrastine and whether the current Homoeopathic use of this remedy, according to the end quantity of these alkaloids, correlated with the Homoeopathic remedy's current uses. Additionally, the research aimed to determine the comparability of the different concentrations. This also allowed the researcher to establish whether there is a difference in the amount of Hydrastine and Berberine present in the end product of the different pharmaceutical preparations of *Hydrastis canadensis.*

Hydrastis canadensis is used as an antimicrobial and antibacterial treatment. This is due to the plant's concentrations of Berberine (up to 6%) and Hydrastine (1.5-4%) (van Wyk and Wink 2015). In an in vitro study conducted by (Budree 2004), *Hydrastis canadensis* 62% v/v tincture was shown to be effective in inhibiting the growth *Candida albicans*. Additionally, *Hydrastis canadensis* was shown to be more effective against *Candida albicans* in vitro than Fluconazole was.

Hydrastis canadensis is dispensed in Homoeopathic community Healthcare Centres in South Africa, for the treatment of upper respiratory tract infections (2019, pers. comm. Dr I Couchman. 21 January 2019). It is used in the treatment of liver disorders, where it is known to improve the liver function and promote bile flow (Potter 1906, cited in Pengelly *et al.* (2012). Bile, which is produced by the liver and stored in the gallbladder, is a fluid that assists with the digestion of fats by breaking them down into fatty acids. Hence, *Hydrastis canadensis* is used as a digestive tonic (Communications 2000). This is due to the bitter constituents in the roots and rhizomes of *Hydrastis canadensis,* as bitterness is known to assist in digestion (Mills and Bone 2000). Furthermore, due to the digestive properties of *Hydrastis canadensis*, the remedy also assists in relieving chronic constipation and haemorrhoids (Communications 2000).

Hydrastis canadensis is also used as an antidiarrheal in the treatment of bacterial infections like gastritis. *E.coli* bacteria is the main cause of this gastric disturbances. A study that was cited in Mills and Bone (2000) showed that >25 mg/kg of orally ingested Berberine in combination with Geranium extract inhibited peristalsis in rats' intestines. Furthermore, oral administration of 0.1 mg of Berberine in combination with *E.coli* enterotoxin was effective in the treatment of *E.coli* induced intestinal fluid accumulation. Reduction of intestinal fluid accumulation as depicted in the study, results in decreased water in stools which is a mechanism of treating diarrhoea. Therefore, quantifying the alkaloids Hydrastine and Berberine that are present in diluted preparations of *Hydrastis canadensis*, allowed the researcher to determine whether these diluted preparations are viable for future Homoeopathic use at free community Healthcare Centres in Durban, in order to save costs for medication.

2.3.5 Therapeutic Uses of *Hydrastis canadensis*

For the purpose of this research, it has been stated that the therapeutic uses of *Hydrastis canadensis* stems from the action of its two main alkaloids, Hydrastine and Berberine. Therefore, the description of the therapeutic uses will be done according to the aforementioned alkaloids.

2.3.6 Pharmacological Actions of *Hydrastis canadensis*

2.3.6.1 Antibacterial Activity

According to Hamon's 1990 study (cited in Pengelly *et al.* (2012), *Hydrastis canadensis* was first used by Native Americans to treat wounds, ulcers, digestive disorders and skin and eye ailments. The root and rhizomes of the plant are mostly used for their medicinal effects (Douglas *et al.* 2013). The presence of the two main alkaloids for which *Hydrastis canadensis* is used, is Hydrastine (1.5% – 4 %) and Berberine (2.5%), the quantities of which are recommended by The British Herbal Compendium (Pengelly *et al.* 2012). There are high concentrations of these alkaloids in the roots and rhizomes of *Hydrastis canadensis* Douglas *et al.* (2013); hence these parts of the plant are commonly used for their medicinal effects as an antimicrobial, particularly an antibacterial.

In order to provide evidence for the antibacterial use of *Hydrastis canadensis,* a study was conducted by Scazzocchio *et al.* (2001) to test the antibacterial activity of major alkaloids, including Berberine and Hydrastine, against gram-positive and gram-

negative bacteria, yeast and protozoa. This was done using various strains of microorganisms including *Staphylococcus aureus, Streptococcus sanguis, Escherichia coli and Pseudomonas aeruginosa*. The bactericidal and bacteriostatic activity of the alkaloids were evaluated; the former was achieved by measuring the amount of time taken for the alkaloids to kill a bacterial inoculum, and the latter by measuring the minimum concentration of alkaloids required to inhibit bacterial activity. The results of the experiment validated the bactericidal activity of *Hydrastis canadensis.*

Gentry *et al.* (1998) showed that *Hydrastis canadensis* has the ability to combat Multidrug-Resistant (MDR) strains of *Mycobacterium tuberculosis* and other *Mycobacterium* species. Junio *et al.* (2011) showed that Berberine was proven to inhibit the growth of *Staphylococcus aureus*. Although Berberine alone has a weak antibacterial activity, when the leaves of the plant are also used in the production of remedies, it increases the effectiveness of the antibacterial activity. Therefore, it is used in modern Phytotherapy in the treatment of respiratory tract infections, which includes whooping cough and pneumonia. Additionally, it is particularly used in the treatment of Tuberculosis because of its anti-tubercular and immuno-stimulant properties.

A study that was conducted by Mahady *et al.* (2003), showed that Berberine and Hydrastine that had been extracted from *Hydrastis canadensis* were shown to inhibit the growth of *Helicobacter pylori* (*H. Pylori)* bacteria *in vitro.* Additionally, crude extracts from the roots and rhizomes of *Hydrastis canadensis* were more effective in inhibiting the growth of *H. Pylori* bacteria than that of *Sanguinaria canadensis.*

According to D'Amico (1950) and Hocking (1977) (cited in Mahady *et al.* (2003), crude extracts from the rhizomes of *Hydrastis canadensis* have been shown to inhibit the growth of *Staphylococcus aureus* and *E. coli in vitro*.

Additional Phyto-therapeutic uses of *Hydrastis canadensis* include an anti-haemorrhagic, which is used to stop bleeding, a digestive stimulant due to its bitter taste and a mild laxative (van Wyk and Wink 2015). Furthermore, *Hydrastis canadensis* is used as part of Western herbal medicine, in the treatment of peptic and duodenal ulcers, gastritis, dyspepsia, colitis, skin disorders, menorrhagia, dysmenorrhea and inflammation of the mucous membranes (Mills *et al.* 2004). Traditionally, *Hydrastis canadensis* is also used to treat skin, mouth and eye infections (Hwang *et al.* 2003).

Extracts of *Hydrastis canadensis* are known to be present in oral washes and toothpastes. In order to investigate this further, a study was conducted by (Hwang *et al.* 2003) to determine the antimicrobial activity of *Hydrastis canadensis* against two oral pathogens, *Streptococcus mutans* and *Fusobacterium nucleatum*. The method of percolation was used to prepare a *Hydrastis canadensis* tincture. The rhizome of the plant was ground into a powder and methanol was used in the preparation of the tincture. The results of this study concluded that from all the alkaloids present in *Hydrastis canadensis*, Berberine is most effective in inhibiting the antimicrobial activity of the two pathogens. Additionally, according to Anders *et al*. (1999) (cited in Maharaj (2006), since *Hydrastis canadensis* is known to promote healing of the mucous membranes, the tincture is applied topically in the treatment of mouth ulcers.

Rehman *et al.* (1999) carried out an *in vivo* investigation in order to determine the antigen-specific immunomodulatory effects of *Hydrastis canadensis* and *Echinacea angustifolia* in the bodies of research subjects (rats). *Hydrastis canadensis* exhibited immunomodulatory effects by stimulating the production of Immunoglobin M (IgM) antibodies in rats within the first two weeks of treatment. The IgM antibody is produced in response to a new antigen that enters the body and causes an infection. This justifies the use of *Hydrastis canadensis* for viral and bacterial infections, including Influenza or the common cold.

2.3.6.2 Antiparasitic Activity

Hydrastis canadensis inhibits the growth of *Entamoeba histolytica, Trichomonas vaginalis, Leishmania donovani* and *Giardia lamblia* (Asmi and Lakshmi 2013). *Giardia lamblia* causes the parasitic infection, giardiasis, which is a disease that is associated with poor quality of drinking water, and is common in tropical regions. Giardiasis is contracted by swallowing water that has been contaminated by this parasite, contact with people who have giardiasis, touching surfaces that are contaminated with faeces of an infected person or animal and ingesting uncooked food that is infested with *Giardia lamblia* (Centers for Disease Control and Prevention - CDC 2015). This disease commonly presents with symptoms of diarrhoea, malabsorption, weight loss, bloating and fatigue (Cernikova, Faso and Hehl 2018).

2.3.7 Contraindications of *Hydrastis canadensis*

Hydrastis canadensis is contraindicated in hypertensive conditions (Mills and Bone 2000). This is probably due to the vasoconstrictive effects of the alkaloid Hydrastine

that is present in the remedy. The remedy is also contraindicated in pregnancy as well as in jaundiced neonates, the latter is due to its effects in increasing bilirubin secretion.

2.4 Active Ingredients or Isoquinoline Alkaloids

Isoquinoline Alkaloids or active ingredients are naturally occurring substances that are derived from flowering plants, microorganisms, animals, cooked food and beverage products (Diaz, Miranda and Diaz 2015). They are known as phytochemicals and display pharmacological and medicinal effects on humans and animals. Alkaloids play an important role in the defence system against pathogens and animals. Hence, plant sourced Homoeopathic remedies take into account the isoquinoline alkaloids of the plant and their therapeutic effects. Some common alkaloids besides Berberine and Hydrastine which do not pertain to this research are papaverine, morphine and atropine. Different alkaloids cause different reactions in the nervous system. Some incite defensive reactions, whilst others incite therapeutic effects in the organism. For the purpose of this research, isoquinoline alkaloids are also referred to as active ingredients.

2.4.1 Berberine

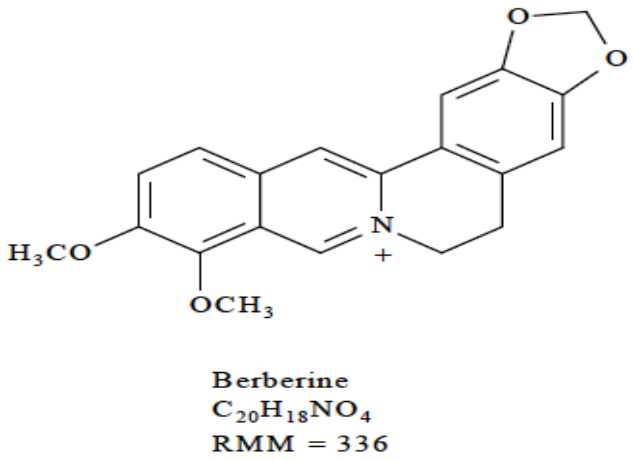


Figure 2-2 Chemical structure of Berberine(Pengelly *et al.* 2012).

Berberine is a yellow isoquinoline alkaloid that occurs in numerous plants, namely *Hydrastis canadensis* (Goldenseal), *Berberis vulgaris* (Barberry), *Coptis chinensis* (Chinese Goldthread), *Berberis aquifolium* (Oregon grape) (Schor 2012). As discussed earlier in this chapter, the recommended quantity of Berberine for its therapeutic effects is 2.5% (Pengelly *et al.* 2012). Berberine's various therapeutic effects will be further discussed in the headings that follow.

2.4.1.1 Therapeutic Uses of *Hydrastis canadensis* Stemming from Berberine

Junio *et al.* (2011) showed that Berberine was proven to inhibit the growth of *Staphylococcus aureus*. Although Berberine alone has a weak antibacterial activity,

when the leaves of the plant are also used in the production of remedies, it increases the effectiveness of the antibacterial activity. For an in-depth review of the therapeutic uses, please see section 2.3.6 Pharmacological actions of Hydrastis canadensis, under the heading 2.3.6.1 Antibacterial Activity.

2.4.1.2 Anti-carcinogenic Effects of Berberine

Berberine displayed anti-cancer properties and can be used be used in the form of palliative treatment for colon cancer. An enzyme called Cyclooxygenase-2 (COX-2) that is present in colon cancer cells. The COX-2 enzyme was consequently known to increase the production of cancer cells in the colon. In a study conducted by Fukuda *et al.* (1999), Berberine was shown to have the ability to suppress the activity of the COX-2 gene, thereby indicating the anti-tumour effects of this alkaloid.

2.4.1.3 Anti-oxidant and Anti-Inflammatory Effects of Berberine in Diabetes Mellitus type 2

According to a study conducted by Li *et al.* (2014), Berberine exhibited anti-oxidant and anti-inflammatory effects when it was administered to diabetic animals. Berberine was shown to decrease the amount of oxidative stress in various different organs of these animals. This was noted by changes in oxidative stress markers. Furthermore, since there is a direct link between oxidative stress, inflammation and Diabetes Mellitus Type 2 with insulin resistance, it was deduced that Berberine can be therapeutically effective in the treatment of insulin resistance and Diabetes Mellitus.

In a study that was conducted by Liu *et al*. (2008) (cited in Kulkarni and Dhir (2010), Berberine was found to be effective in the treatment of diabetic nephropathy in rats. Diabetic nephropathy is a complication of Diabetes mellitus which causes damage to the kidneys, as a result of the excess levels of glucose circulating in the blood.

2.4.1.4 Berberine's Anti-bacterial and Anti-fungal Activity

Hydrastis canadensis has been used by the Native American Indians in the treatment of stomach related illnesses, namely gastritis, dyspepsia and indigestion (Mahady *et al.* 2003). Furthermore, it is also used in the treatment of peptic ulcers and colitis (Mills *et al.* 2004). A study that was conducted by Mahady *et al.* (2003) showed that Berberine and Hydrastine that had been extracted from *Hydrastis canadensis* were shown to inhibit the growth of *Helicobacter pylori* (*H. Pylori)* bacteria in vitro. However, Berberine was more effective since it was shown to inhibit all fifteen strains of *H. Pylori. H. Pylori* is the organism that is responsible for the development of gastric related diseases (Bik *et al.* 2006).

Junio *et al.* (2011) showed that Berberine was proven to inhibit the growth of *Staphylococcus aureus*. Although Berberine alone has a weak antibacterial activity, when the leaves of the plant are also used in the production of remedies, it increases the effectiveness of the antibacterial activity. Therefore, it is used in modern Phytotherapy in the treatment of respiratory tract infections, which include whooping cough and pneumonia. Additionally, it is particularly used in the treatment of Tuberculosis because of its anti-tubercular and immuno-stimulant properties. The

Pharmacological actions of Berberine in *Hydrastis canadensis* are discussed in more detail under the heading 2.3.6.1 Antibacterial activity.

Gentry *et al.* (1998) showed that *Hydrastis canadensis* has the ability to combat Multidrug-Resistant (MDR) strains of *Mycobacterium tuberculosis* and other *Mycobacterium* species.

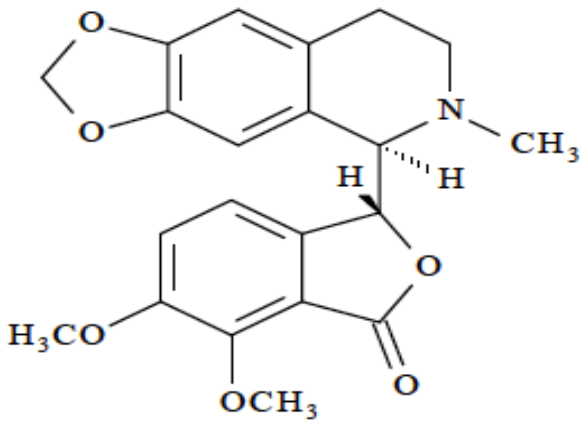
Berberine was also shown to be toxic against certain species of bacteria and fungi. In studies that were conducted *in vitro* by D'Amico (1950) and Hocking (1977) (cited in Mahady *et al.* (2003), Berberine that was extracted from the rhizomes of *Hydrastis canadensis*, was shown to inhibit the growth of *Staphylococcus aureus* and *E. coli.*

2.4.1.5 Anti-ischemic and Cardiovascular Effects of Berberine

Wu and Liu's 1995 study (cited in Mills and Bone (2000) showed that Berberine displayed anti-ischemic properties in rats with reversible cerebral artery occlusion. The rats that were administered with a dose of Berberine displayed a decrease in platelet aggregation and a reduction in Thromboxane $B_{2,}$ which causes vasoconstriction and facilitates platelet aggregation. These factors contribute to thrombus formation, which consequently result in artery occlusions and ischemia. Therefore, Berberine administration resulted in a combined decrease in these thrombus-formation factors, thereby displaying anti-ischemic effects in arteries.

A clinical trial that was conducted by Marin-Neto et al (1988) (cited in Asmi and Lakshmi (2013) showed that Berberine demonstrated ventricular antiarrhythmic activity in patients, particularly ischemia induced arrhythmia. Berberine was also shown to induce contractility of the heart (Asmi and Lakshmi 2013), thereby improving cardiac function and also decreasing blood pressure.

2.4.2 Hydrastine



Hydrastine
$C_{21}H_{21}NO_6$
RMM = 383

Figure 2-3 Chemical structure of Hydrastine (Pengelly *et al.* 2012).

Hydrastine is used as an astringent, vasoconstrictor and a uterine stimulant (Chadwick, Wu and Kinghorn 2001). This alkaloid is known to have antibacterial and choleretic effects. The latter indicates that since Hydrastine is known to increase bile

flow in the liver, it may be used in the treatment in liver disorders. Additionally, Hydrastine has vasoconstrictive properties and can also be used as a sedative (Mills and Bone 2000). For an in depth review of the therapeutic uses of *Hydrastis canadensis*, please refer to the subheading, 2.3.5 Therapeutic Uses of *Hydrastis canadensis.*

2.5 Introduction to High Performance Liquid Chromatography (HPLC)

The term chromatography originated from the Greek word, ‘chroma’ meaning colour and ‘graphia’ meaning writing (Gault and McClenaghan 2013). In simpler terms, chromatography is the use of liquid or gas to separate components in a mixture, which are produced as coloured bands. These results are displayed on a computer and can be interpreted for qualitative and quantitative analysis, of individual constituents in a mixture. This is particularly useful in pharmaceutical companies where the ingredients of medications or chemical preparations want to be quantified or analysed (Neue and El Fallah 1997). HPLC was previously known as high-pressure liquid chromatography. This is a more accurate term as this method makes use of a column through which the solvent or sample mixture, in this case, *Hydrastis canadensis*, was pumped through at high pressure. This is known as the mobile phase of HPLC. The autosampler contains a pump that uses high pressure to move the liquid through the chamber, during the mobile phase, to the stationary phase (Gault and McClenaghan 2013).

High-performance liquid chromatography (or High-pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. It is a technique from analytical chemistry that physically separates liquid compounds of a dissolved mixture into individual components, in order to identify each component and quantify it (Carter 2017). HPLC mainly utilises a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interaction between the stationary phase, the molecules being analysed, and the solvent(s) used. The sample to be analysed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. The retention time is used to identify peaks on a chromatogram (Ahuja and Dong 2005).

Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile). Separation has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend

on the nature of the stationary phase and the analyte. HPLC is particularly suitable for the separation of compounds having one or more of the following characteristics: (a) high polarity (b) high molecular weight (c) thermal instability (d) a tendency to ionize in solution.

A liquid solvent containing the sample or mixture that needs to be separated, is pumped at a high pressure through a narrow stainless steel column that is packed with an adsorbent material. In this study, silica was the adsorbent that was packed into a C_{18} stainless steel column (2020, pers. comm. Dr V Mohanlall. February 2020). When the individual components of the sample mixture or analyte came into contact with the adsorbent in the column, they separate due to their varying degrees of interaction (Abourashed and Khan 2001). Therefore, HPLC allows molecules to be separated into their constituents within a reasonable timeframe of approximately forty minutes or less (Abourashed and Khan 2001). HPLC is a particularly suitable method for isolating chemical compounds as it allows for a high resolution and fairly quick analysis of results, which are displayed on a computer that is connected to the system (Gault and McClenaghan 2013)**.** Time is an important factor to consider when determining the practicality of a research project. This process was repeated three times in order to ensure the validity and accuracy of HPLC results. Therefore, this would have been an impractical and tiresome process if the method of conducting this practical was long and drawn out.

2.5.1 Types of HPLC

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified: adsorption chromatography, ion-exchange chromatography and size exclusion chromatography (Palamareva 2005).

2.5.1.1 Adsorption Chromatography

In adsorption chromatography the stationary phase is an adsorbent, such as silica gel, alumina or magnesium oxide or any other silica-based packings, and the separation is based on repeated adsorption-desorption steps (Meyer 2013). An adsorbent is a substance that is adsorbed or bound to a surface.

