# FRACTIONATION OF THE PHYTEXPONENT POLYHERBAL FORMULATION BY COLUMN CHROMATOGRAPHY AND QUALITATIVE ANTIOXIDANT SCREENING

**ELIZABETH NJOKI**

**BPHARM/54311/2016**

**A RESEARCH PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A BACHELOR OF PHARMACY DEGREE OF**
**MOUNT KENYA UNIVERSITY**

**COLLEGE OF HEALTH SCIENCES**

**SCHOOL OF PHARMACY**

**DEPARTMENT OF PHARMACEUTICS AND PHARMACOGNOSY**

**DECEMBER 2020**

## DECLARATION

This project is my original work and has not been presented for the award of a degree in any other university or any other award.

Signature _____ Date_____

ELIZABETH NJOKI

BPHARM/54311/2016

This project has been conducted and submitted for examination with my approval as University supervisor.

Signature _____ Date_____

PROF. EPAPHRODITE TWAHIRWA

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

SCHOOL OF PHARMACY

## DEDICATION

I dedicate this project to my parents and loving husband for their prayers, financial support and encouragement throughout my research work.

## ACKNOWLEDGEMENT

I would like to express my deep and sincere gratitude to the Almighty God, for His grace, favor and good healthy throughout the research work.

I am extremely grateful to my parents for their prayers, sacrifices and moral support. My heartfelt thanks go to my husband for his love, understanding, prayers and continuing support to complete the research successfully.

My sincere thanks go to professor Epa Twahirwa for his motivation, guidance, immense knowledge and assistance.

Finally, I would like to extend my appreciation to Mr. Gervason Moriasi and Elias for their immense support and guidance during the planning and development of this research work.

## ABSTRACT

Medicinal plants are a rich source of natural healthcare products for alleviating various diseases. They have been reported to be of great importance due to their endless therapeutic properties that are useful in curing many chronic diseases. Their usage in the management of many profound compared with synthetic drugs that have been associated with side effects. Many natural products including polyherbal formulations are derived from plants. Various synthetic products presently in the market are arguably costly and associated with adverse side effects, therefore warranting a need for viable alternatives. Medicinal plants have various bioactive compounds, which exhibit diverse pharmacological properties. However, many of the bioactive compounds present in these plants have not been separated making their isolation and characterization a challenge. Phytexponent, a polyherbal formulation, is composed of alcoholic extracts of *Viola Tricolor, Echinacea purpurea, Allium sativum, Triticum repens*, and *Matricaria chamomilla.* Phytexponent has been widely used in the complementary medicine to treat inflammatory disorders, mange stress, boost the body immunity, manage blood pressure and many more other conditions. However, the components present in the phytexponent have not been separated and the pure compounds isolated and characterized. Hence this study, evaluated the column chromatographic fractionation and qualitative antioxidant evaluation of the phytexponent. The phytexponent was fractionated by adopting the normal phase column chromatography. Column was packed with the silica gel slurry prepared in the petroleum ether, followed by loading of the phytexponent mixed in few grams of silica and then eluted with various organic solvents starting with those of lower polarity to high. The collected fraction that were pooled together based on their properties and then qualitatively evaluated for the antioxidant activity using 1,1-diphenyl-2-picryhydrazyl free radical assay. The silica gel pre-coated thin layer chromatography plates were spotted with the collected fractions and then developed in the mobile phase and then air dried followed by spraying with 1,1-diphenyl-2-picryhydrazyl methanolic solution. Chromatographic separation of the Phytexponent yielded 285 fractions which were further separated by thin layer chromatography. Based on their thin layer results, they were combined in 13 fractions. Visualization was achieved under Ultraviolet light at 254 nm and 365 nm, respectively, whereby various colours were observed. The minerals could be barite, calcites, corundum and halite that fluorescents red, pectolite and margarite that fluorescents blue and apatite that fluorescents purple. Under short ultraviolet albite, bentonite and calcite that fluorescents blue was present. The antioxidant activity was only present in the petroleum ether: Dichloromethane (50:50) and Dichloromethane (100 %) fractions in which yellow spots on the purple background was noted. Therefore, the phytexponent polyherbal formulation contain various compounds that are non-polar in nature as most of the isolated compounds were from the non-polar solvent. Similarly, the phytexponent has antioxidant compounds and can be used as a natural source of free radical scavengers.