Two modes of adsorption chromatography are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography (Palamareva 2005).

2.5.1.2 Normal Phase Chromatography

In normal phase chromatography, the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials (Palamareva 2005).

2.5.1.3 Reverse Phase Chromatography

Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as

mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

2.5.1.4 Ion-Exchange Chromatography

In ion-exchange chromatography the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

2.5.1.5 Size Exclusion Chromatography

In size exclusion chromatography the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel".

2.5.2 Two Components formed the basis of the HPLC technique for this research:

- Mobile phase (solvent) - this phase is usually composed of a liquid or gas solvent. In this study, a liquid solvent was used (Coskun 2016).

- Stationary phase (silica) - silica, which is a solid, was packed into a C18 stainless steel column. Silica crystals are made to withstand high pressure. A C18 column is a steel column that is packed with a Carbon-18 substance which was used in the stationary phase, to analyse individual components in a mixture. A C18 column is non-polar but the substance that it was coated with, which is silica, is a polar molecule. The mechanism of separation of the *Hydrastis canadensis* was partition and adsorption. Adsorption is the adhesion or binding of liquid particles to the surface of the object. In this research, the liquid, which is the mother tincture of Hydrastis canadensis, will adhere to the silica-coated walls of the C18 column (Adamson and Gast 1967).

2.5.3 Principle of separation of chemical compounds

In this research, the various liquid samples which included different concentrations of pure Hydrastine and Berberine, the different diluted preparations of *Hydrastis canadensis* and 60% Alcohol, adhered to the silica-coated walls of the C_{18} column (Adamson and Gast 1967).

In adsorption chromatography, the liquid particles of the analyte bind to the surface of the silica-coated steel column. Additionally, alumina or magnesium oxide may also be used to separate compounds instead of silica (Forgács and Cserháti 1997).

The chemical properties of the individual components of the analyte affect the rates at which these components pass through the column in the mobile phase. Some components of the analytes adhere more strongly to the silica-coated walls of the C_{18} steel column, due to the intermolecular dipole-dipole forces that are present between molecules (Forgács and Cserháti 1997)**.** Hence, these components that display stronger adhesive properties pass through the column at a slower rate (Guerrero 2014). Since each component has different chemical properties, it passes through the column or the stationary phase at different times and this causes them to separate into individual components. This process of separating the solvent is called elution (Gault and McClenaghan 2013).

HPLC is currently one of the most accurate and comprehensive methods of quantifying and analysing components of a mixture. It widely used throughout pharmaceutical industries and yields reliable results. Therefore, it is often used in phytochemistry to isolate and identify chemical compounds in medicines and as a method of quality control to ensure that the isolated compounds are uncontaminated and pure (Gupta and Shanker). Although there are a few different active ingredients that are present in *Hydrastis canadensis,* research showed that Berberine and Hydrastine were the two main ingredients that yielded the curative results of the remedy. However, there was a lack of research to show whether the different diluted preparations of Homoeopathic remedies, especially *Hydrastis canadensis*, contained a sufficient concentration of these active ingredients. Hence, this research aimed to bridge that gap. The researcher was trained on the use of HPLC by the Biotechnology lab technician and Dr Viresh Mohanlall and also

received training on method development, data analysis and interpretation of results (2019, pers. comm. Dr V. Mohanlall. April 2019).

2.5.4 Components of an HPLC system

There are five basic components of an HPLC, which are shown below in Figure 2.4. Schematic diagram of a typical HPLC set up (IDEX Health & Science 2020). These are:

- Pumping system - This delivers the solvent from a reservoir, in the mobile phase, through a filter which pressurises the solvent so that it may pass through the HPLC column and into the sample injector (Gault and McClenaghan 2013).
- Injection system - This lies between the solvent pump and the HPLC column. It delivers the sample that is being analysed, which has been dissolved in a solvent, into the column.
- Column (Separating system) - A C_{18} stainless steel column is usually used. This tube is packed with materials that allow it to separate analytes, based on polarity.
- Detecting and Data collecting system - As the sample in the solvent is passed through the column, the analytes are separated according to their retention time. The solvent passes through the column into a detector (Gault and McClenaghan 2013). There are different types of detectors, but a UV detector is most commonly used. Different wavelengths are used to detect different types of samples, namely 280 nm for aromatic amino acids, 220 nm for specific

peptide bonds and 260 nm for nucleic acids. The HPLC system is linked to a computer, which receives and processes the data.

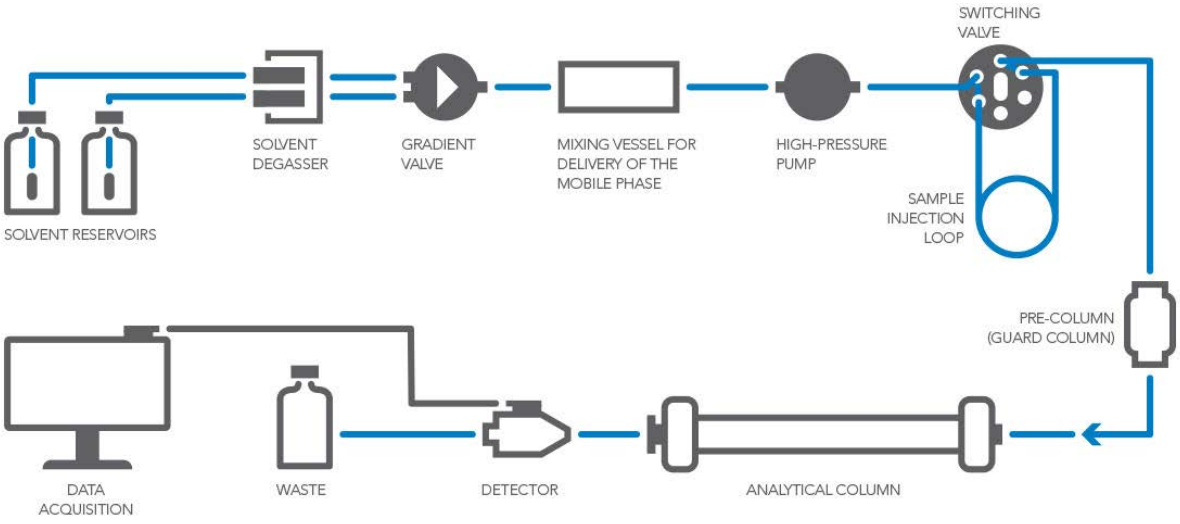


Figure 2-4 Schematic diagram of a typical HPLC set up (IDEX Health & Science 2020)

Figure 2-5 HPLC System (Shimadzu Group 2020b)

2.5.5 Le Chatelier's Principle and HPLC

According to Le Chatelier's principle, as discussed further under the heading 2.1.5, a system will shift in order to achieve equilibrium. pH is an important factor in maintaining the normal function of a system. The pH of a solution is regulated by a biochemical buffer, which resists internal and external changes and keeps the environment at a constant, so that biochemical reactions may occur efficiently. When a sample or solution is added to the mobile phase of the HPLC analysis, the pH is altered. In order to resist any changes in the pH and regulate it in order to achieve equilibrium, a buffer is added to the mobile phase to allow the analyst or researcher to maintain control over the sample being analysed (Anonymous 2014).

The pH of a solution describes how acidic or alkaline the solution is, where a low pH of 0 – 6.9 is Acidic, a pH of 7 is Neutral and a high pH of 7.1 – 14 is Basic (Siyavula 2015b). An acidic solution, with a low pH of less than 7 has a high concentration of hydrogen ions (H^+). Consequently, a basic solution with a pH of greater than 7.1 has a high concentration of hydroxide ions (OH^-). Hence, the pH of a solution (represents / is the measure of)* the concentration of hydrogen ions (H^+) in a solution. The pH scale has no unit of measurement. In HPLC, the pH of the mobile phase is important as it ensures efficient separation methods of analysed compounds, and because it protects the C_{18} column so that it may be reused multiple times.

A buffer solution is made up of a combination of weak acids and their salts (sodium salts, etc.) or of weak bases and their salts. Additionally, a buffer solution allows for good separation and reproducibility of the experiment. The most popular buffers for HPLC with UV detection are phosphate and acetate (Shimadzu Group 2020a)

Ionisation is the degree of charge of the substance. Ionised compounds are hydrophilic and consequently less lipophilic and therefore unable to pass through a non-polar lipid membrane easily (Hale and Abbey 2017). Ionised compounds elute at a faster rate from the HPLC column. The pH of the mobile phase determines whether the compounds that are being analysed are in an ionised or non-ionised form (Anonymous 2020). Moreover, non-ionised compounds are less polar, hydrophobic and lipophilic. They are strongly retained (in reverse phase) to the HPLC column. Acids are more strongly retained to the HPLC column at a low pH whereas bases are more strongly retained at a high pH (Dolan 2016). For the purpose of this research, the pH of the buffer, sodium acetate, was set to 4.8. Buffers maintain the pH of solutions when acids or bases/analytes are added. This decreased the retention time and peaks of Berberine and Hydrastine were seen in half the expected time. Furthermore, ammonium acetate may have stripped the silica in the C_{18} column, thereby damaging the column and producing inaccurate results in the experiment (Schwartz 2013).

2.6 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel. On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (Rf) expressed as:

$$Rf = \frac{distance\ of\ sample\ from\ baseline}{distance\ of\ solvent\ front\ from\ baseline}$$

The factors affecting retardation factor are the solvent system, amount of material spotted, absorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatography technique.

2.6.1 TLC Principle

Like other chromatographic techniques, thin layer chromatography depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

2.6.2 TLC Diagram

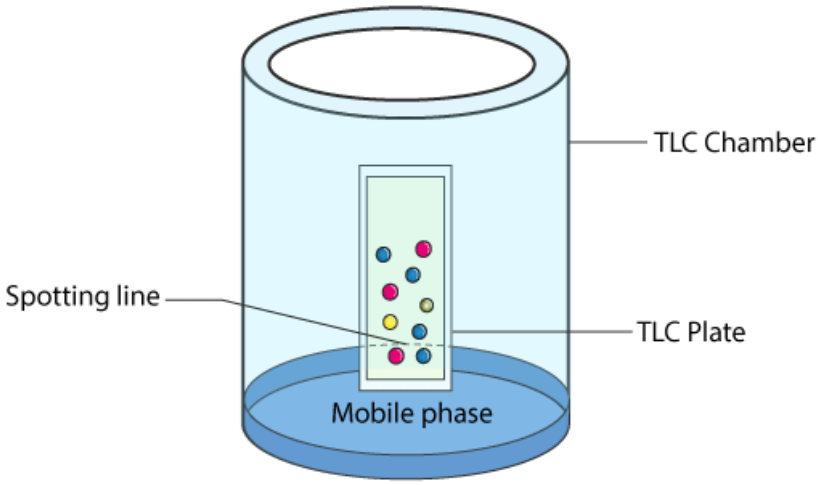


Figure 2-6 Principle of TLC (BYJU'S 2020)

2.6.3 Studies Conducted Using TLC

- The qualitative testing of Various medicines such as sedatives (Street and Perry 1968) local anaesthetics (Schmidt and Bracher 2006), anticonvulsant tranquilizers (Pippenger, Scott and Gillen 1969) , analgesics (Elder 1995), antihistamines (Morrison and Chatten 1964), steroids and hypnotics (Pushpalatha *et al.* 2009).
- TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids and serum (Sherma and Fried 2005).

- Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes and alkaloids (Danciu, Hosu and Cimpoiu 2018).
- It is widely used in separating multicomponent pharmaceutical formulations (Bebawy and El-Kousy 1999).
- It is used to purify of any sample and direct comparison is done between the sample and the authentic sample (Spangenberg, Poole and Weins 2011).
- It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives (Sengar and Sharma 2014).
- It is used in the cosmetic industry (De Kruijf *et al.* 1987).
- It is used to study if a reaction is complete (Susan *et al.* 2018).

2.6.4 Rationale for HPLC

HPLC is more advantageous over other forms of classical chromatography as it is a more advanced system of automated chromatography. HPLC encompasses the major methods of liquid chromatography and ensures more precise and improved methods of data analysis. It is therefore used by pharmaceutical companies to ensure quality assurance of new drug products that are being produced (Ahuja and Dong 2005).

2.6.4.1 Rationale for Using HPLC Over TLC

HPLC is the successor of TLC and has made significant contributions to the growth of analytical science and its diverse applications in pharmaceuticals, foods, polymers and plastics, environmental monitoring and clinical fields. HPLC is used by

pharmaceutical companies to ensure quality assurance of new drug products that are being produced (Ahuja and Dong 2005). The Researcher did not utilise TLC due to the important parameter of quantification. The Researcher required a method that can quantify the content of Hydrastine and Berberine and not just detect the presence of these alkaloids.

Parameter	**Thin Layer Chromatography (TLC)**	**High Performance Liquid Chromatography (HPLC)**
Resolution	In TLC separations it becomes difficult to differentiate between overlapping bands and spots	The peaks in HPLC can be easily resolved and evaluated by controlling operational parameters such as flow rate of mobile phase, buffer control of the mobile phase and column oven temperature,
Speed of analysis	In TLC separations the carrier liquid progression on the plate is dependent on gravitational force or capillary action	In HPLC the flow of mobile phase through the column is fast as it is pushed through with the help of a pump. Different pump pressure ranges can be achieved using normal as well as ultra-high-pressure systems
Quantification of results	TLC quantifications are based on visual comparisons or spot intensity matching techniques	HPLC software is capable of reporting precise and accurate

	which can be less quantitative in practice	results based on area counts of peaks
Sensitivity	Such large and sensitive detection options are not available in TLC separations	HPLC offers a large number of detector options to choose from such as UV, refractive index, electrochemical, fluorescence, conductivity, mass selectivity detector. Simultaneous detection is also possible using the photodiode array detection system
Software	No software is utilized.	HPLC software is capable of reporting precise and accurate results based on area counts of peaks. In addition, software also helps set and monitor operational parameters such as flow rate, detector wavelength and column temperature.
Choice of stationary phase and columns	This unique facility is not available on TLC plate separations.	A large number of stationary phases are available to suit a different range of applications. Similarly, columns of different bores and lengths are available for

		micro-level determinations to preparative scale preparations.
Storage of results	TLC plates cannot be stored due to fading of spots over time and requirement of proper storage conditions. However, pictures of TLC plates can be taken and stored as permanent records.	For HPLC it is possible to keep a permanent record of generated chromatograms as hard or soft copies.
Hyphenated techniques	This option is not possible in TLC separations.	HPLC has been successfully coupled with mass spectrometers and FT-IR systems and this has resulted in significant improvement in both resolution and sensitivity.
Reuse of Columns	New TLC plates are required for each analysis	The columns if maintained properly and operated under carefully controlled conditions without overloading can be used repeatedly for significant time periods.

2.6.5 Studies on Phytochemistry Conducted Using HPLC

Withania somnifera, commonly known as Ashwagandha, is a herb that is widely prescribed in Ayurveda and Homoeopathy (Joshi 2013). It is used in the treatment of

insomnia, mild depression, anxiety and fatigue and inflammatory conditions (Umadevi *et al.* 2012). In a study conducted by Ganzera, Choudhary and Khan (2003) , the HPLC technique was used to determine the presence and quantities of the two main withanolides, withaferin A and withanolide D, in different parts of the plant. The roots, stem and leaves were analysed, and it was discovered that each part of the plant had different quantities of withanolides, with the roots containing the highest concentrations. In this study, the method of HPLC analysis was conducted, at ambient temperature, as follows: *Withania somnifera* was analysed using Waters Alliance 2690 HPLC system, equipped with a 996 photodiode array detector (Waters, Milford, MA, USA) (cited in Ganzera, Choudhary and Khan (2003). A Synergi MAX-RP 80 A˚ column (150X4.6 mm, 4 mm particle size) from Phenomenex (Torrance, CA, USA) was used to separate the chemical compounds in *Withania somnifera*.

The mobile phase consisted of water (A) and a mixture of Methanol and reagent alcohol in the ratio of 1:1 (B), which were applied in the following gradient elution: from 65 Ay35 B in 25 min to 55 Ay45 B. Each run was followed by a 5-min wash with 100 B and an equilibration period of 10 minutes. The separation temperature was kept at a constant 50 °C. The reason for performing it at this temperature was that it significantly reduced the separation time and the column backpressure, without affecting the peak resolution. The flow rate was set at 1 ml/min with a sample/injection volume of 10 ml. However, results showed that in order to obtain similar retention times and good detection sensitivity the flow rate was reduced to 0.5 ml/min and the solvent gradient modified (55 Ay45 B to 45 Ay55 B in 25 min). All separations were

monitored at 230 nm. Peaks were assigned by spiking the samples with authentic samples of 1 and 2, and comparison of the UV-spectra and retention times.

For peak confirmation, a Liquid chromatography–mass spectrometry LC-MS experiment, using an AQA mass spectrometer from Finnigan (San Jose, CA, USA), together with a Finnigan HPLC (AS3000) autosampler, P4000 pump and UV6000LP detector) was performed. Best results were obtained in positive ESI mode, with ionization voltage set to 50 V, source voltage to 3.0 kV and probe temperature to 350 8C. An LC-MS experiment was performed to confirm the identity of the peaks of interest. The HPLC conditions were slightly changed as the separation had to be performed at room temperature and a flow rate of 1.0 ml/min did not allow sensitive detection of the compounds. With modifications in the solvent composition and flow rate, the MS signals were readily assignable.

The results of the HPLC study conducted by Ganzera, Choudhary and Khan (2003) showed the presence of Withaferin A and withanolide D in the root, stem and leaf of *Withania somnifera*, with differences in the ratio of both the compounds. The roots were shown to contain a greater quantity of Withanolide D. Furthermore, this same compound, Withanolide D, was significantly less in the leaves of the plant, consequently showing that a higher quantity of Withaferin A was present in the leaves of the plant. The stems of the plant were shown to contain the smallest quantities of both compounds, Withaferin A and Withanolide D.

In another study that was conducted, (Abourashed and Khan 2001) aimed to improve the method of HPLC analysis by accelerating the time taken to analyse different products of *Hydrastis canadensis* that were purchased in America and quantify Berberine and Hydrastine that were present in these products. They referred to two previous HPLC analyses where the analytical run time for each sample was 40 minutes. The results of their study showed that they were able to decrease the analytical run time to 20 minutes whilst still being able to quantify Berberine and Hydrastine in different products of Hydrastis canadensis. The setup for this experiment was as follows: the HPLC system consisted of a 600E controller, a 600 pump, a 712 WISP autoinjector, and a 996 UV Photodiode Array Detector. A pre-packed Luna, 15 cm_4.6 mm (5 mm particle size), C_{18} HPLC column and SecurityGuard cartridge system (Phenomenex, Torrance, CA) were operated. The mobile phase consisted of solvent A (0.10 M sodium acetate/acetic acid, pH 4.0, in water) and solvent B (acetonitrile/methanol, 90/10, v/v). Helium was used to continuously degas the system. Elution was run at a flow rate of 1.0 mL/min with a linear gradient of 80±40% A in B over 20 min and ultraviolet (UV) detection at 290 nm. A Quantex computer with Millennium 32 software on was used to process the chromatographic data. The standard solutions of Berberine and Hydrastine were prepared in a 10 mL volumetric flask. 1.0 mg each of Berberine and Hydrastine and 10 mL of methanol were added to the flask and this standard solution was used to prepare solutions of known concentrations by serial dilutions with methanol. Each sample was analysed three times using HPLC in order to validate the method and analysis. The retention time for berberine was at 14.92 min and hydrastine was at 10.87 minutes. The concentrations of alkaloids in the analysed samples of Hydrastine canadensis were up to 2.51% for hydrastine and up to 4.35% for berberine. The

retention time is the time at which the peaks for each alkaloid is visible. These results prove that (Abourashed and Khan 2001) successfully improved and sped up the HPLC analysis process. Additionally, by effectively decreasing the time required to run each sample to 20 minutes, Abourashed and Khan (2001)'s method showed that it would be practical for the Researcher to conduct an HPLC analysis of different Homoeopathic diluted preparations of Hydrastis canadensis in triplicate, without this process being tedious. If each sample analysis had required a 40-minute run time, this research practical would have been a long and drawn-out process.