## TABLE OF CONTENT

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

## ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| **DCM** | Dichloromethane |
| **DPPH** | 1,1-diphenyl-2-picryhydrazyl |
| **EA** | Ethyl Acetate |
| **HPLC** | High Pressure Liquid Chromatography |
| **NSAIDs** | Non-Steroidal Anti-Inflammatory Drugs |
| **RF** | Retention Factor |
| **ROS** | Reactive Oxygen Species |
| **TLC** | Thin Layer Chromatography |
| **UV** | Ultraviolet |

# CHAPTER ONE: INTRODUCTION

## 1.1 Background Information

Medicinal plants are the source of most of the naturally occurring compounds that are now on demand due to their therapeutic values. These bioactive compounds have been used as medicines and perfumes, food preservatives and flavor in the cosmetic and food industries respectively. In the folk medicine they have been employed in management of many chronic and infectious diseases (Altemimi et al., 2017). These bioactive constituents include alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids (Nigussie, 2020).

Extraction is the first and crucial step in the process of evaluating pharmacologic efficacy and discovery of drugs. The extraction process involves the separation of the active constituents from the rest of the material such as plant powder (Majekodunmi, 2015). During extraction, effective and selective methods, such as using organic and aqueous solvents, modern methods that depend on pressure or elevated temperature are employed (Zhang et al., 2018). The extraction methods such maceration, digestion, Soxhlet extraction, percolation and infusion are used. All these extraction methods are used for specific components that need to be extracted. For instance, the thermolabile components are extracted with methods such as maceration and percolation while the heat stable components are extraction methods such as digestion and Soxhlet extraction are the best (Majekodunmi, 2015).

Upon completion of extraction process, extract obtained is usually a mixture of many different compounds. These compounds need further separation, isolation and purification to obtain pure compounds. In separation various separation such as chromatographic techniques aid separation basing on the size, shape and charge of the extract are employed.

In chromatography, mobile phase which is a solvent, or gas and stationary phase such as silica gel are used (Tradit et al., 2007). The chromatographic techniques involve various mechanism such as adsorption, partition, affinity, ion exchange or size exclusion are involved during compound separation (Tradit et al., 2007). The chromatographic techniques such as thin layer chromatography, paper chromatography, column chromatography and high pressures liquid chromatography are used in separation of compounds mostly from plants (Deepika et al , (2016). All these techniques depend on the different polarities to achieve successful separation.

Many pure compounds from natural sources such as penicillin, morphine and paclitaxel from plants have been isolated and purified. These compounds have been further tested for their biological activities through clinical trials and further incorporated into pharmaceutical products, dietary products and cosmetics. However, the high demand from the rising population that depends on medicinal plants as the source of relive of the many both chronic and infectious diseases that have emerged, there is a need to study more about the plants. This involves obtaining the pure compounds that have been reported to be potent against the many emerged diseases such cancer. Pure compounds as well have an advantage against the mixture as it's easy to use for specific purpose. However, many natural products that are in use as remedies of various conditions have not been separated hence making it a challenge for the elucidation and characterization of the present components (Tradit et al., 2007).

Phytexpoent is a polyherbal herbal preparation of Belgium origin that is composed of five medicinally known plants; *Viola Tricolor, Echinacea purpurea, Allium sativum, Triticum repens and Matricaria chamomilla.* These plants are mixed in various portions to make the final product with 62.1 % of its content being ethanol. This herbal preparation is marketed globally as an immunomodulator. The respective plants have been individually

used in the management of various conditions such as microbial infection, tumor, oxidative stress related disorders, inflammation and pain. Since these plants are only extracted and combined together, the final product contains many bioactive components that have different therapeutic properties. The compounds present in the respective plant extracts have not been separated, isolated and characterized. Therefore, separation of these compounds is paramount and will aid in identifying the compounds present in the phytexponent. This study aimed at separating the compounds present in the phytexponent through the column chromatography and further separation using thin layer chromatography and evaluation of the antioxidant activity of the isolated compounds.

### 1.2 Problem statement and justification

The association of severe side effects and reduced efficacy of the modern medicine has greatly impacted negatively the advances made in management and treatment of various chronic condition. Additionally, the modern drugs are characterized by short shelf life, expensive and in some instances toxic as well (Bajpai et al., 2016).The only remaining option has been the remedies from medicinal plants and other natural sources. The increased reliance on these plants has been due to the lower cost incurred and high potency with low toxicity (Arika et al., 2019). However, the larger part of materials of the natural products are the inactive components that covers the active ingredients reducing their efficacy (Sticher, 2008). Similarly, high content of the inactive material makes the isolation of the target component and screening of its pharmacological properties extremely a cumbersome process (Sticher, 2008). Also, separation techniques for these bioactive constituents are not well explored and there is need for an urgent development of effective and selective methods for extraction and isolation of the bioactive compounds. This has seen many researchers exploring various efficient extraction, isolation and

separation techniques that will aid in obtaining pure active ingredients from their natural sources.

The medicinal plant extracts have a combination of various bioactive compounds that are responsible for various activity(Arika et al., 2019). Hence the extraction, isolation, separation and characterization of these active ingredient is important. Many techniques both older and modified ones for extraction, isolation, separation and characterization of medicinal extracts exist. Modified extraction methods such as percolation and microwave assisted digestion have greatly resulted in the extraction of the active compounds from plants. Cost effective chromatographic techniques such as column chromatography and thin layer chromatography have effectively enhanced the separation of many components of medicinal plant extracts. These techniques have ensured the exploration of many medicinal plants for their therapeutic properties. However, this represent a smaller percentage of the many medicinal plants available and whose pharmacological activities have been studied. This study aimed at separation of the compounds of the phytexponent preparation by column chromatography and further separation using TLC and evaluation of qualitative antioxidant activity of the consolidated fractions.

## 1.3 Objectives

### 1.3.1 General objective

Column chromatographic fractionation and qualitative antioxidant evaluation of the phytexponent

### 1.3.2 Specific objectives

I. To fractionate the polyherbal formulation through column chromatography
II. To evaluate the antioxidant properties of the eluted fractions of phytexponent polyherbal formulation by thin layer chromatography.

## 1.4 Research questions

I. Does the phytexponent polyherbal formulation have various compounds

II. Do the eluted fractions of the phytexponent polyherbal formulation have antioxidant properties?

# CHAPTER TWO LITERATURE REVIEW

## 2.1 Extraction of the natural products

Extraction is the process that involves separation of the target active compounds from a mixture of material. This process is very important as it helps in separation of active components from the rest non-active materials (Kothari & Gupta, 2014). This process is procedural and effective and selective methods are employed. Briefly, the extraction solvent is left to penetrate in the solid matrix dissolving the solute, the dissolved solute diffuses out of the solvent and lastly the collection of the extracted solute is done (Zhang et al., 2018).The extraction process involves use of various techniques that use various solvents both organic and aqueous to accomplish the process. The extraction technique of choice depends on various aspects that range from the polarity and stability of the compounds and the solvent of choice whose toxicity, volatility, viscosity and its purity is considered (Zhang et al., 2018).

### 2.1.1 Extraction methods

The extraction of the natural compounds mainly from plants involves the use of various methods both traditional and modern. many more methods that aid in extraction of plant material are stilling evolving while the traditional ones are being modified due to the advances being made in the science of herbal medicines. The choice of the appropriate method is critical as it depends more of the nature of the target compound. Other factors such as stability to heat, type of solvent, duration of extraction and final volume of the extract need are put in consideration during the choice of the extraction method (Azwanida Nn, 2015);(Majekodunmi, 2015).

The selection of solvent is usually a crucial step in extraction in which the selectivity, cost, solubility and the safety must be first determined prior to using the solvent (Zhang et al., 2018). Basing on the law of similarity and impermissibility the solvent with polarity

comparable to that of the active compounds to be extracted results into efficient extraction process. The more polar solvents such as methanol and ethanol have been regarded as the universal solvents for extraction of various phytochemicals.

Temperature is as well very vital in the extraction process. High temperatures result into more efficient extraction due into the increased rate of solubility and diffusion. Very high temperatures have been noted to cause decomposition of the volatile and heat sensitive compounds (Majekodunmi, 2015). The extraction is efficient with increased percentage yield when there is great solvent-solid material ratio. The extraction duration results into the increased extraction as the duration of extraction increases to certain limits.