A study was conducted by Weber *et al.* (2003) using HPLC to compare the weight percentages of alkaloids, Berberine and Hydrastine that were present in the root powder of Hydrastis canadensis obtained from three different commercial suppliers. According to monographs from the American Herbal Pharmacopoeia and Therapeutic compendium (cited in Weber *et al.* (2003), the alkaloid content calculated on dry weight basis should be no less than 2% for hydrastine and 2.5% for berberine. The three sample extracts from suppliers A, B and C were stored at ambient temperature. The results of the study concluded that the weight percentages of alkaloids in the root powder sample obtained from suppliers B and C correlated with the literature, with ~3.5% berberine and ~2.5% Hydrastine. However, the sample obtained from supplier A had an insufficient Hydrastine content of 1.38% (< 2.0%). Further investigations revealed that the sample obtained from supplier A was not pure goldenseal and therefore was not a suitable sample to have been used for the study.

The Mobile phase for the study conducted by Weber *et al.* (2003) consisted of solvent A, 10 mM ammonium acetate/acetonitrile (90:10, v/v) and solvent B, 10 mM ammonium acetate/acetonitrile (10:90, v/v). The ammonium acetate buffer (10 mM) was prepared using acetic acid and this was used to adjust the pH to 4.85 (Weber *et al.* 2003). Furthermore, the column temperature for this experiment was kept at 30 ^{O}C with an injection volume of 10 μL ; elution was run at a flowrate of 1.0 mL/min; a diode array detector (DAD) was used to record absorption spectra from 200 to 400 nm, with 235 nm being the chosen wavelength for all peaks (Weber *et al.* 2003). The HPLC analysis was set at 40 minutes. The chromatogram labelled as Figure 2, for the experiment conducted by (Weber *et al.* 2003) depict the presence of Hydrastine and Berberine present in Goldenseal root powder from Supplier A. The results show that Berberine peaked first, between 11 and 12 minutes, and Hydrastine peaked between 14 and 15 minutes, with an analysis time set at 40 minutes.

3 CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

This was a quantitative study that aimed to determine whether there were sufficient concentrations of Hydrastine and Berberine in different pharmaceutical preparations of *Hydrastis canadensis* mother tincture. This study was conducted using High-Performance Liquid Chromatography (HPLC). The practical aspects of this study were conducted at the Durban University of Technology. The Homoeopathic remedies were prepared with the assistance and supervision of a specialist Laboratory Technician and Homoeopath, Dr Shraddha Brijnath, in the lamina flow room at the Department of Homoeopathy. The HPLC practical was conducted in the Biotechnology Laboratory, under the supervision of the co-supervisor and Biochemist, Dr Viresh Mohanlall. Permission was obtained from the Head of Department, Professor Swalaha and Dr. Viresh Mohanlall (pers comm. Professor Swalaha and Dr V. Mohanlall. July 2018).

3.2 Preparation of tinctures

3.2.1 Hydrastis canadensis Mother Tincture

Different dilutions of *Hydrastis canadensis* mother tincture were obtained from a Homoeopharmaceutical compnay. They prepared *Hydrastis canadensis* mother tincture in 60% ethanol (*Hydrastis canadensis* is stable in 60% ethanol), according to

methods HAB 3a and HAB 4a of the German Homoeopathic Pharmacopoeia (Appendix A). The dilutions that were obtained were dependent on the stock that was available at the time of purchase. The reason for this, is that *Hydrastis canadensis* is prepared from a plant that is only available in North America and Canada, and thus, difficult to obtain at short notice and in small quantities. Different dilutions of Homoeopathic remedies are prepared in the same manner, according to the standard procedures that are in accordance with Good Manufacturing Practise. The remedies were safely packaged with dry ice, and were couriered to Durban in a specialised, refrigerated vehicle.

3.2.2 Commercial Preparation of *Hydrastis canadensis*

Different dilutions of *Hydrastis canadensis* were prepared in a laboratory of a pharmaceutical company, following standard procedures of the German Homoeopathic Pharmacopoeia (Appendix A). The French Homoeopathic Pharmacopoeia was not readily available to the researcher, nor is it commonly used in South Africa. A 1:10 dilution was prepared at a pharmaceutical company, according to method HAB 4a. The roots and rhizomes of the fresh plant of *Hydrastis canadensis* were used to prepare the mother tincture. 10 parts of the dried plant were used, to 100 parts of alcohol (60% m/m).

The base substance of the starting tincture was prepared according to method HAB 4a. Thereafter, the manufacturers diluted this stock tincture into different dilutions. This ensured that there were no discrepancies between the different preparations, since

the stock was the same. Additionally, the use of the same tincture for both preparation decreased any variability. Hence, the only difference between the different preparations was the dilution.

3.2.3 Researcher Preparation of *Hydrastis canadensis* at the Durban University of Technology

The dilution that was purchased from the Homoeopharmaceutical company, was used to prepare a 1:10 dilution of *Hydrastis canadensis.* These Homoeopathic remedies were prepared by the Researcher, with the assistance and supervision of a specialist Laboratory Technician and Homoeopath, Dr Shraddha Brijnath, in the lamina flow room at the Department of Homoeopathy. The remedies were prepared in a temperature-controlled room, under lamina flow conditions, according to Good Manufacturing Procedures (Appendix B). The prepared 1:10 dilution was compared to the 1:10 dilution that was purchased from a Homoeopharmaceutical company, using HPLC. This enabled the researcher to determine whether the two different preparations, namely the 1:10 dilution that was prepared at the laboratory in the Durban University of Technology and the 1:10 dilution that was prepared by a Homoeopharmaceutical company, yielded similar concentrations of Berberine and Hydrastine.

3.3 Materials and Methods

3.3.1 Materials

Berberine chloride and β-hydrastine were purchased from Merck (Sigma-Aldrich, Durban, South Africa). Berberine chloride and Hydrastine containing products were

purchased either from local retailers or via the Internet: ``Sample 1'' (1:10 tincture of *Hydrastis canadensis*, Natures Way Products, Inc., Springville, UT); ``Sample 2'' (1:10 tincture of *Hydrastis canadensis*, Student preparation); and “Sample 3” (1:3 tincture of *Hydrastis canadensis*, Natures Way Products, Inc., Springville, UT). HPLC grade acetonitrile and methanol were purchased from Merck (Durban, South Africa). Deionized water was obtained from a Milli-Q Plus analytical deionization system (Bedford, MA). Sodium acetate and glacial acetic acid was purchased from Merck (Sigma-Aldrich, Durban, South Africa).

3.3.2 Instrumentation and Chromatographic Conditions

A component HPLC system (Shimadzu, Kyoto, Japan) consisted of a DGU-20A_3 controller, a LC20AB pump, a SIL 20A autoinjector, and an SPD-M20A Photodiode Array detector. A pre-packed Sunflare, 12.5 cm X 4.6 mm (5 µm particle size), C18 HPLC column and SecurityGuard cartridge system (Phenomenex, Torrance, CA) were operated with a mobile phase consisting of solvent A (0.10 M sodium acetate/acetic acid, pH 4.8, in water) and solvent B (acetonitrile/methanol, 90/10, v/v). Elution was run at a flow rate of 1.0 mL/min with a linear gradient of 80±40% A in B over 20 min and ultraviolet (UV) detection at 254 nm (Table 1). Chromatographic data were processed with LabSolutions V5.42 SP6 software (Shimadzu Corporation, Kyoto, Japan) installed on a Hewlett Packard PC.

Figure 3-1 Percentage gradient flow rate of solvent A (0.10 M sodium acetate/acetic acid, pH 4.8, in water) and solvent B (acetonitrile/methanol, 90/10, v/v) over 11 minutes

Time (minutes)	%A	%B
0	80	20
11	40	60

3.3.3 Standard Solutions and Sample Preparation

Hydrastine and Berberine (1.0 mg each) were placed in a 1 mL graduated centrifuge tube and the volume was brought to 1 mL with methanol resulting in a final concentration of 1 mg/ml. Solutions with known concentrations (500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml) were prepared from the standard stock solution by serial dilution with methanol. These standard solutions were injected separately into the chromatograph. The commercial samples (1:10 tincture of *Hydrastis canadensis*, Natures Way Products, Inc., Springville, UT; 1:3 tincture of *Hydrastis canadensis*, Natures Way Products, Inc., Springville, UT) and DUT prepared sample by the Researcher (1:10 tincture of *Hydrastis canadensis*) were prepared by aliquoting 1 ml of the sample into HPLC vials in triplicate and were injected separately into the chromatograph.

3.3.4 Method Validation

Calibration curves were constructed by plotting peak areas for the external standards, Hydrastine and Berberine, versus a 4-fold concentration range of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml. Each solution was injected three times beginning with the most dilute concentration. The slope, intercept, and correlation coefficients were calculated by linear regression analysis on the GraphPad Prism software. Extracts were injected in the chromatograph and the alkaloid concentrations were determined. The retention time and relative standard deviation (RSD) values for measured alkaloids in all samples were used to determine method precision. Peak purity was established by spectral overlap.

4 CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter highlights the results that were obtained from the HPLC analysis of different pharmaceutical preparations of *Hydrastis canadensis*, some of which were purchased from a Homoeopharmaceutical company and some of which were prepared by the Researcher from these commercial samples. Furthermore, HPLC analysis was conducted on standard samples of pure Hydrastine and Berberine obtained from Sigma-Aldrich and the analysis of the negative control, 60% Alcohol.

The HPLC analysis was done on various pharmaceutical preparations of *Hydrastis canadensis*, in order to determine the quantities of Hydrastine and Berberine that are present in these preparations. This analysis was conducted on 1:3 and 1:10 diluted preparations of *Hydrastis canadensis* that were purchased from a Homoeopharmaceutical company. Additionally, the analysis was conducted on the Researcher's 1:10 dilution of *Hydrastis canadensis* that was prepared from the commercial 1:10 dilution of the remedy. The Researcher prepared the 1:10 dilution of *Hydrastis canadensis* in the Homoeopharmaceutical laboratory at the Durban University of Technology. A comparison is drawn between the quantities of Hydrastine and Berberine that are present in the Researcher's 1:10 diluted preparation and the commercial 1:10 preparation of *Hydrastis canadensis*.

The results of the HPLC analysis are depicted in this chapter and relevant appendices, using a chromatogram. The peak areas are plotted on a Peak Area versus Concentration graph. This graph is used to formulate the Standard Curve. The Standard Curve for Berberine and Hydrastine is achieved by using different dilutions of the standard sample including 50 µg/ml, 100 µg/ml, 200 µg/ml and 500 µg/ml. The Standard Curve is plotted using SigmaPlot and the former is used to calculate the concentration of the Hydrastine and Berberine in the diluted tincture samples, using the straight-line equation – y=mx+c.

The statistical analysis of data was conducted using the Analysis of Variants, also known as the ANOVA test. ANOVA is used to compare the mean values that have been obtained from two or more groups in a study (Kim 2014). The R-squared value is a statistical measure of variation that explains what percentage of a dependent variable can be explained by the independent variable. This is calculated from the linear regression. The R-square value shows the sensitivity of the HPLC analysis. Its value ranges between 0 and 1 but it is interpreted as a percentage. The closer to 1 the R-squared value is, the greater the sensitivity of the experiment. For example, in the case of this research, the R-squared value for the Hydrastine standard, is 0.9940, as seen in in this Chapter 4, under the heading Figure 4.7 B – Statistical evaluation of dataset for Hydrastine standard. This means that 99.4% of the results can be explained. Therefore, this is a good value as most of the variation can be explained. The confidence limits should ideally be greater than 95% (Pers comm. Dr Mohanlall 2020).

The HPLC analyses for the standards, Berberine and Hydrastine were conducted in triplicate. Alcohol, which was the negative control, was analysed once. The 1:3 commercial preparation, 1:10 commercial preparation and 1:10 Researcher preparation of *Hydrastis canadensis* were all analysed in triplicate. The results of the first analysis of each sample are displayed in the chromatograms below. Please refer to Appendix F, G and H for duplicate and triplicate chromatograms of the respective analyses.

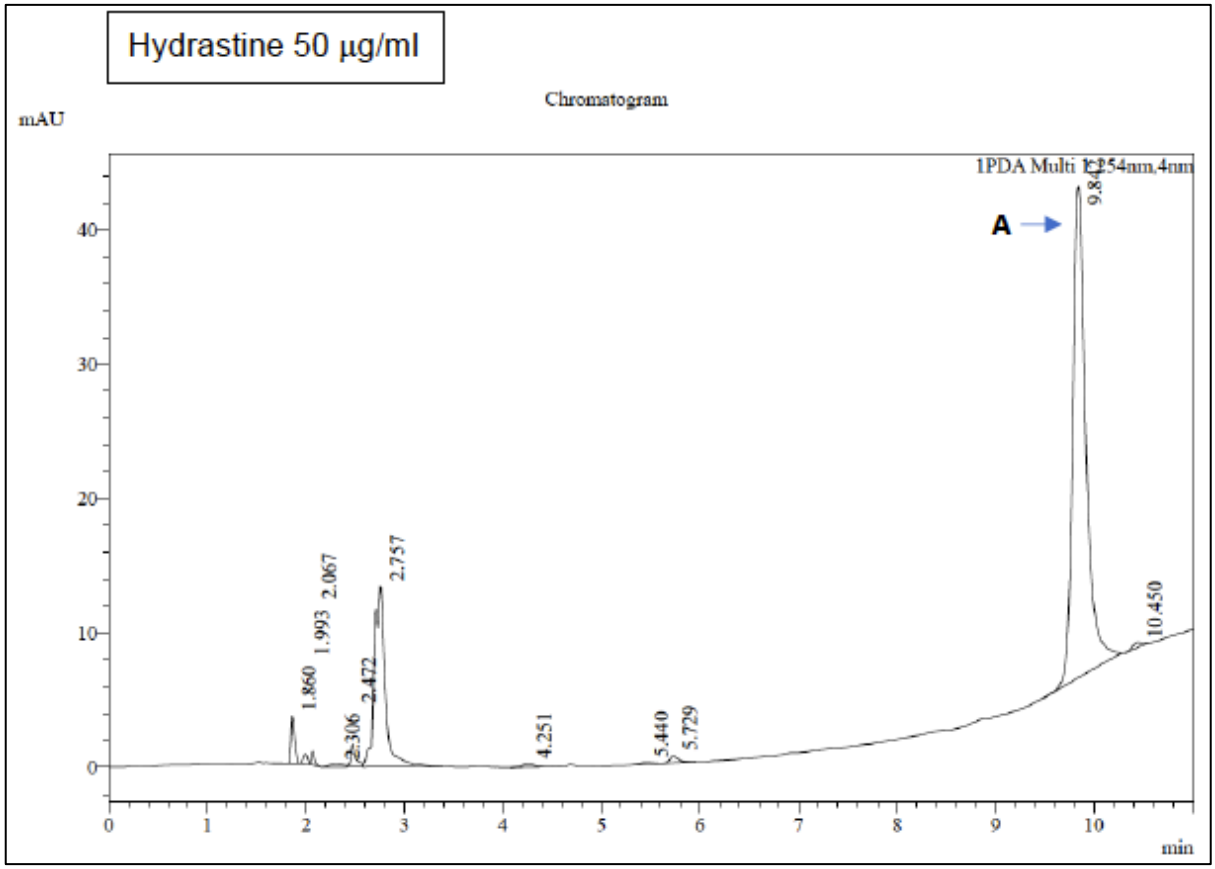


Figure 4-1 Representative chromatogram showing the standard

A – Hydrastine 50 µg/ml. Retention time - 9.842 minutes.

This figure shows that the sample of Hydrastine 50 µg/ml peaks at 9.842 minutes. This retention time is used to identify Hydrastine present in different Pharmaceutical preparations of *Hydrastis canadensis* that are analysed.

Sample duplicates and triplicates are available in Appendix C.

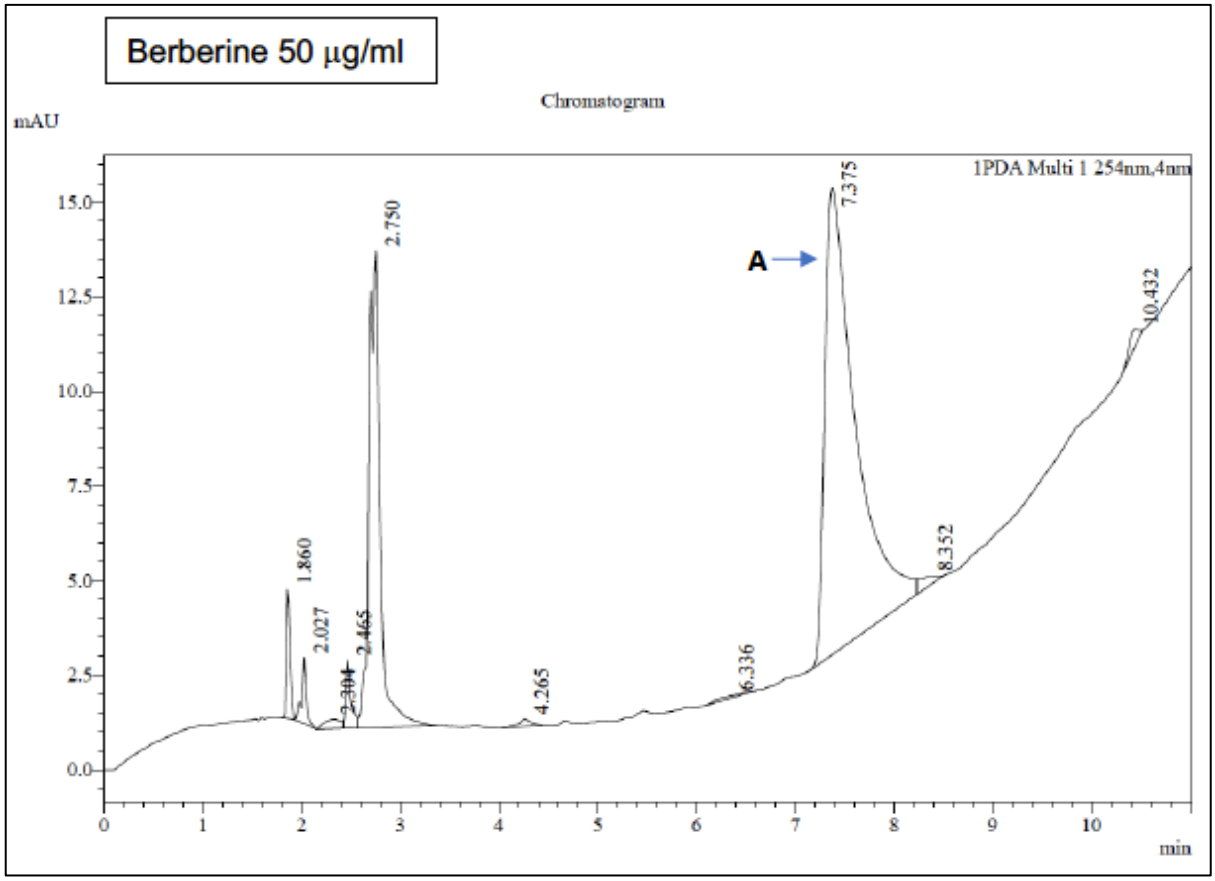


Figure 4-2 Representative chromatogram showing the standard

A – Berberine 50 µg/ml. Retention time - 7.375 minutes.

This figure shows that the sample of Berberine 50 µg/ml peaks at 7.375 minutes. This retention time is used to identify Berberine present in different Pharmaceutical preparations of *Hydrastis canadensis* that are analysed.

Sample duplicates and triplicates are available in Appendix D.

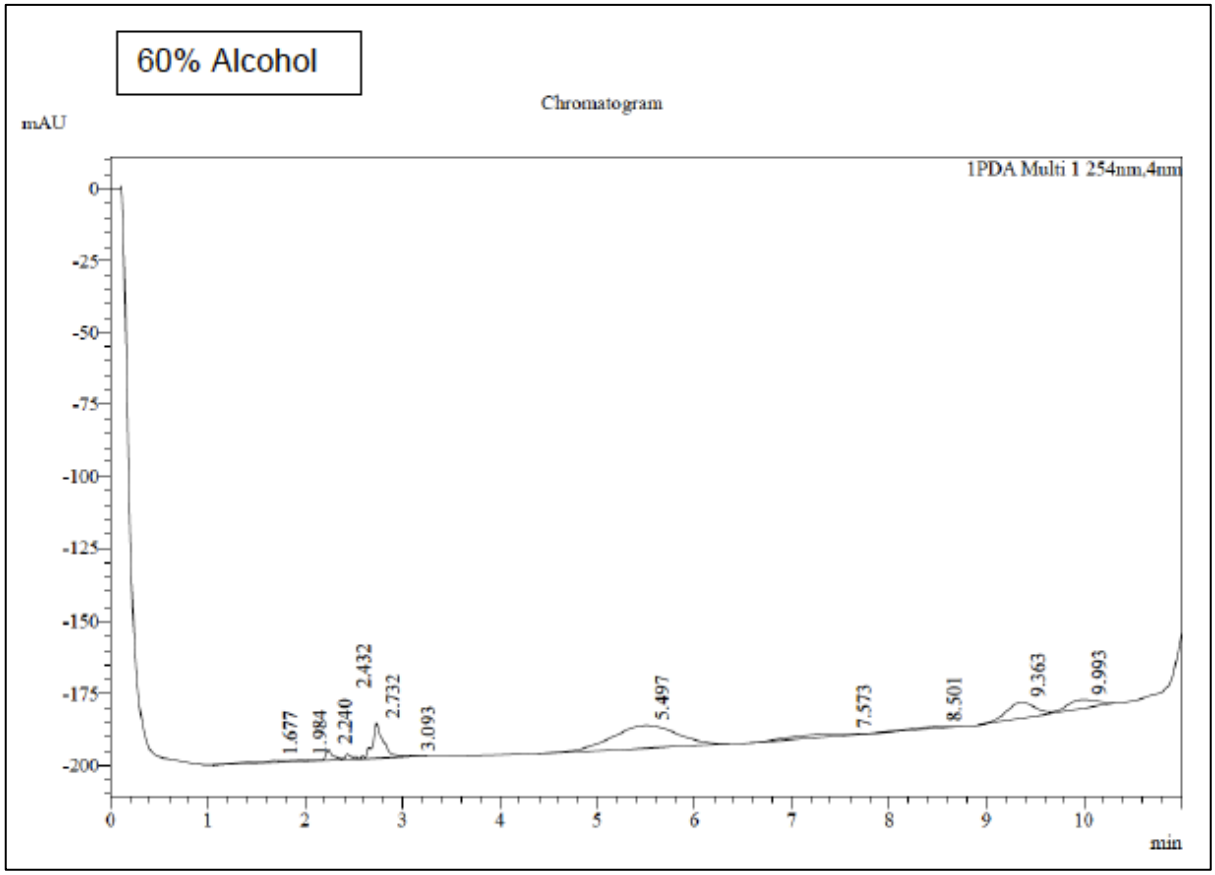


Figure 4-3 Representative chromatogram showing the absence of peaks in the Standard, 60% Alcohol preparation, used as a negative control

This figure shows an absence of peaks, which means that there are no other alkaloids or major contaminants present in the standard, 60% Alcohol preparation (Appendix E).

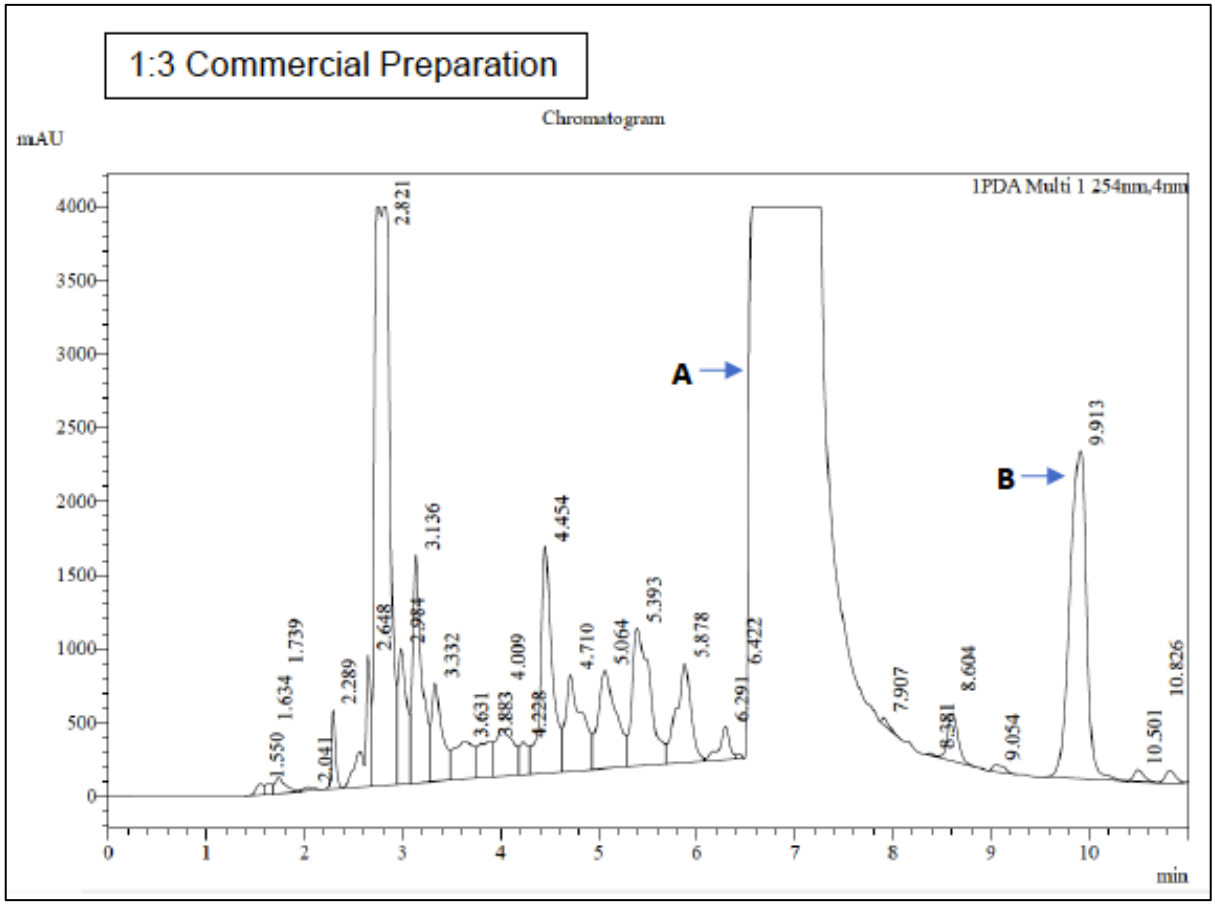


Figure 4-4 Representative chromatogram showing the presence of Berberine and Hydrastine in the first 1:3 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 6.422 minutes with results beyond maximum quantifiable limits.

B – Hydrastine. Retention time - 9.913 minutes.

Sample duplicates and triplicates are available in Appendix F.

This figure shows the concentrations of Berberine and Hydrastine that are present in the 1:3 commercial preparation of *Hydrastis canadensis* that was obtained from a

Homoeopharmaceutical company. In the chromatogram, **A** represents Berberine, which peaked at 6.422 minutes with results beyond maximum quantifiable limits. **B** represents Hydrastine, which peaked at 9.907 minutes.

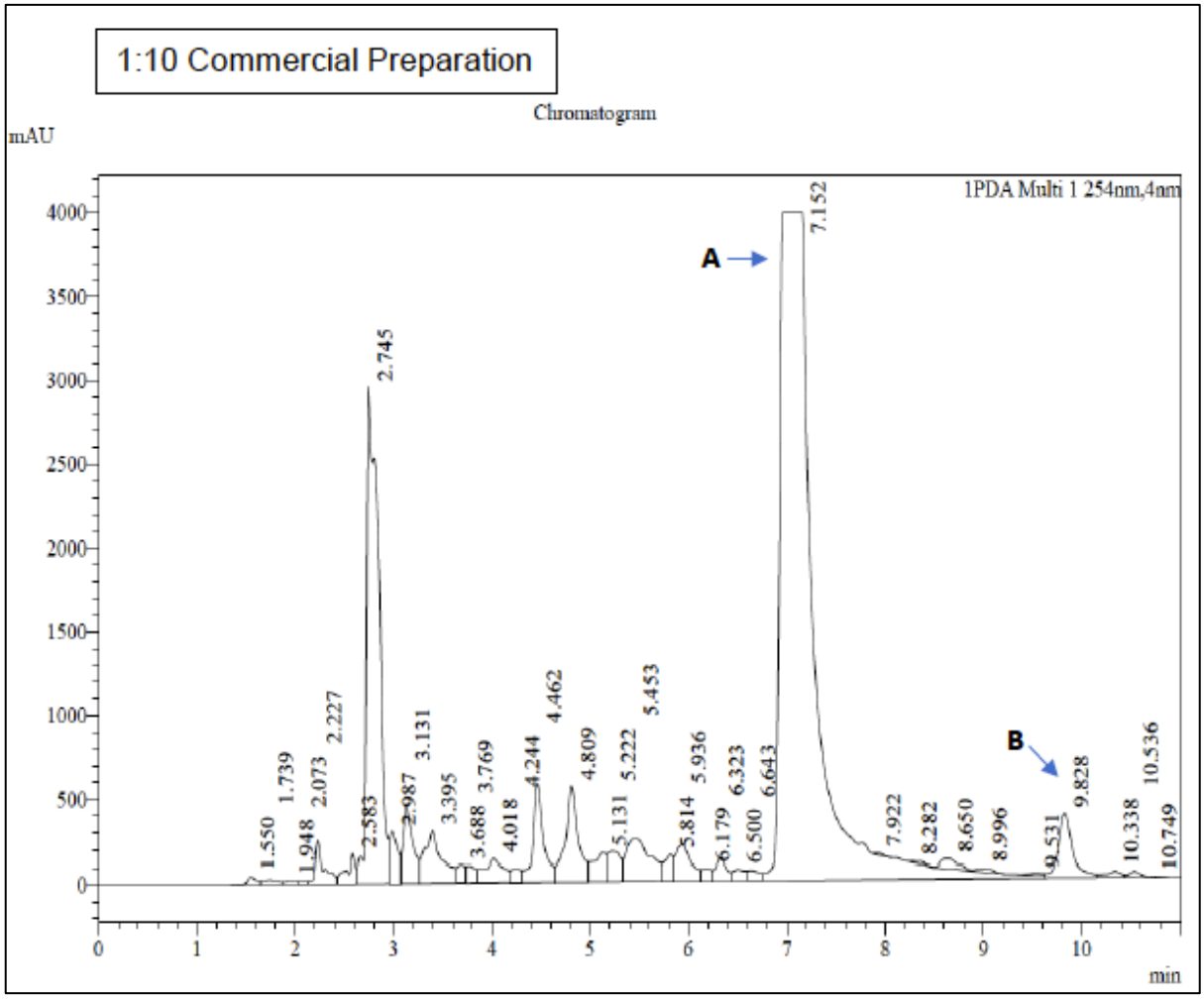


Figure 4-5 Representative chromatogram showing the presence of Berberine and Hydrastine in the first 1:10 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 7.152 minutes.

B – Hydrastine. Retention time - 9.828 minutes.

Sample duplicates and triplicates are available in Appendix G.

This figure shows the concentrations of Berberine and Hydrastine that are present in the 1:10 commercial preparation of *Hydrastis canadensis* that was obtained from a

Homoeopharmaceutical company. In the chromatogram, **A** represents Berberine, which peaked at 7.152 minutes and **B** represents Hydrastine, which peaked at 9.828 minutes.

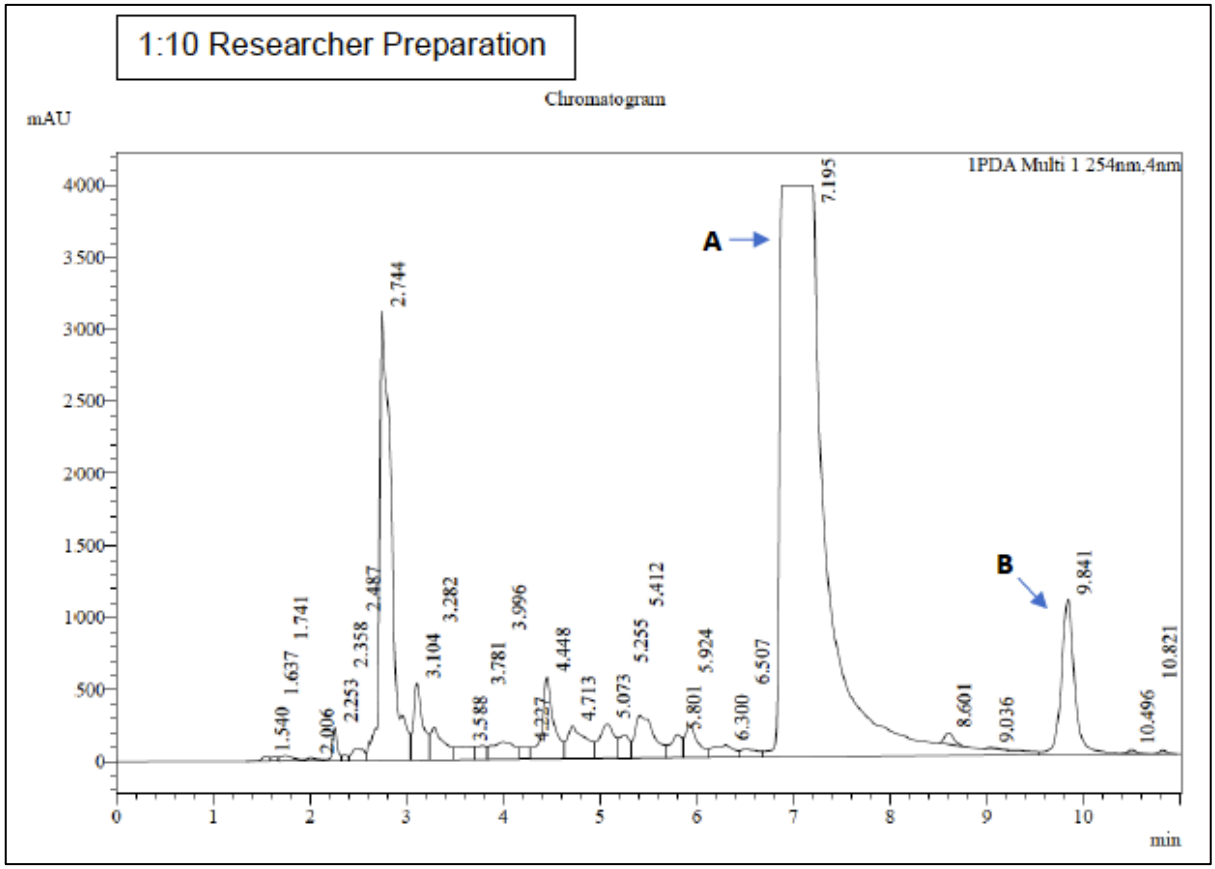


Figure 4-6 Representative chromatogram showing the presence of Berberine and Hydrastine in the first 1:10 Researcher preparation of *Hydrastis canadensis*

Sample duplicates and triplicates of 1:10 Researcher preparations are available in Appendix H.

A – Berberine. Retention time - 7.195 minutes.

B – Hydrastine. Retention time - 9.841 minutes.

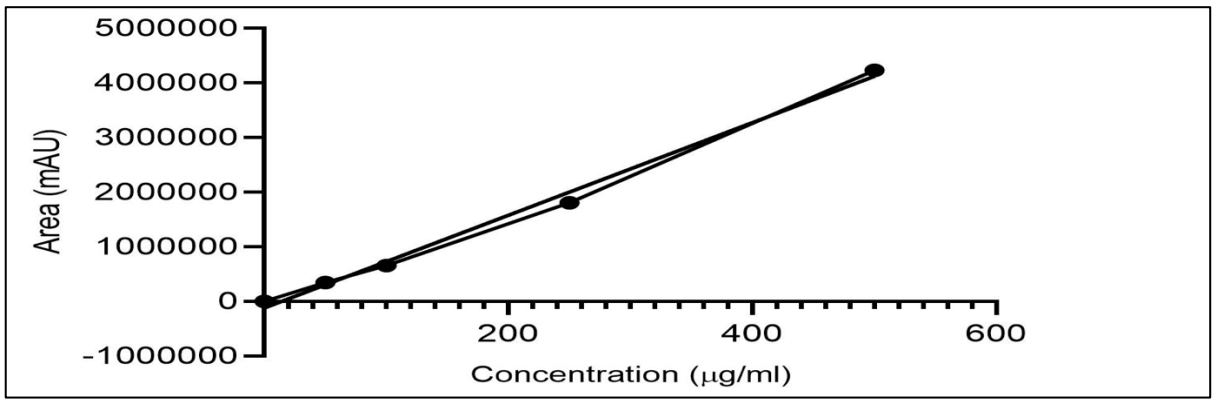
This figure shows the concentrations of Berberine and Hydrastine that are present in the 1:10 Researcher's preparation of *Hydrastis canadensis* that was obtained from a Homoeopharmaceutical company. In the chromatogram, **A** represents Berberine,

which peaked at 7.195 minutes and **B** represents Hydrastine, which peaked at 9.841 minutes.

4.2 Concentrations of Hydrastine and Berberine from HPLC Analysis

Standard Curves of Hydrastine and Berberine

A. Peak Area Vs Concentration in four samples of Hydrastine (50 μg/ml, 100 μg/ml, 250 μg/ml, and 500 μg/ml)



B. Statistical evaluation of dataset for Hydrastine standard

0	0
50	346497
100	658797
250	1806338
500	4231436

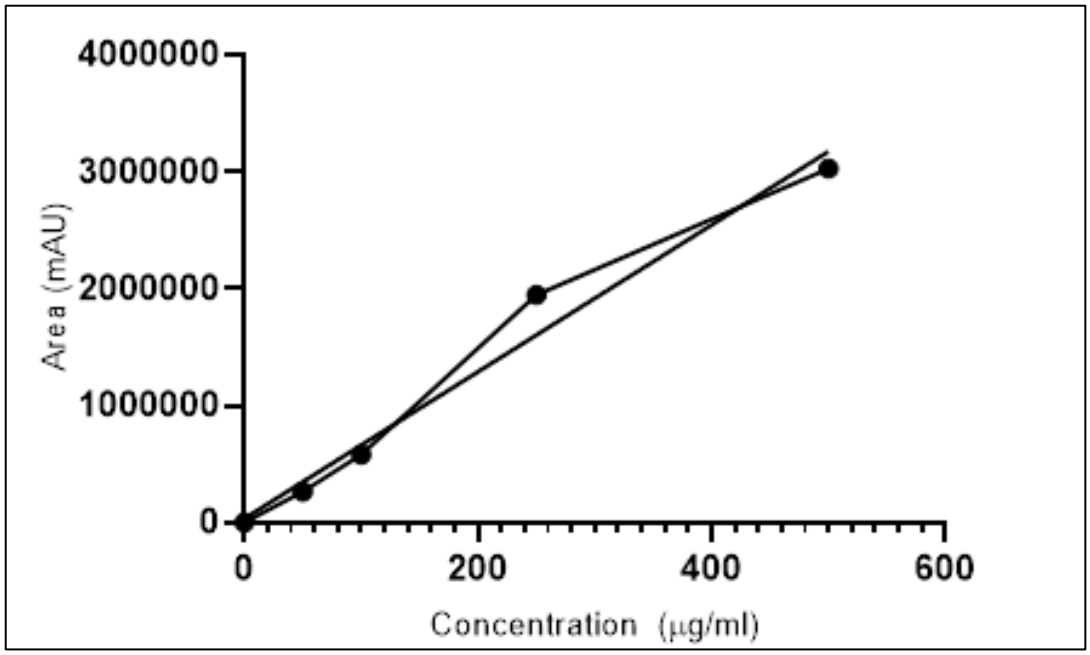
Best-fit values	
Slope	8483
Y-intercept	-118355
X-intercept	13.95
1/slope	0.0001179
Std. Error	
Slope	380.8
Y-intercept	97073
95% Confidence Intervals	
Slope	7271 to 9695
Y-intercept	-427286 to 190576
X-intercept	-25.08 to 46.06
Goodness of Fit	
R square	0.9940
Sy.x	153723
Is slope significantly non-zero?	
F	496.4
DFn, DFd	1, 3
P value	0.0002
Deviation from zero?	Significant
Equation	Y = 8483*X - 118355
Data	
Number of X values	5
Maximum number of Y replicates	1
Total number of values	5
Number of missing values	0

Figure 4-7 Standard curve and statistical evaluation of standard Hydrastine

A – Peak Area Vs Concentration in four samples of Hydrastine (50 μg/ml, 100 μg/ml, 250 μg/ml, and 500 μg/ml).

B – Statistical evaluation of dataset for Hydrastine standard.

A. Peak Area Vs Concentration in four samples of Berberine (50 μg/ml, 100 μg/ml, 250 μg/ml, and 500 μg/ml).



B.