#### 2.1.1.1 Maceration

Maceration is the most commonly solvent extraction method that is mostly considered for the thermolabile compounds. In this method the solid materials are soaked in the organic solvent and allowed extraction duration of two days before collection of the extracted compounds(Ahmad Dar et al., 2020). However, this method has been associated with reduce efficiency and long extraction period which forms the demerits of this method (Zhang et al., 2018)

#### 2.1.1.2 Percolation

This extraction method is most effective and mostly used in the preparation of tinctures and fluid extracts. A specific apparatus known as a percolator which is a narrow cone-shaped glass vessel that is opened at both ends is used. This method briefly involves moistening of the solid ingredients with enough extraction solvent and then allowed to standard for about four hours in a closed vessel (Azwanida Nn, 2015). The entire content is then transferred in the percolator and extra solvent added then upper end closed. The mixture is then allowed humble time of 24 hours to macerate. The lower part of outlet the percolator is then opened and the liquid contained therein is allowed to drip slowly. An

additional extraction solvent is added until the percolate measures about 75% of the required volume of the finished product (Pandey et al., 2014).

#### 2.1.1.3 Digestion

This extraction method involves use of moderate heat during the extraction process. Its more suitable for the heat stable and water-soluble compounds (Majekodunmi, 2015);(Pandey et al., 2014);(Deshmukh Krishi Vidyapeeth et al., 2017). The crude plant material is boiled with specified volume of water and at certain period of time after which the mixture is cooled and strained or filtered. The boiling is done by placing the boiling flask in a water bath or in an oven at $50^{0}$ C (Deshmukh Krishi Vidyapeeth et al., 2017).

#### 2.1.1.4 Soxhlet extraction

This extraction process also identified as continuous hot extraction involves use of Soxhlet extractor. This apparatus consists of round bottom flask, extraction chamber, siphon tube and condenser at the top all made of glass. Finely powdered material is placed in the thimble (porous sample holder bag) and tightly plugged with cotton wool (Hossain et al., 2014). The extraction solvent is then poured in the round bottom flask and connected onto the extraction chamber with the thimble. The entire apparatus is then transferred to the heating mantle and the condenser connected to the water source. The extraction solvent is hen heated from the round bottom flask and upon evaporation is then condensed as it passes through the condenser. The condensed solvent flows back in the extraction chamber where it dissolves the components of interest in the materials. When the extraction solvent with the extracted components in the extraction chamber reaches the top of the siphon, it flows back in the round bottom flask (Hossain et al., 2014). This process is repeated two more times, after which the extraction solvent is heated and upon collection in the extraction chamber it is recovered prior to flowing back in the flask. This leaves the extracted components in the flask. This method is suitable for the heat stable components and it can be used to extract larger materials with small volume of solvent (Pandey et al.,

2014). However, shaking that ensure the complete interaction of the solvent with the material is not applicable and this method is not suitable for the thermolabile materials (Hossain et al., 2014).

### 2.2 Separation and isolation of pure compounds.

The various bioactive compounds present in the crude extract after the various extraction methods are a mixture of the many natural compounds with each particular compound having a different polarity. To remain with pure compounds various separation and purification techniques are involved. These techniques include Thin layer chromatography, column chromatography, HPLC, gas chromatography which have been the widely used(Deepika et al., (2016).

#### 2.2.1 Thin layer chromatography

This is the widely used chromatographic separation technique in the separation of the natural products. Due to its cheapness and less time consuming it has become the most preferred as it as well helps in setting the parameters that are to be used in the column chromatography. The technique uses either silica or alumina as the stationary phase on which the separation of the various compounds occurs and the various organic solvents are the mobile phase. In this technique two phases are recognized; the normal phase and the reverse phase. The use of the either alumina or silica pre-coated plates and the organic solvents as the mobile phase is regarded as the normal phase. On the other hand, the use of the use of the stationary phases as alkyl bonded silica or alumina (less polar) with polar solvents such as water as the mobile phase is referred to as the reverse TLC (Tradit et al., 2007).

### 2.2.2 Column chromatography

This has been regarded as the most effective technique that is applicable in the separation of the crude extracts in their respective components. This usually involves the use of a cylindrical glass column in which the silica gel the stationary phase is packed and the mobile phase (eluent) is allowed to flow through the column under the influence of gravity. In this technique the stationary phase is first packed in the column and then the sample is then loaded at the top of the column. The mobile phase is then allowed to flow down through the column as components in the crude extract will interact differently with the stationary phase due to the difference in the polarities (Ahmad Dar et al., 2020). The different fractions are then containing specific separated compounds are collected in test tubes and allowed to evaporate under room temperature prior to further analysis such as structure elucidation.

The separation in this technique is usually based on the adsorption properties. The differences in the adsorption affinities of the natural compounds for the surface of the adsorbents. Efficient separation of natural compounds is achieved by appropriate selection of the adsorbent material and mobile phase.

### 2.2.3 Gas chromatography

This is the chromatographic technique that separates the various compounds based on their volatility. In this method both qualitative and quantitative data about the compounds under investigation is provided. The stationary phase is usually the organic solvent whereas the mobile phase is an inert gas. In this technique the sample is usually vaporized and is then injected in the head of chromatographic column. While in the column the sample is transported by the flow of the gaseous mobile phase. The column usually has a liquid stationary phase that is adsorbed to the inert solid surface.

### 2.3 Phytexponent preparation

A phytexponent is a polyherbal herbal extract that is made from five different medicinal plants that are extracted in the ethanol prior to combination. The respective plant extracts are then mixed in specific percentage ratios that depend on the dry weight yield to the final product; *Viola Tricolor – 3.77% Echinacea purpurea- 26.42% Allium sativum- 11.32% Triticum repens- 26.42% Matricaria chamomilla- 32.08%*. the specific plants are extracted by maceration by soaking them in ethanol for two days before filtering and combing to came up with the final product. The final product is usually in liquid form with 62.1% part being ethanol and the 37.9 % the plant extracts.

#### 2.3.1Phytexponent composition

***Viola Tricolor*** this medicinal plant taxonomically belongs to the family violaceae. It Hs been commonly identified by various names; referred to as Heartsease, Johnny Jump up, Call-me-to-you, or Bird's Eye (Lim, 2014). Viola tricolar has been reported to have various pharmacological activities that have validated its traditional use in managing conditions such as skin diseases such as ulcers, itching, scabs, psoriasis, and eczema. In the Europe it has been reported to be efficient in treating anti-inflammatory disorders and skin diseases (Hellinger et al., 2014). The phytochemistry of *Viola tricolar* has revealed the presence of many bioactive constituents; flavonoids, polysaccharides, phenyl carbonic acids, coumarins, catechins, and salicylic acid derivatives (Hellinger et al., 2014) and it has been reported to be a rich source of macrocyclic peptides such as cyclotides which have been reported to act as immunosuppressive peptides inhibiting T-cell proliferation.

***Echinacea purpurea,*** is a perennial herb of medicinal value that belongs to the family Asteraceae. This plant goes by the common name eastern coneflower or purple coneflower. It has been reported to be indigenous to north America. Manayi *et al.* (2015) reported that this herb has both the anti-inflammatory and immunostimulatory properties.

It has commonly been used in the management of cold symptoms. It has found much attention and favor from many researchers as a result of its characteristic of being used for many purposes. Many studies have reported its various biological activities such as antimutagenicity, cytotoxicity, antidepression, and antianxiety. However, it's not 100 % safe as it has shown adverse side that include urticaria, erythema, rash, prutitus, nausea, dyspnea, angioedema, and abdominal pain (Manayi et al., 2015). The studies of Sharma et al. (2010) and Ahmad *et al.* (2014) have reported its anti-inflammatory properties. This study showed that the Echinacea extract was able to counter the inflammation inducted by bacteria in the epithelial cell culture by reducing the cytokines while the root powder inhibited the formation of edema in mice as a result of inhibiting the COX-1 and 2 by alkamides.

***Allium sativum*** (Garlic) is both a herb and food additive used as an aphrodisiac and a spice that is taxonomically paced in the family Alliaceae the same onion family (Moutia et al., 2018). Its globally distributed and best sold herbal product (Majewski, 2014). Garlic extracts have more than 200 chemicals entities that have been identified to recent date and characterized to various biological activities responsible for treating various conditions such as certain types of cancer. Its preparation can been in two forms; liquid or solid (Arreola et al., 2015). This herb has been reported to have various anti-inflammatory effects such as anticancer, antiangiogenic, and free radical-mediated anti-inflammatory effects. In model animals, Garlic showed positive effects in improving dyslipidemia, hyperglycemia, and allergy response. Furthermore, aqueous garlic extracts have been shown high antioxidant activities that include inhibiting reactive oxygen species (ROS) and enhancing antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (Arreola et al., 2015).