0	0
50	265963
100	583193
250	1947685
500	3026771

Line	
Best-fit values	
YIntercept	34285
Slope	6280
Std. Error	
YIntercept	143024
Slope	561.0
95% CI(profile likelihood)	
YIntercept	-420883 to 489452
Slope	4495 to 8066
Goodness of Fit	
Degrees of Freedom	3
R squared	0.9766
Sum of Squares	153891843466
Sy.x	226489
Number of points	
# of X values	5
# Y values analyzed	5

Figure 4-8 Standard curve and statistical evaluation of standard Berberine

A – Peak Area Vs Concentration in four samples of Berberine (50 μg/ml, 100 μg/ml, 250 μg/ml, and 500 μg/ml).

B – Statistical evaluation of dataset for Berberine standard.

Sample (x-axis) Vs Area (y-axis) Graph of Hydrastine and Berberine

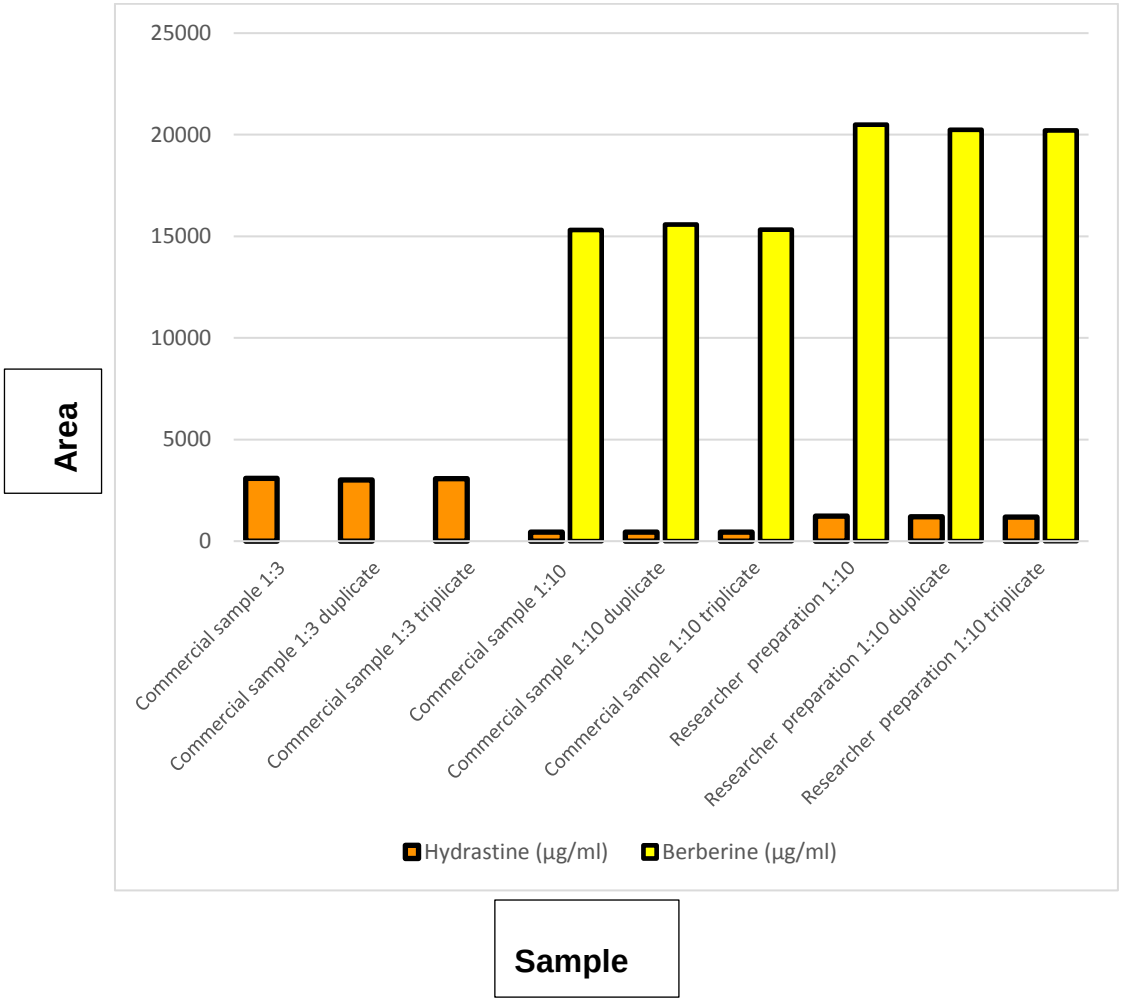


Figure 4-9 Graph showing concentrations (µg/ml) of Hydrastine and Berberine present in 1:3 commercial preparation, 1:10 commercial preparation and 1:10 Researcher preparation of *Hydrastis canadensis*

Table 4-1 Hydrastine and Berberine

	Hydrastine (µg/ml)	Berberine (µg/ml)
Commercial sample 1:3	3090	MQL*
Commercial sample 1:3 duplicate	3012	MQL*
Commercial sample 1:3 triplicate	3079	MQL*
Commercial sample 1:10	442	15308
Commercial sample 1:10 duplicate	443	15584
Commercial sample 1:10 triplicate	450	15337
Researcher preparation 1:10	1235	20494
Researcher preparation 1:10 duplicate	1195	20242
Researcher preparation 1:10 triplicate	1181	20210

* MQL- maximum quantifiable limit

The table above shows the concentrations (μg/ml) of Hydrastine and Berberine present in 1:3 commercial preparations, 1:10 commercial preparations and 1:10 Researcher preparations of *Hydrastis canadensis.*

4.3 Mean Calculations of Hydrastine and Berberine Concentrations from HPLC Analysis

4.3.1 The Effect of HPLC on a Commercial Sample 1:3 Dilution of *Hydrastis canadensis*

The HPLC triplicate analyses of a commercial sample 1:3 dilution of *Hydrastis canadensis* showed that the mean concentration of Hydrastine in this preparation was 3 060 μg/ml .

The mean concentration (μg/ml) of Hydrastine that was present in the commercial sample 1:3 dilution was calculated as follows:

3090 μg/ml + 3012 μg/ml + 3079 μg/ml = 9 181 μg/ml

Therefore, 9 181 μg/ml / 3 samples = 3 060 μg/ml .

Furthermore, the concentration of Berberine that was present in the commercial sample 1:3 dilution was beyond the Maximum Quantifiable Limit (MQL), and therefore too large to have attached a numerical value.

4.3.2 The Effect of HPLC on a Commercial Sample 1:10 Dilution of *Hydrastis canadensis*

The HPLC triplicate analyses of a commercial sample 1:10 dilution of *Hydrastis canadensis* showed that the mean concentration of Hydrastine in this preparation was 445 μg/ml .

This was calculated as follows:

442 μg/ml + 443 μg/ml + 450 μg/ml = 1 335 μg/ml / 3

The mean concentration (μg/ml) of Berberine that was present in the commercial sample 1:10 dilution was 15 409.67 μg/ml .

(1 508 μg/ml +15 584 μg/ml + 15 337 μg/ml = 46 229 μg/ml / 3)

4.3.3 The Effect of HPLC on the Researcher's Sample 1:10 Dilution of *Hydrastis canadensis*

The HPLC triplicate analyses of the Researcher's sample 1:10 dilution of *Hydrastis canadensis* showed that the mean concentration of Hydrastine in this preparation was 1 203, 67 μg/ml .

(1 235 μg/ml + 1 195 μg/ml + 1 181 μg/ml = 3 611 μg/ml / 3)

The mean concentration (μg/ml) of Berberine that was present in the Researcher's sample 1:10 dilution was 20 315.33 μg/ml.

(2 094 μg/ml + 20 242 μg/ml +20 210 μg/ml = 60 946 μg/ml / 3)

4.3.4 The Effect of HPLC on 60% Alcohol as a Negative Standard for the Analysis

The HPLC analysis of the negative standard, 60% Alcohol preparation, showed an absence of peaks in the chromatogram. Hence, no traces of Hydrastine or Berberine were present in the negative control, as expected.

4.3.5 The Effect of HPLC on Standard Hydrastine in 50 μg/ml, 100 μg/ml, 250μg/ml and 500 μg/ml samples

The HPLC analyses of the standard Hydrastine were conducted on 50 μg/ml, 100 μg/ml, 250 μg/ml and 500 μg/ml samples. The chromatogram showed that the concentration of Hydrastine 50 μg/ml peaked at 9.842 minutes, Hydrastine 100 μg/ml peaked at 9.835 minutes, Hydrastine 250 μg/ml peaked at 9.856 minutes and Hydrastine 500 μg/ml peaked at 9.845 minutes. Hence, the concentrations of Hydrastine were seen to peak between 9 minutes and 10 minutes.

4.3.6 The Effect of HPLC on Standard Berberine in 50 μg/ml, 100 μg/ml, 250μg/ml and 500 μg/ml Samples

The HPLC analyses of the standard Berberine were conducted on 50 μg/ml, 100 μg/ml, 250 μg/ml and 500 μg/ml samples. The chromatogram showed that the concentration of Berberine 50 μg/ml peaked at 7.375 minutes, Berberine 100 μg/ml peaked at 7.314 minutes, Berberine 250 μg/ml peaked at 7.279 minutes and Berberine 500 μg/ml peaked at 7.268 minutes. Hence, the concentrations of Berberine were seen to peak between 7 minutes and 7.5 minutes.

5 CHAPTER 5

DISCUSSION

5.1 Introduction

This study aimed to determine the quantities of active ingredients, Hydrastine and Berberine, that are present in different pharmaceutical preparations of *Hydrastis canadensis* mother tincture, using High-Performance Liquid Chromatography (HPLC). It also aimed to compare the differences in concentrations of the aforementioned active ingredients, that are present in a 1:10 dilution that is prepared by a Homoeopharmaceutical company, with that which is prepared by the Researcher, In Chapter 4, Figures 4.4, 4.5 and 4.6 show the concentrations in micrograms per millilitre, or μg/ml, of the aforementioned active ingredients. Each sample was run through the HPLC system at least three times, which ensured standardisation and accuracy of the results obtained.

5.1.1 The Effect of HPLC on a Commercial Sample 1:3 Dilution of *Hydrastis canadensis*

The results in Chapter 4 show that the Commercial sample 1:3 dilution of *Hydrastis canadensis* contained a significant concentration of Hydrastine, which amounted to an average of 3 060 μg/ml . Furthermore, the concentration of Berberine in the same sample was too high to be quantified, that is, Berberine's concentration was beyond the Maximum Quantifiable Limit (MQL). This means that the 1:3 preparation strongly

retained the alkaloid Berberine despite being diluted. As discussed in Chapter 1, one of the objectives of this research is to compare to determine the quantities of Hydrastine and Berberine in a 1:3 dilution of *Hydrastis Canadensis* obtained from a Homoeopharmaceutical company, using HPLC. This 1:3 dilution is used by the Researcher to prepare the 1:10 dilution of *Hydrastis canadensis,* therefore these high concentrations of alkaloids are favourable to this research.

5.1.2 The Effect of HPLC on a Commercial Sample 1:10 Dilution of *Hydrastis canadensis*

The results in Chapter 4 show that the Commercial sample 1:10 dilution of *Hydrastis canadensis* contained a significant concentration of Hydrastine, which amounted to an average of 445 μg/ml. Furthermore, the concentration of Berberine in the same sample was an average of 15 409.67 μg/ml . Despite the 1:10 sample being more diluted than the 1:3 sample, this sample of *Hydrastis canadensis* still retains a significant amount of alkaloids. An objective of this research, as discussed in Chapter 1, is to determine the quantities of Hydrastine and Berberine in a commercial sample 1:10 dilution of *Hydrastis*, using HPLC. This will be compared to the Researcher's 1:10 preparation of *Hydrastis canadensis,* from the commercial 1:3 dilution, in terms of the concentrations of alkaloids that are present.

5.1.3 The Effect of HPLC on the Researcher's Sample 1:10 Dilution of *Hydrastis canadensis*

The results in Chapter 4 show that the Researcher's sample 1:10 dilution of *Hydrastis canadensis* contained a mean concentration of 1 203, 67 μg/ml of Hydrastine. Furthermore, the concentration of Berberine in the same sample was an average of 20 315.33 μg/ml . An objective of this research is to compare the difference between the quantities of Hydrastine and Berberine that are present in the Commercial sample 1:10 dilution and the Researcher's sample 1:10 dilution of *Hydrastis canadensis.* The Researcher's 1:10 dilution is shown to contain higher concentrations of Berberine and Hydrastine than the Commercial sample 1:10 dilution. The Researcher's sample 1:10 dilution of *Hydrastis canadensis* was prepared from the Commercial sample 1:3 dilution. The latter contained an average concentration of 3 060 μg/ml of Hydrastine and the concentration of Berberine was beyond the Maximum Quantifiable Limit (MQL). These concentrations of alkaloids in the Commercial sample 1:3 dilution show that it is expected that the further dilutions of *Hydrastis canadensis* will still retain much of the alkaloids. It is possible that the reason the alkaloids in the remedies are so well preserved even after undergoing dilutions, is that manner in which it was initially prepared by the Homoeopharmaceutical company followed strict and rigid protocol. As discussed in Chapter 2, under the heading 2.2.2 Standardisation of Homoeopathic Remedies, GMP guidelines are enforced to preserve the integrity of Homoeopathic remedies so that they are safe and effective for patient consumption (World Health Organization 2009). This includes storage and transportation of remedies and this possibly indicates that the Homoeopharmaceutical company from which the

Homoeopathic remedy *Hydrastis canadensis* was purchased, adhered to GMP since the remedies retained such high concentrations of alkaloids.

Additionally, in Chapter 4 under Figure 4.6, the chromatogram of the Researcher's sample 1:10 dilution shows that **A** represents Berberine which peaks at 7.195 minutes, whilst **B** represents Hydrastine which peaks at 9.841 minutes. Comparatively, the Abourashed and Khan (2001) study shows that in Sample 2, the Goldenseal analysis showed Hydrastine to have peaked earlier than Berberine.

The Researcher's study differs from that of Abourashed and Khan (2001) favourably since Hydrastine peaked between 10.5 minutes and 11.5 minutes, as opposed to the Researcher's showing Hydrastine peak at 9.841 minutes, whilst Berberine peaked between 14.5 minutes and 15.5 minutes and the Researcher's peak for Berberine is 7.152 minutes.

5.1.4 The Effect of HPLC on 60% Alcohol as a Negative Standard for the Analysis

The HPLC analysis of the negative standard, 60% Alcohol preparation, showed an absence of peaks in the chromatogram. Hence, no traces of Hydrastine or Berberine were present in the negative control, as expected. This shows the Negative Standard was fairly uncontaminated and is a good control in the experiment.

5.1.5 The Effect of HPLC on Four Samples of Standard Hydrastine

The chromatograms of the HPLC analyses that were conducted on four different samples of the standard Hydrastine showed that their concentrations were seen to peak between 9 minutes and 10 minutes. This allowed the Researcher to determine when to expect Hydrastine to peak in the analysis of *Hydrastis canadensis* dilutions.

5.1.6 The Effect of HPLC on Four Samples of Standard Berberine

The chromatograms of the HPLC analyses that were conducted on four different samples of the standard Berberine showed that their concentrations were seen to peak between 7 minutes and 7.5 minutes. This allowed the Researcher to determine when to expect Berberine to peak in the analysis of *Hydrastis canadensis* dilutions.

5.2 Summary of the Effects of HPLC Analyses

The HPLC analysis of the negative control, 60% Alcohol, showed that the standard contained no traces of Hydrastine or Berberine as the Researcher had predicted. The concentrations of Hydrastine samples were shown to peak between 9 and 10 minutes. Additionally, the concentrations of Berberine were shown to peak between 7 and 7.5 minutes. The chromatograms for these results are depicted in Chapter 4 and Appendix C and Appendix D, respectively.

The HPLC analysis of the Commercial sample 1:3 dilution of *Hydrastis canadensis,* showed that it contained a mean concentration of 3 060 μg/ml Hydrastine, while the

concentration of Berberine in the sample was beyond the Maximum Quantifiable Limit (MQL). The mean concentration of Hydrastine in the Commercial sample 1:10 dilution of *Hydrastis canadensis* was 445 μg/ml, whilst the mean concentration of Berberine was 15 409.67 μg/ml. Comparatively, the Researcher's sample 1:10 dilution of *Hydrastis canadensis* contained a mean concentration of 1203, 67 μg/ml Hydrastine and 20 315.33 μg/ml Berberine. As discussed in Chapter 1, a rationale for conducting this study is to investigate whether a Homoeopathic remedy that is purchased from a Homoeopharmaceutical company and then further diluted in a Homoeopathic laboratory, are comparable to that of the same dilution that could be purchased from the same company, based on their concentrations of end alkaloids. The HPLC results in Chapter 4 show that the Researcher's sample 1:10 dilution of *Hydrastis canadensis* contained significantly higher concentrations of the two alkaloids than that of the Commercial sample 1:10 dilution of *Hydrastis canadensis.* These chromatograms in Chapter 4 show a higher concentration of Hydrastine and Berberine in the Researcher's sample 1:10 dilution than the Researcher had anticipated, as this was prepared from a 1:3 dilution. Hence, these results show that not only are they comparable but also favourable, as this Homoeopharmaceutical method of preparation yielded higher concentrations of the end alkaloids.

Although this is not the aim of the study, it has been observed that the study conducted by Abourashed and Khan (2001) showed that Hydrastine peaked at 10.8 minutes, and Berberine peaked at 14.92 minutes. Their study proved that they were able to conduct the HPLC analysis in half the time of that conducted in other studies, whilst still obtaining significant peaks. This study inadvertently verifies Abourashed (2001)'s

study. This shows that by altering the HPLC method of analysis, the Researcher was able to see peaks at a much faster rate. This resulted in an increased speed and accuracy of results as larger peaks were seen in the chromatograms in chapter four, in a shorter time frame.

5.3 Discussion of the Results of HPLC Analyses

This study aimed to determine the quantities of active ingredients Hydrastine and Berberine that were present in 1:10 and 1:3 pharmaceutical preparations of *Hydrastis canadensis* mother tincture, using HPLC.

The HPLC analyses were conducted at least three times, which further verified the accuracy of the results obtained. This study also proved that the Researcher's preparation of a 1:10 dilution of *Hydrastis canadensis* from the commercially prepared 1:3 dilution of *Hydrastis canadensis* contained higher concentrations of Hydrastine and Berberine, than that of the commercially prepared 1:10 dilution of the remedy.

The HPLC experiments were conducted on the same day, in the same laboratory and under the same conditions. The same HPLC machine was used to run the standards of the experiment and all the sample preparations. The temperature of the laboratory was ambient, and the samples were run one after another, albeit allowing the system to clean between experiments. The former is ensured because even though the HPLC

system contains a column oven which controls the column temperature and temperature of the analysis, the temperature of the laboratory will still affect the temperature of the column oven (Poppiti and Sellers 1994). The temperature of the HPLC column directly affects the retention time of the analysis. Hence, it is important to maintain a constant column temperature so that results may be reproducible (Bélanger, Paré and Sigouin 1997). Alcohol was used as a standard for this experiment. This 60 % ethanol was part of the same batch and of the same percentage of ethanol that was used by the Researcher to prepare the 1:10 diluted preparation of *Hydrastis canadensis* (pers. comm. Dr Shraddha Brijnath. 2019). This was important as the HPLC machine needed an accurate baseline which could be used to compare the peaks seen in different dilutions of Hydrastis canadensis containing 60% Alcohol, so that this could be eliminated as the purpose of the experiment is only to compare the peaks produced as a result of Hydrastine and Berberine.

5.3.1 Comparison of Dilution

The results in Chapter 4, as seen in Figure 4.3, show that in the Researcher's 1:10 preparation of *Hydrastis canadensis,* the average concentration of Hydrastine is 1 203, 67 μg/ml, and the average concentration of Berberine is 20 315.33 μg/ml. These results prove that it is justified to prepare a 1:10 dilution from a 1:3 dilution following standard operating procedures. Moreover, a 1:10 dilution prepared in this manner is preferable as it would remove the need for practitioners to order a variety of Homoeopathic dilutions and therefore save costs in the future, whilst still providing a more concentrated and higher quality remedy of *Hydrastis canadensis* to

Homoeopathic patients, particularly in community Homoeopathic Healthcare Centres in Durban. The same principle may be applied to other remedies that are prepared in 1:10 dilutions, from a 1:3 dilution.

As discussed in Chapter 2, the active ingredients Berberine and Hydrastine in *Hydrastis canadensis* exhibit antibacterial, antifungal and antiparasitic activities. Therefore, it is used in the treatment of gastritis, respiratory conditions and certain cardiovascular conditions, to name a few. For an in-depth review of Berberine and Hydrastine, please refer to Chapter 2, under the heading 2.3.6 Pharmacological actions of *Hydrastis canadensis.*

The results in Chapter 4 showed that the commercial 1:3 diluted preparation of *Hydrastis canadensis* contained such high levels of Berberine, that its concentration was considered to be beyond the Maximum Quantifiable Limit (MQL). It was deduced that this could have been the reason for such a significant concentration of Berberine (20 315.33 μg/ml) being present in the Researcher's 1:10 dilution of *Hydrastis canadensis,* which was prepared from the commercial 1:3 diluted preparation of the remedy. This is favourable as the high concentration of Berberine justifies the current use of *Hydrastis canadensis* as an antimicrobial, antibacterial as well as in the treatment of respiratory conditions. As stated in Chapter 2, according to The British Herbal Compendium, the recommended quantities of the alkaloids in *Hydrastis canadensis* is 1.5% – 4 % for Hydrastine and 2.5% for Berberine (Pengelly *et al.* 2012). Furthermore, the results in Chapter 4 showed that the commercial sample 1:3 dilution

contained 3 060 μg/ml of Hydrastine. Consequently, the Researcher's preparation of the 1:10 dilution that was prepared from the commercial 1:3 dilution of *Hydrastis canadensis,* contained 1203, 67 μg/ml of Hydrastine. This is a promising value to see as this is just less than half the concentration, despite the remedy having been diluted further. These concentrations of the active ingredients, Berberine and Hydrastine were significantly higher than the Researcher had anticipated before conducting the HPLC analysis. As stated in the Abstract, these results provide a justification for using a 1:3 dilution to prepare a 1:10 dilution, which is commonly used in Homoeopathic practice.

This research further proved that a 1:3 diluted preparation of *Hydrastis canadensis* yielded favourable results when it was used as a start-up potency to produce consequent diluted preparations of the same remedy. This is based on the high concentrations of alkaloids Hydrastine and Berberine that were depicted in the chromatograms in Chapter 4 and the relevant appendices. This is seen in the concentrations of alkaloids Berberine and Hydrastine, that were yielded in the Researcher's 1:10 preparation, depicted in the chromatogram in Chapter 4, under the heading, Figure 4.6. Representative chromatogram showing the presence of Berberine and Hydrastine in the first 1:10 Researcher preparation of *Hydrastis canadensis*.

The Researcher's preparation of *Hydrastis canadensis* 1:10 dilution showed significantly higher concentrations of Hydrastine and Berberine than that of the commercially prepared 1:10 dilution of the remedy. As stated in Chapter 2, the

Researcher prepared this 1:10 dilution from the commercial 1:3 dilution of *Hydrastis canadensis*, using 60% Alcohol that had been stored in the DUT Homoeopathic Lamina flow room (Pers. comm. Dr Brijnath 2019.). It is worth mentioning that DUT Homoeopathic department would be storing the medication under optimal conditions and using sanitary equipment for preparing medications, as remedies that are produced are of high quality as seen by the concentrations of active ingredients that are present in the Researcher's 1:10 dilution of *Hydrastis canadensis*.

5.3.2 Quality Control

As it is shown in the results of the chromatograms in Chapter 4, it is interesting to note that such a high amount of active ingredients was preserved in the commercial 1:3 diluted preparation of *Hydrastis canadensis*. This is commendable to the Homoeopharmaceutical company that prepared these tinctures of *Hydrastis canadensis*.

The Researcher confirmed with the staff at the Homoeopharmaceutical company from which the commercial remedies were purchased, that the different pharmaceutical preparations of *Hydrastis canadensis* were prepared from pure source material that was imported from Canada and then preserved according to strict protocol and following GMP (MediHerb 2020). These remedies were prepared in segments by more than one person, whilst still following strict protocol. Comparatively, at DUT, one Laboratory Technician who is also a qualified Homoeopath prepares the entire remedy

to ensure that there is less room for contamination and possible errors. As discussed in Chapter 2, in the case of this Research, the Researcher prepared the remedies under the supervision and guidance of a specialist Laboratory Technician and Homoeopath, following GMP. Furthermore, the commercial remedies were packaged safely to prevent the glass bottles from breaking and were delivered to the Researcher in a refrigerated vehicle, which ensured that the integrity of the remedy remained. A remedy that is prepared from poor quality source materials may compromise the effectiveness of the remedy (Fusion Homoeopathics 2020). Moreover, as mentioned in Chapter 2, storage and transportation of raw materials require optimal temperatures to prevent damaging or denaturing their active components (MediHerb 2020).

5.3.3 Alkaloid Concentration Compared to Medicinal Uses

As discussed in Chapter 2, Hydrastine (1.5% – 4 %) and Berberine (2.5%) are the two main active ingredients in *Hydrastis canadensis,* for which this remedy is used (Pengelly *et al.* 2012). Hydrastine is known for its astringent, antibacterial and choleretic properties (Chadwick, Wu and Kinghorn 2001). Berberine also has antibacterial effects and is also used for its antifungal properties (Mahady *et al.* 2003). These two active ingredients contribute to *Hydrastis canadensis'*s use in the treatment of bacterial infections, liver disorders and dyspepsia, to mention a few. The results in Chapter 4 show that mean concentration of Hydrastine present in the commercial sample 1:3 dilution of *Hydrastis canadensis* is 3 060 μg/ml. Furthermore, the mean concentration of Hydrastine in the commercial sample 1:10 dilution is 445 μg/ml, whilst that of the Researcher sample 1:10 dilution is 1 203, 67 μg/ml Hydrastine. This shows that there are high concentrations of Hydrastine present in *Hydrastis canadensis* even

after dilution, and therefore the remedy may be used as an astringent, antibacterial and choleretic, as discussed earlier in this paragraph.

It is also seen in Chapter 4, that the mean concentration of Berberine that is present in the commercial sample 1:3 dilution *Hydrastis canadensis* is beyond the Maximum Quantifiable Limit (MQL). Furthermore, the commercial sample 1:10 dilution contains a mean concentration of 15 409.67 μg/ml of Berberine, whilst the mean concentration of Berberine in the Researcher sample 1:10 dilution is 20 315.33 μg/ml. The large concentrations of Berberine that are seen in these results in Chapter 4, show that *Hydrastis canadensis* may be used for the aforementioned conditions. The therapeutic effects of *Hydrastis canadensis* are discussed in more detail in Chapter 2, under the heading 2.3.5 Therapeutic Uses of *Hydrastis canadensis*.

5.3.4 Wavelengths

It was seen in Chapter 2, (Joshi 2013) found that the wavelength for the separations of *Withania somnifera* were recorded at 230 nm, whereas the results of this study on *Hydrastis canadensis* showed that the absorption spectra was recorded at 254 nm. The wavelength affects the absorption of the analyte. This means that the higher the wavelength, the greater the absorption of the analyte and the higher the peak. In future HPLC studies that will be conducted, it will be useful to note that using a higher wavelength in nanometres will result in more accurate and clear results.

5.3.5 Sensitivity of HPLC Detection

As discussed in Chapter 2, under the heading 2.6.5 Studies on Phytochemistry Conducted Using HPLC, the study conducted by Ganzera, Choudhary and Khan (2003) used a flow rate of 1 ml/min with a sample/injection volume of 10 ml. However, their results showed that the flow rate had to be reduced to 0.5 ml/min to in order to improve their retention times and improve the sensitivity of the analysis. In HPLC, the flow rate affects the pressure in the system, thereby affecting the peak width and the retention time of the analysis. Sharp peaks are indicative of increased sensitivity and resolution. In this experiment, the flow rate was set at 1 ml/min and this produced sharp peaks in the chromatogram. It further affected the retention time positively as Hydrastine and Berberine peaked between 7 and 9 minutes.

The R-squared value shows the sensitivity of the experiment. As discussed in the introduction of Chapter 4, the closer to 1 that the R-squared value is, the greater the sensitivity of the experiment. In this research, the R-squared value of the Hydrastine standard is 0.9940 and that of the Berberine standard is 0.9766. As stated previously in Chapter 4, since the confidence limits should be greater than 95% and these are above 97%, this indicates a good level of sensitivity in the HPLC analysis that was conducted in this research.

5.3.6 Technological Advances

Although this was not part of the study, while conducting this experiment the Researcher deduced that the HPLC system that was used is more sensitive to

alkaloids due to improved technology. This is seen in the increased speed at which peaks are formed on the chromatogram and alkaloids are analysed. Reference is made to the HPLC method used by (Abourashed and Khan 2001) where the analytical run was set to 20 minutes, Berberine only peaked at 14.92 min for sample 1 and Hydrastine peaked at 10.87 minutes for the same sample. Although sodium acetate was also used in their mobile phase, their pH was only set to 4.0. On the other hand, for this HPLC analysis the Researcher set the pH of the buffer, sodium acetate, to 4.8. Buffers maintain the pH of solutions when analytes are added. This decreased the retention time and peaks of Berberine and Hydrastine were seen in half the expected time. Furthermore, the Researcher opted for sodium acetate as a buffer as ammonium acetate may have stripped the silica in the C_{18} column, thereby damaging the column and producing inaccurate results in the experiment (Pers comm. Dr Mohanlall 2020).

As seen in Chapter 2, the time for the HPLC analysis conducted by (Weber *et al.* 2003) which detected the presence of alkaloids present in Goldenseal root powder from Supplier A, was set at 40 minutes. Berberine peaked between 11 and 12 minutes and Hydrastine peaked between 14 and 15 minutes. However, the reason for their 40 minute run time was to allow for other alkaloids to be detected, which was not the case in this research project. In this research project, the Researcher only required the concentrations of Hydrastine and Berberine and according to (Abourashed and Khan 2001) both these alkaloids were shown to peak in under 20 minutes.

The Researcher initially set the analytical run for this experiment at 20 minutes as well. However, as shown in Chapter 4, Berberine peaked at around 7 minutes and the

analytical run time was adjusted to 11 minutes and the samples of Berberine were run again. Berberine again peaked at around 7 minutes and therefore the analytical run time was set at 11 minutes for all standards and samples of Homoeopathic remedies. It can be deduced that there is accuracy to the study conducted by (Abourashed and Khan 2001) as the Researcher found that the alkaloids reached its peak time much quicker than the allocated time, which is 40 minutes. Hence, the study of (Abourashed and Khan 2001) is verified because the Researcher further proved that a 20 minute run time is acceptable.

5.3.7 Time Differences

The protocol for 40 minutes probably allows excess time to ensure accuracy of the results. This experiment verifies the reasoning of (Abourashed and Khan 2001) that a substance can peak at a faster rate, as once the peak is reached at 9 minutes, there are no further significant changes or peaks. In accordance with Abourashed and Khan (2001) regarding the development of their “fast and simple” HPLC method with a run time of 20 minutes, the Researcher observed that during this HPLC procedure, the alkaloids peaked at 7 minutes (Berberine) and at 9 minutes (Hydrastine). This supports Abourashed and Khan’s (2001) “fast and simple” method of HPLC and also suggests that the time can be further decreased to produce significant peaks.

In comparison to the study mentioned in Chapter 2, conducted by (Abourashed and Khan 2001), this study resulted in Berberine and Hydrastine producing equations of $Y = 6280*X + 34285$ and $Y = 8483*X – 118355$ respectively. The correlation coefficient (R^2) was 0.9766 for Berberine and 0.9940 for Hydrastine. The mean retention time for

Hydrastine standard is 9.842 minutes (9.842 min + 9.835 min + 9.846 min + 9.845 min) and for Berberine standard is 7.309 minutes (7.375 min + 7.314 min + 7.279 min + 7.268 min). This retention time was used to observe and identify the peaks for Berberine and Hydrastine in the chromatograms of different preparations of *Hydrastis canadensis*.

6 CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

This research proved that a 1:3 diluted preparation yielded favourable results when used as a start-up potency to produce consequent diluted preparations of the same remedy, based on the high concentrations of alkaloids Berberine and Hydrastine that were depicted in chromatograms. A recommendation would be to further investigate whether a different dilution of *Hydrastis canadensis*, such as a 1:5 dilution would be a good start-up potency to produce a 1:10 dilution. However, it should be noted that a 1:5 diluted preparation of *Hydrastis canadensis* is not always readily available for purchase. Furthermore, since the plant is native to North America and is transported to South Africa, it is not always possible for commercial suppliers to produce various diluted preparations, unless it is commonly used by Homoeopaths.

6.2 Recommendations

As discussed in Chapter 2, Gentry *et al.* (1998) showed that *Hydrastis canadensis* has the ability to combat Multidrug-Resistant (MDR) strains of *Mycobacterium tuberculosis* and other *Mycobacterium* species. Perhaps further research could be done to investigate the Homoeopathic or Phyto-therapeutic use of *Hydrastis canadensis* in the

treatment of Malaria and Tuberculosis, particularly in South Africa where these diseases are rife.

As discussed in Chapter 2, according to van Wyk and Wink (2015), *Hydrastis canadensis* is used Phytotherapeutically as anti-haemorrhagic. However, the use of *Hydrastis canadensis* in the treatment of ischemia could be further investigated due to the remedy's substantial Berberine content in 1:3 and 1:10 diluted preparations of this remedy, as depicted in the chromatograms in chapter 4. A study may be conducted on a group of patients that suffer from haemorrhagic conditions, to determine the therapeutic effects of *Hydrastis canadensis* 1:10 dilution.

According to the literature discussed in Chapter 2, a study that was conducted by Scazzochio *et al*. (2001) validated the anti-bacterial effects of Berberine and B-hydrastine in *Hydrastis canadensis* based on the bactericidal activity that was exhibited against gram-positive and gram-negative bacteria, yeast and protozoa. Additionally, the results of this study as depicted in Chapter 4, showed high concentrations of Berberine in 1:3 and 1:10 pharmaceutical preparations. A suggested study would be to determine whether 1:3 and 1:10 diluted preparations of *Hydrastis canadensis* that are added to an aqueous cream base and applied topically, would be effective in the treatment of microbial infections, such as bacterial impetigo.

A further study that could be conducted in the future is to analyse the concentrations of Berberine in *Berberis aristata*. According to the study cited in Chapter 2 under 2.3.3 Toxicology of Hydrastis canadensis, despite Berberine's ability to exhibit phototoxic properties *Berberis aristata* was shown to be safer than *Hydrastis canadensis* (Karim *et al.* 2015).

6.2.1 Wavelengths

In future HPLC studies that will be conducted, it will be useful to note that using a higher wavelength in nanometres will result in more accurate and clearer results (Joshi 2013).The reason for this is discussed further in Chapter 5, under the heading 5.4.4 Wavelengths.

6.2.2 Pharmacopoeias

A further study could be done to compare the content of active ingredients in remedies of *Hydrastis canadensis*, or active ingredients in any other Homoeopathic remedy, that are prepared according to different Homoeopathic Pharmacopoeias. An example of such is comparing the contents of active ingredients in a remedy that has been prepared according to the French Homoeopathic Pharmacopoeia, with the same remedy that has been prepared according to the German Homoeopathic Pharmacopoeia. However, the Researcher found that unlike the German Homoeopathic Pharmacopoeia, the French Homoeopathic Pharmacopeia is difficult

to source in South Africa, despite many attempts whilst in the process of finalising a research topic.

6.2.3 Dilutions

This study showed that the concentrations of Hydrastine and Berberine were the highest in the commercial 1:3 preparation of *Hydrastis canadensis*. A recommended study would be to further assess 1:3 and 1:10 dilutions of *Hydrastis canadensis* in vitro, to compare their effectiveness. This would enable a Researcher to see whether higher concentrations of the active ingredients Hydrastine and Berberine in a 1:3 dilution, increase the therapeutic effects of the remedy.

6.2.4 Quality Control

Another important factor in Quality Control of plant-sourced remedies is Good Agricultural Practise (GAP). However, Homoeopathic Practitioners often purchase Homoeopathic remedies from pharmaceutical companies. These Pharmaceutical companies often import the plant substance if it is only available in other countries, and thus, it is difficult to ensure that GAP has been implemented. A recommendation would be to conduct a survey amongst the different Homoeopharmaceutical companies, to determine whether they have ensured that the plant material that they are purchasing from other countries has been planted and harvested according to the criteria set out in GAP. GAP is a process which covers all aspects planting, such as choosing the best quality seeds, sewing them into fertile soil of an optimal pH, environmental conditions in which the seeds are grown, the use of pesticides and fertilisers as well as time of year and day at which the plants are harvested.

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APPENDICES

Appendix A

German Homoeopathic Pharmacopoeia

Method: HAB 4a for dried herbal drugs

Monograph: *Hydrastis canadensis*

The roots and rhizomes of the plant will be used to prepare the mother tincture. These dried underground parts of *Hydrastis canadensis,* contain no less than 3 per cent of alkaloids, calculated as berberine (M 353.4)

Tests:

Foreign matter:

- Not more than 5 per cent of residues of aerial parts.
- Rhizomes and roots that contain whitish or brown fracture and material in which the vascular bundles are separated only by narrow medullary rays or where the xylem forms an unbroken ring, or a solid core are not present.
- Not more than 2 per cent of other foreign matter.

Assay:

0.2000 g of the powdered form of *Hydrastis canadensis* will be diluted in 50.0 ml of Ethanol (60 per cent) *R.* This mixture will be shaken for 30 minutes and the drug will be filtered. 10.0 ml of the filtrate will be diluted to 100.0 ml of 0.05 M of methanolic sulfuric acid RH (relative humidity) as the compensation liquid. The absorbance *A* of the solution will be measured at 425 nm against 0.05 M methanolic sulfuric acid *RH* as the compensation liquid. The per cent alkaloid content will be determined x%, calculated as berberine, from the specific absorbance $A_{1cm1}^{\%} = 163$, according to the following equation: X% = 3.07 A/*m* where *m* is the mass of the herbal drug in grams.

Storage:

The mother tincture will be stored away from light.

Dosage forms:

Production:

The production of Hydrastis canadensis mother tincture will be prepared according to method HAB 4a. The German preparation is a 1:10 dilution. One part of the dried herbal drug will be diluted using ten parts of Ethanol (62 per cent *m/m*). The mother tincture will be prepared by maceration or percolation.

Production by maceration:

The plant material will be crushed into a coarse powder or cut into small pieces. Thereafter, it will be mixed with Ethanol (62 percent *m/m*) and then it will be left to stand in a closed container for five days. After this time, the residue from the plant material will be separated from the Ethanol and if necessary, the plant material will be pressed out in order to remove excess residue. If the latter is done, the residue will then be combined with the Ethanol.

Production by percolation:

If necessary, the herbal drug will be comminuted. It will be mixed thoroughly with a portion of ethanol of the appropriate concentration, and then it will be left to stand in a closed container for five days. After this time, it will then be transferred to a percolator and the percolate will be left to flow slowly, at room temperature, while making sure that the herbal drug to be extracted is always covered with the remaining ethanol. The residue will be pressed out and the expressed liquid will be combined with the percolate.

Characteristics:

The mother tincture is a dark yellow liquid that has an odour.

Identification:

Solution S will be the mother tincture.

Tests:

Relative density: 0.890 to 0.905.

Dry residue: Not less than 1.8 per cent.

Assay:

2.000 g of the mother tincture of *Hydrastis canadensis* will be diluted in 100.0 ml of Ethanol (60 per cent) with 0.05 M methanolic sulfuric acid *RH.* 5.0 ml of the resulting solution will be diluted to 25.0 ml with 0.05 M methanolic sulfuric acid *RH.* The absorbance *A* of the solution will be measured at 425 nm against 0.05 M methanolic sulfuric acid *RH* as the compensation liquid. The per cent alkaloid content will be determined x%, calculated as berberine, from the specific absorbance $A_{1cm1}^{\%} = 163$, according to the following equation: X% = 3.07 A/*m* where *m* is the mass of the herbal drug in grams.

Storage:

The mother tincture will be stored away from light.

Appendix B

Researcher Preparation of 1:10 Dilution of *Hydrastis canadensis* from Commercial 1:3 Dilution of *Hydrastis canadensis*

Aim:

To produce 150 ml of *Hydrastis canadensis* 1:10 dilution, from *Hydrastis canadensis* 1:3 dilution.

Apparatus:

Measuring cylinder

Consumables:

150 ml amber glass bottles (AGB)

Labels

Stationery

Ingredients:

60% ethanol ; 45% ethanol

Distilled water

Hydrastis canadensis 1:3 dilution from CoMed health

Method:

All apparatus and utensils will be (washed and flamed) cleaned and odourless.

1. To prepare 1:10 dilution of *Hydrastis canadensis* from the purchased 1:3 dilution:

   - By means of (BMO) a measuring cylinder add 45ml (3/10 x 150ml) of *Hydrastis canadensis* 1:3 dilution to a 150 ml AGB.

   - BMO a measuring cylinder, add 105ml (7/10 x 150ml OR 150ml – 45ml) of 45% ethanol (ROH) to the same 150 ml AGB. Cap and mix gently.

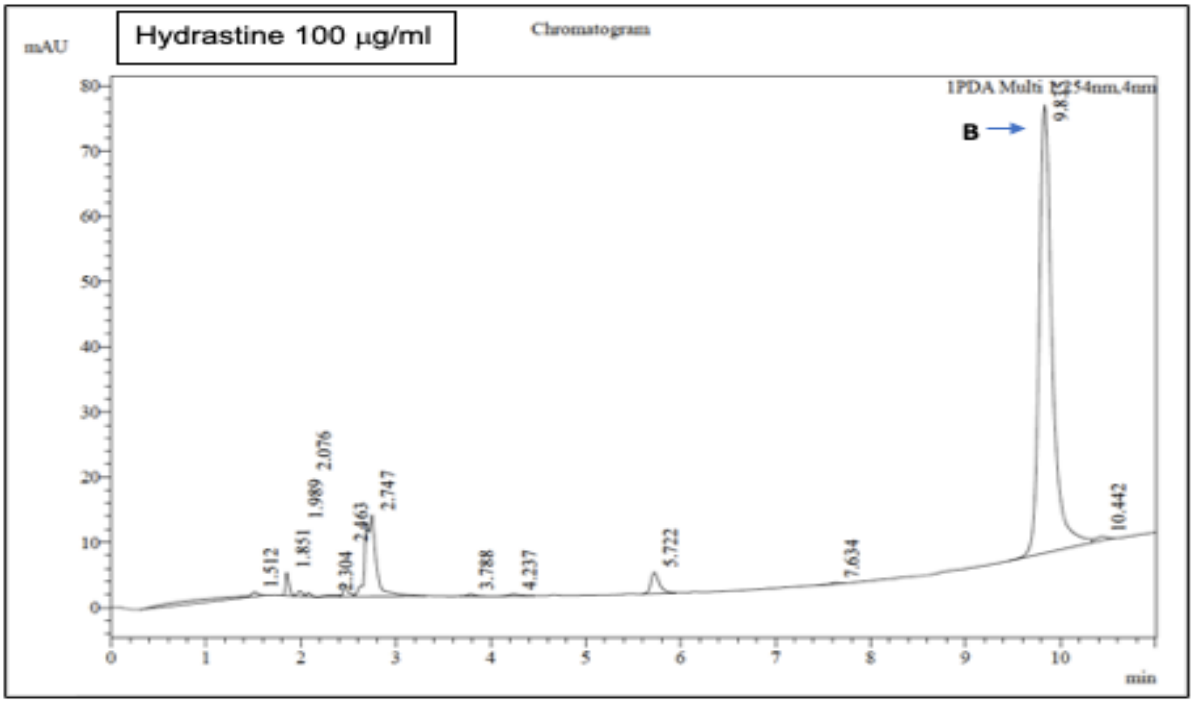
   - Label: *Hydrastis canadensis* 1:10 dilution

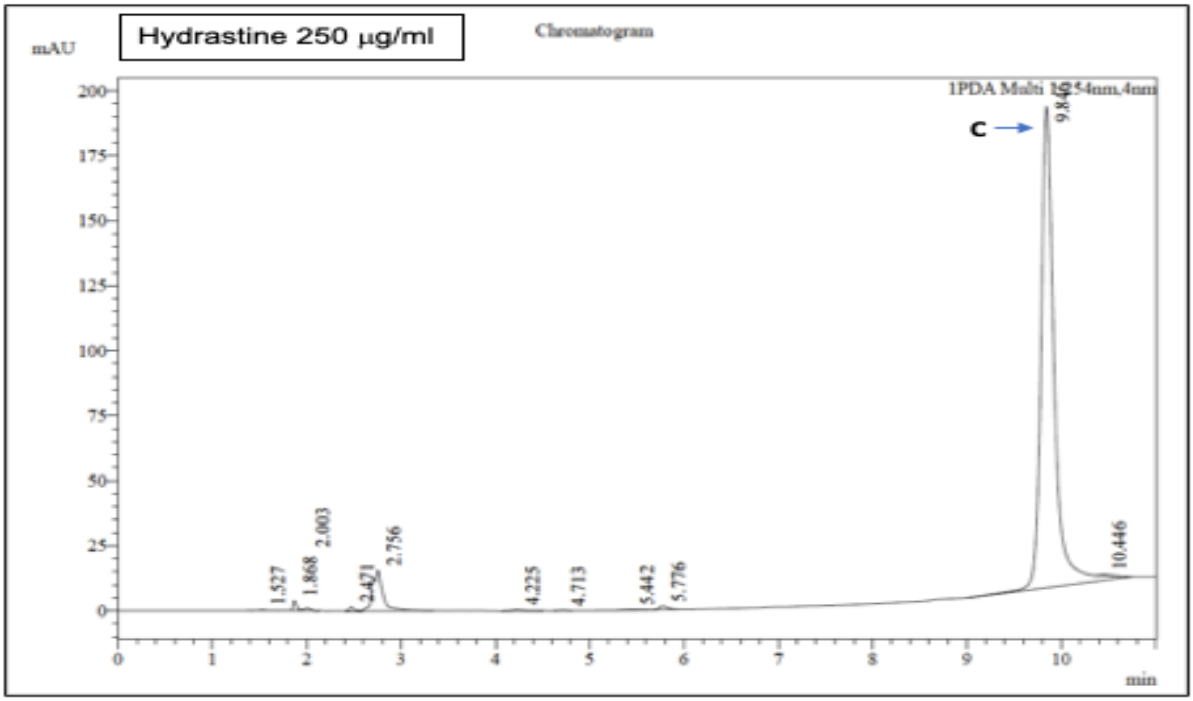
   Formula used: Potency X Volume

   Calculations:
   To prepare 1:10 dilution from a 1:10 dilution
   - 1:3 = 1:10 (divide by 1)
   - Cross multiply: (3): (10)
   - Answer: *3:10*
   - Therefore, 1:3 can be written as 3:10 or 3/10
   - Potency X Volume
   - 3/10 x 150ml = 45ml
   - 7/10 x 150 = 105ml (150 ml – 45 ml = 105 ml)

Appendix C

HPLC Analysis of the Standard Hydrastine using samples of 100 µg/ml, 200 µg/ml and 500 µg/ml





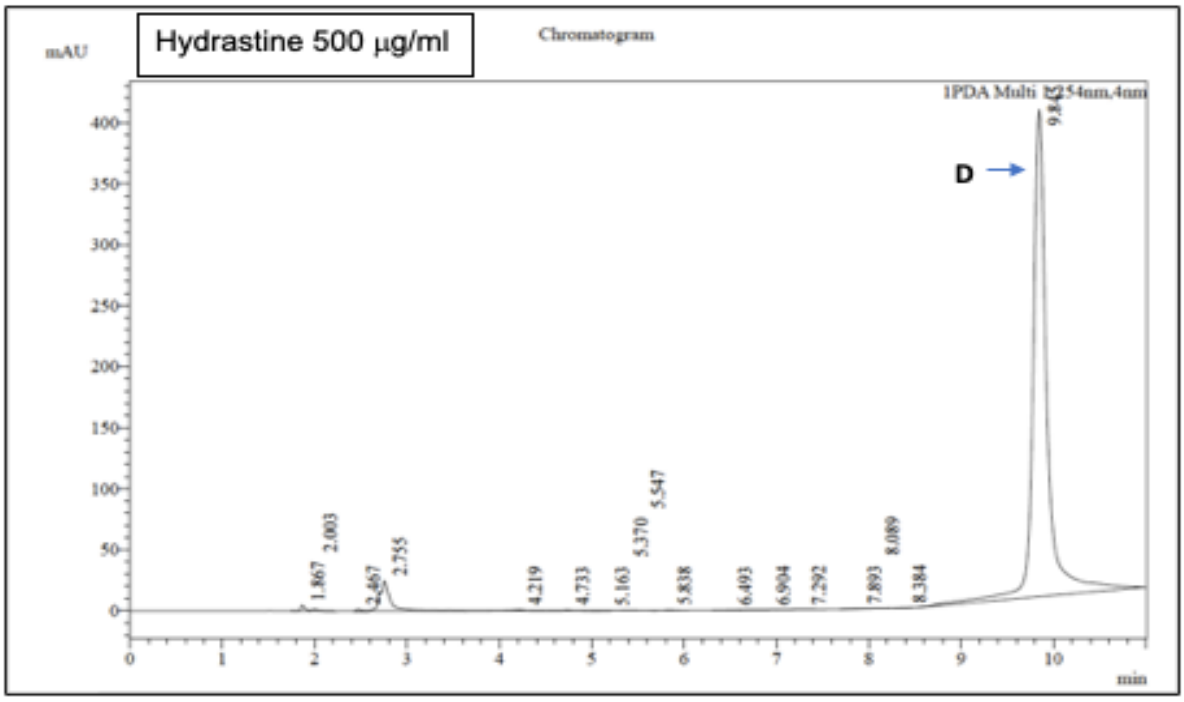


Figure 4.1. Representative chromatogram showing the standard

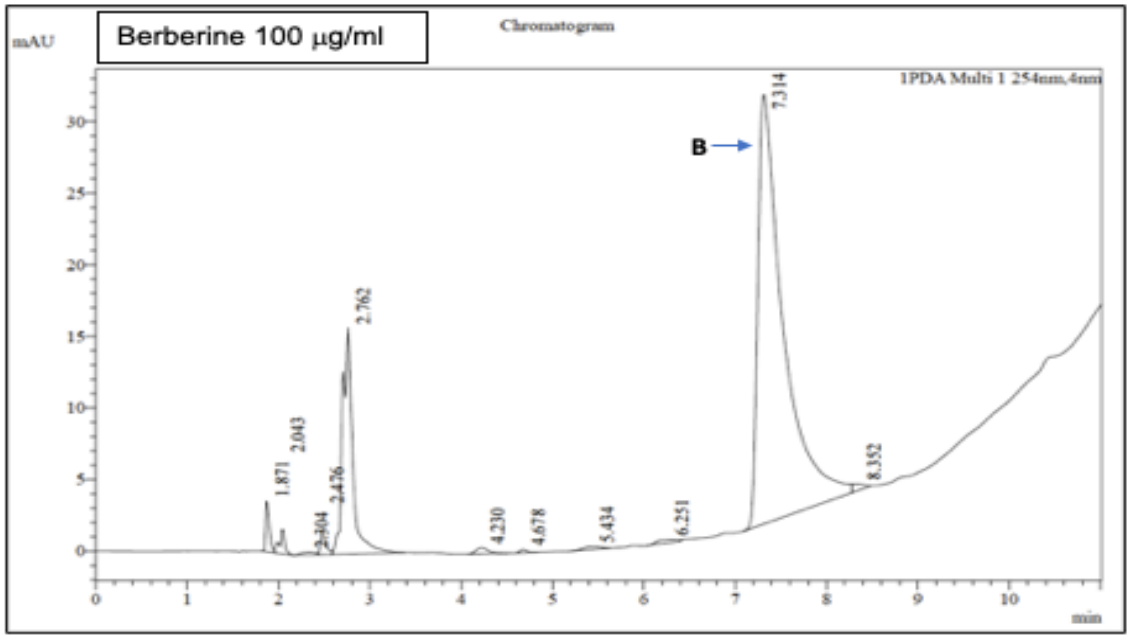
B – Hydrastine 100 µg/ml. Retention time - 9.835 minutes

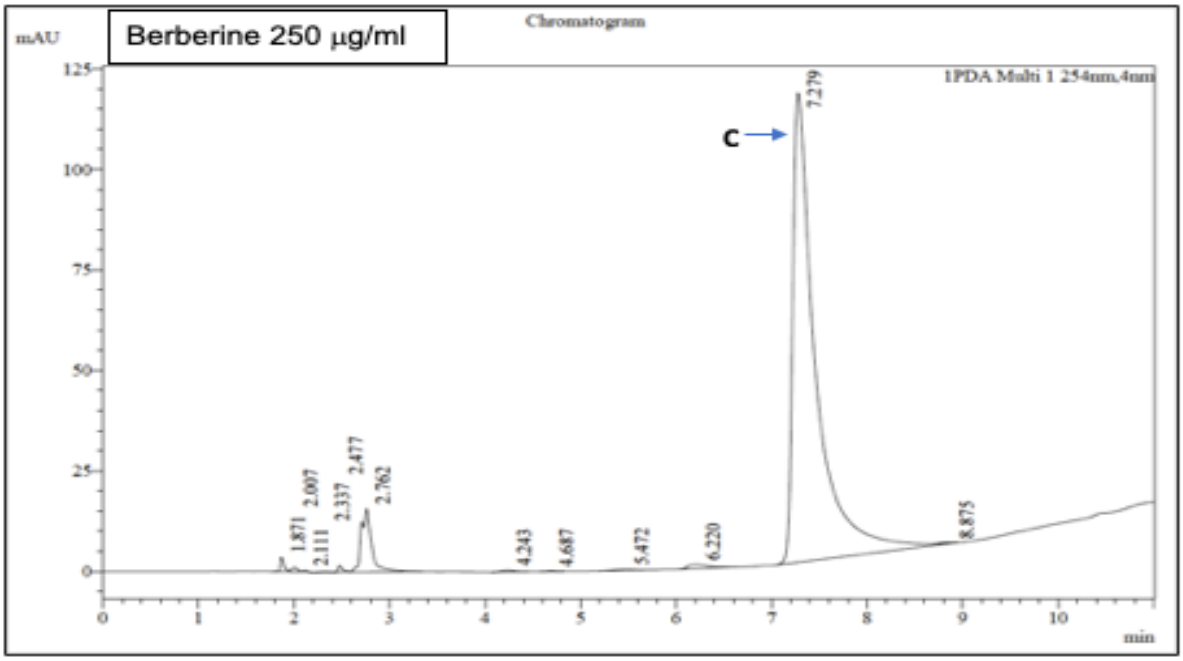
C – Hydrastine 250 µg/ml. Retention time - 9.846 minutes.

D – **Hydrastine** 500 µg/ml. Retention time - 9.845 minutes

Appendix D

HPLC Analysis of the Standard Berberine using samples of 100 µg/ml, 200 µg/ml and 500 µg/ml





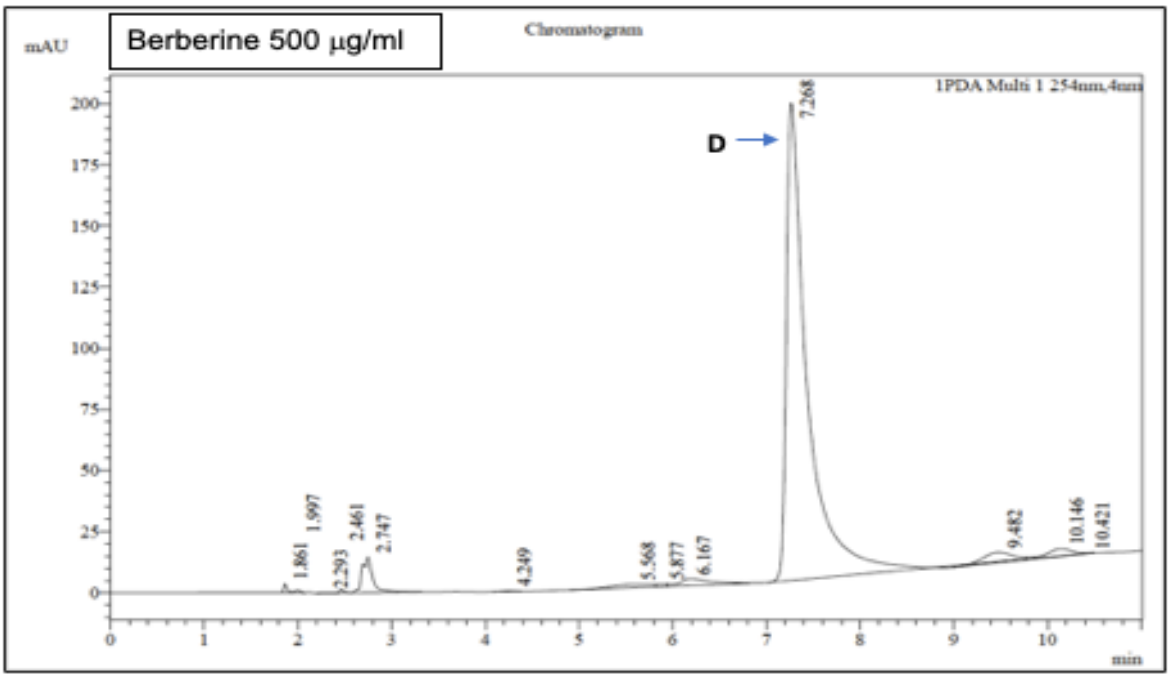


Figure 4.2. Representative chromatogram showing the standard

B – Berberine 100 µg/ml. Retention time - 7.314 minutes

C – Berberine 250 µg/ml. Retention time - 7.279 minutes

D – Berberine 500 µg/ml. Retention time - 7.268 minutes

Appendix E

HPLC Analysis of 60% Alcohol Preparation, the Negative Control

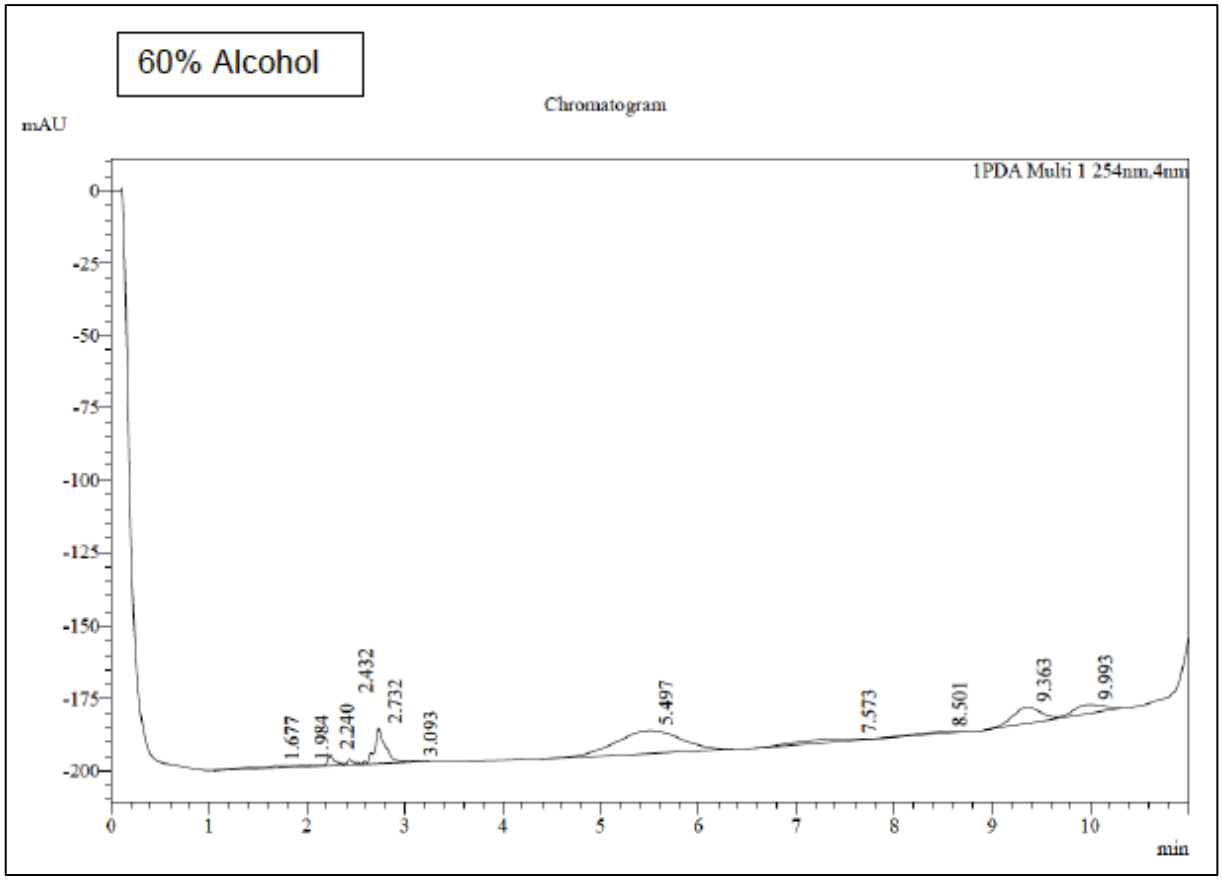


Figure 4-1. Representative chromatogram showing the absence of peaks in the Standard, 60% Alcohol preparation, used as a negative control

This figure shows an absence of peaks, which means that there are no other alkaloids or major contaminants present in the standard, 60% Alcohol preparation (Appendix E).

Appendix F

Triplicate HPLC analysis of 1:3 Commercial Preparation of *Hydrastis canadensis*

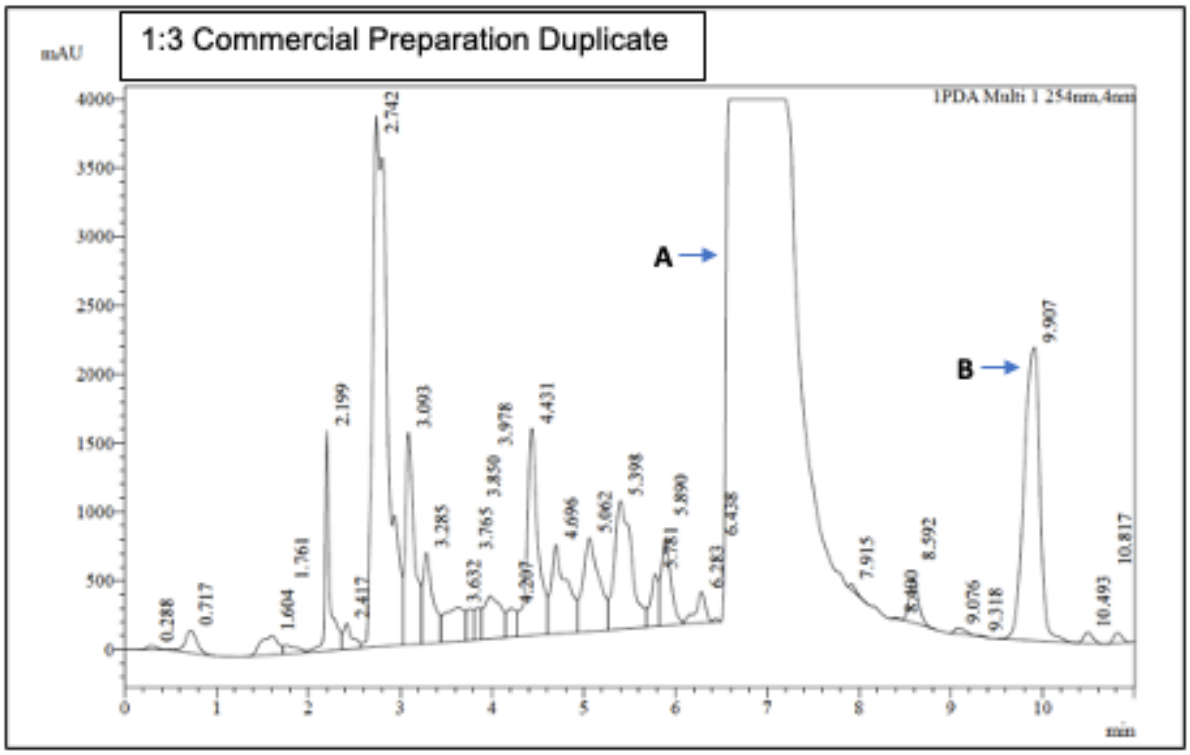


Figure 4.4.1. Representative chromatogram showing the presence of Berberine and Hydrastine in the second 1:3 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 6.438 minutes with results beyond maximum quantifiable limits

B – Hydrastine. Retention time - 9.907 minutes

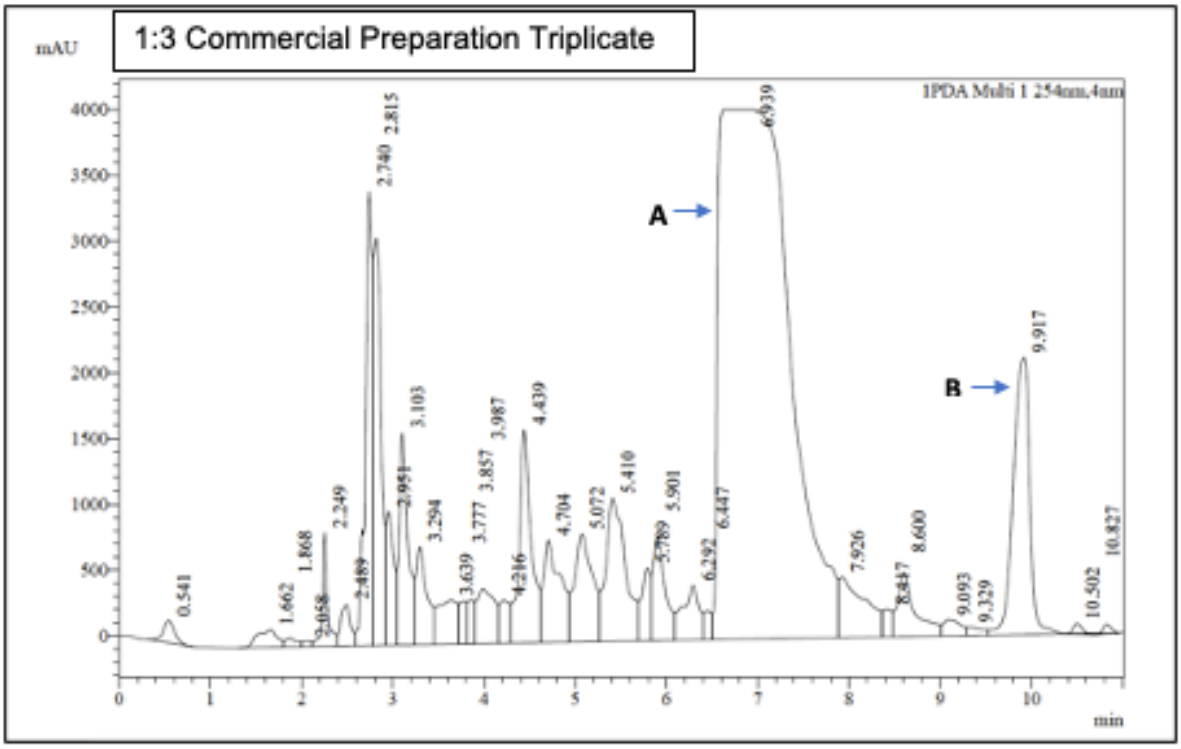


Figure 4.4.2. Representative chromatogram showing the presence of Berberine and Hydrastine in the third 1:3 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 6.447 minutes with results beyond maximum quantifiable limits

B – Hydrastine. Retention time - 9.917 minutes

Appendix G

Triplicate HPLC analysis of 1:10 Commercial Preparation of *Hydrastis canadensis*

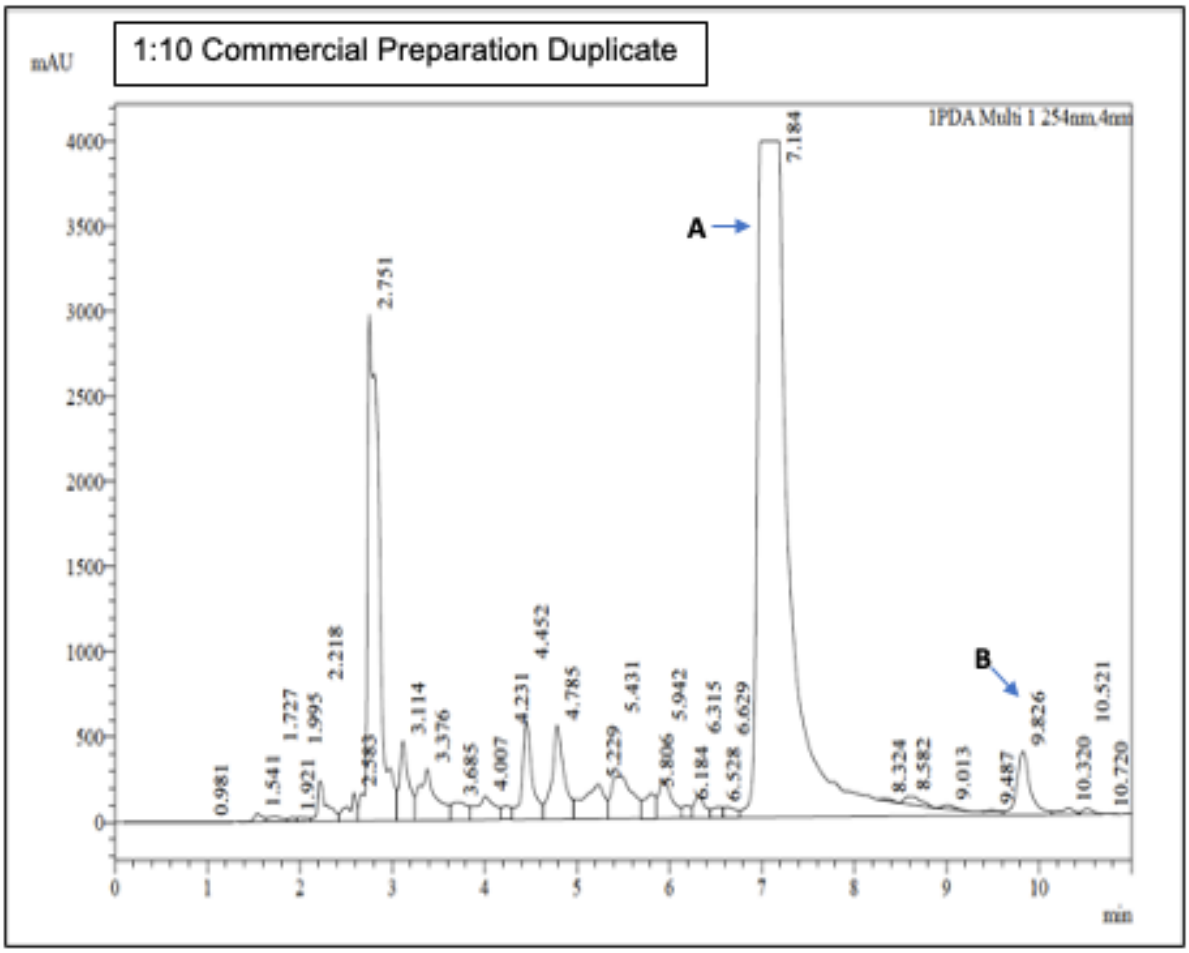


Figure 4.5.1. Representative chromatogram showing the presence of Berberine Hydrastine in the second 1:10 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 7.184 minutes

B – Hydrastine. Retention time - 9.826 minutes

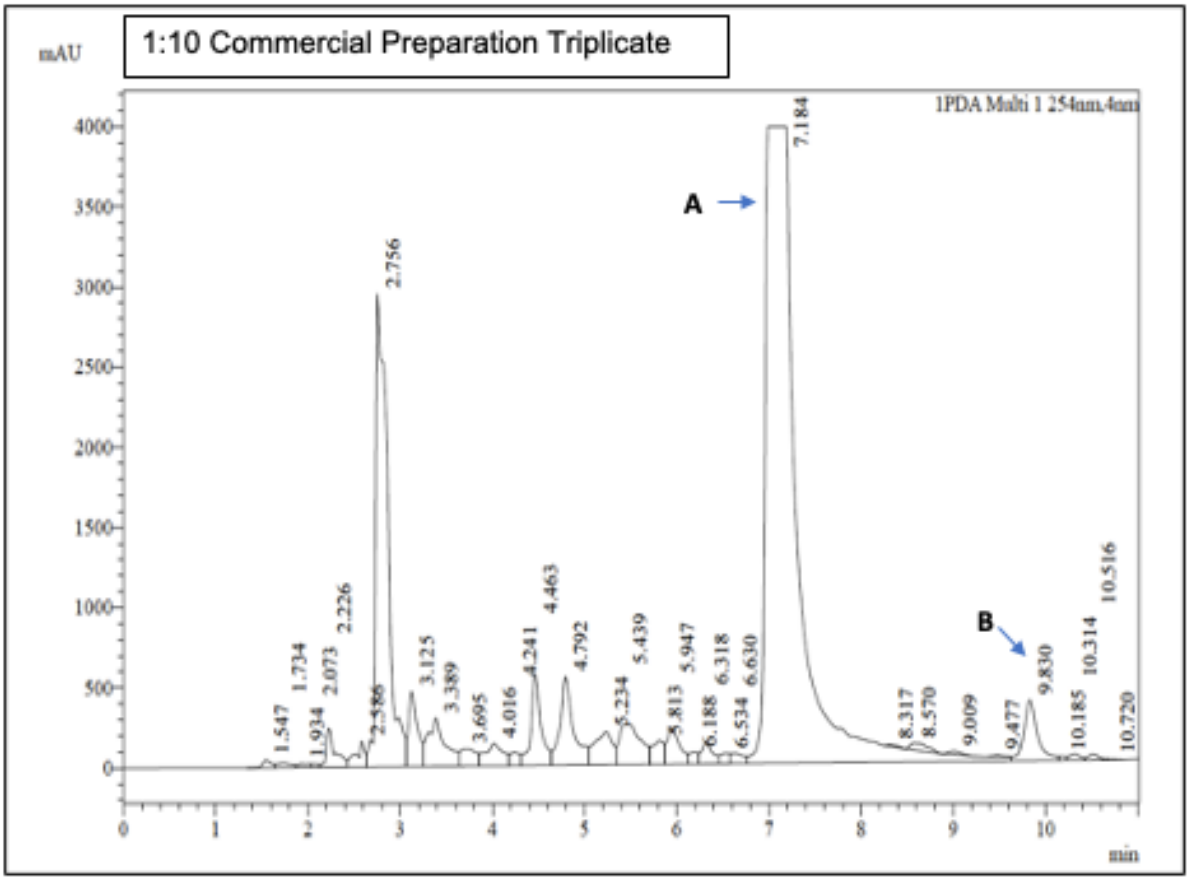


Figure 4.5.2. Representative chromatogram showing the presence of Berberine Hydrastine in the third 1:10 commercial sample of *Hydrastis canadensis*

A – Berberine. Retention time - 7.184 minutes

B – Hydrastine. Retention time - 9.830 minutes

Appendix H

Triplicate HPLC analysis of 1:10 Researcher Preparation of *Hydrastis canadensis*

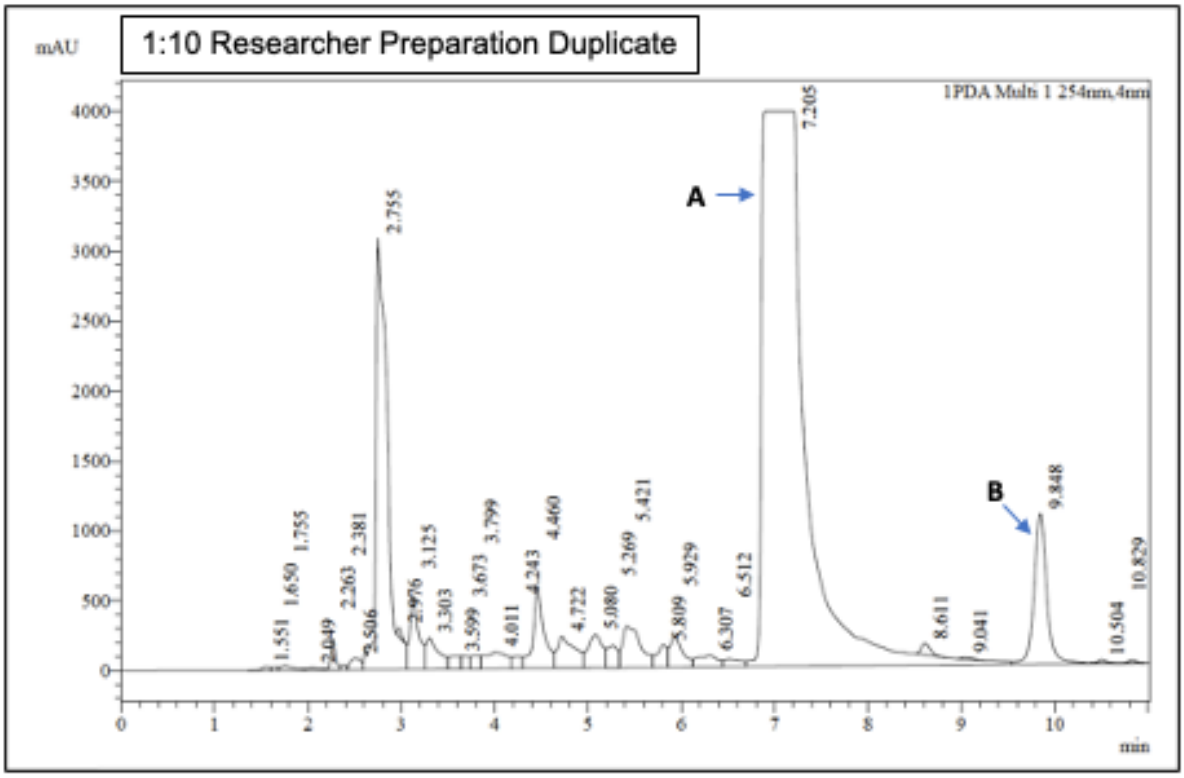


Figure 4.6.1. Representative chromatogram showing the presence of Berberine and Hydrastine in the second 1:10 Researcher preparation of *Hydrastis canadensis*

A – Berberine. Retention time - 7.205 minutes

B – Hydrastine. Retention time - 9.848 minutes

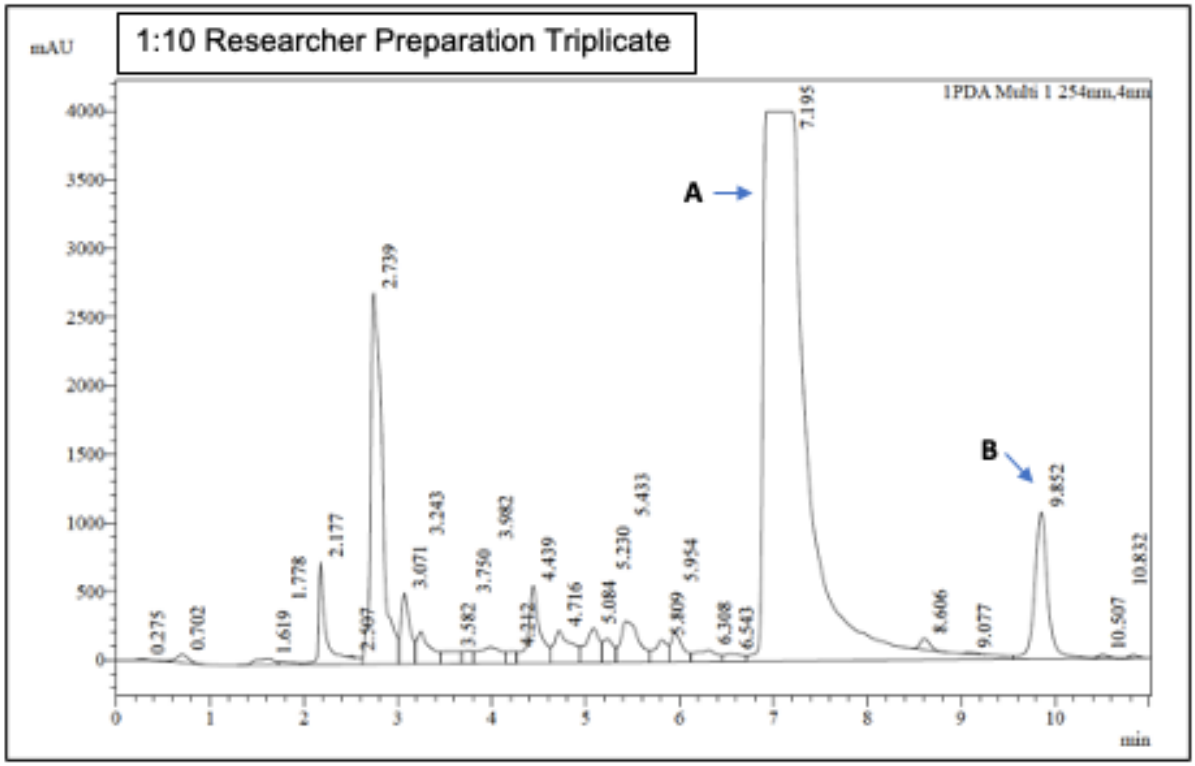


Figure 4.6.2. Representative chromatogram showing the presence of Berberine and Hydrastine in the third 1:10 Researcher preparation of *Hydrastis canadensis*

A – Berberine. Retention time - 7.195 minutes

B – Hydrastine. Retention time - 9.852 minutes