***Matricaria chamomilla*** is a commonly known medicinal plant in *Asteraceae* family (Singh, Khanam, Misra, & Srivastava, 2011). As aherbal remedy it has been in use for quite some years and incorporated in in more than 26 drugs. It is also forming one of the ingredients in several traditional homeopathy and unani medicinal preparations. Chamomile is also used as an anti-inflammatory and antispasmodic drug. Additionally, it's also an analgesic with the ability of can alleviating pain associated with disturbances in the stomach. Chamomile is also used extensively as a tea or tonic, as well as for treating hysteria, anxiety, insomnia, and nightmares (Murti, Mayank, Gajera, & Solanki, 2012).

***Tricum vulgare,*** is an invasive weed whose roots and leaves are used as medicine. Its commonly to as cough grass. It is used in managing constipation, bladder swelling, cough, fever, hypertension, kidney stones, and inflammation (Sanguigno et al., 2018). It is already found in some of the pharmaceutical formulations for treatment of burns, skin lesions, and decubitus ulcers. In current years, the focus has been entirely on its anti-inflammatory properties.

# CHAPTER THREE: MATERIALS AND METHODS

## 3.1 Source of Phytexponent

The phytexponent preparation (Pharmapath 27, Belgium; LOT NO:17E19) was sourced from Maendeleo Pharmacy – Nairobi and stored according to the manufacture's instruction until the experiment day.

## 3.2 Materials and methods

### 3.2.1Equipment and apparatus

The equipment used in this study included, the UV- lamp, the apparatus consisted of test-tubes and test tube racks, beakers, conical flask, glass column and capillary tubes.

### 3.2.2 Reagents and solvents

Analytical grade solvents and reagents were used. Solvents included; Ethyl acetate, Ethanol, Petroleum Ether, Methanol and Dichloromethane all from Loba Chemie. The reagents included L-ascorbic acid, DPPH, iodine and silica gel.

### 3.2.3 Preparation of the phytexponent for column chromatography

About 10 ml of the phytexponent preparation at 100 % was added into 20 grams of silica gel and mixed before loading in the packed column. This was to ensure sufficient interaction of the phytexponent herbal with silica gel prior to loading in the column.

### 3.2.4. Column packing

The glass column with the measurements 98 cm long and 2.5 cm diameter was rinsed with petroleum ether and allowed to completely dry before packing. The silica gel slurry was first prepared by adding 72.12g of the silica gel (mesh size 60-120 Finar®) in petroleum ether before packing. The column was then packed according to the standard procedures(Deepika et al., (2016). This involved blocking the column at the lower point using cotton wool and then packed with the silica gel slurry under constant tapping to

ensure continues in packing. The already prepared phytexponent product was then loaded at the top of the column and about 10 grams of soft sand added to prevent back extraction in the column.

### 3.2.5 Fractionation of the phytexponent product

The phytexponent was then eluted with the increasing polarity of various solvents. The solvents Petroleum ether, Ethyl acetate, Dichloromethane and ethanol in the increasing polarity were used in various ratios with 285 fractions being collected (table 3.1). The column was eluted at a flow rate of 0.05 ml per second with 10 ml of each fraction being collected in specific tube and then numbered consecutively for further analysis on the thin layer chromatography. The collected fractions were allowed to dry under room temperature.

**Table 3. 1: The solvent system for fractionation of the phytexponent polyherbal formulation**

| Solvent system | Ration (%) | Fractions |
|---|---|---|
| Petroleum ether | 100 | F1-F20 |
| Petroleum ether:DCM | 90:10 | F21-F46 |
| Petroleum ether:DCM | 80:20 | F47-63 |
| Petroleum ether:DCM | 50:50 | F64-84 |
| DCM | 100 | F85-122 |
| DCM: EA | 90:10 | F123-F143 |
| DCM: EA | 50:50 | F144-F175 |
| EA | 100 | F176 -F190 |
| EA: ETHANOL | 90:10 | F191-F216 |
| EA: ETHANOL | 50:50 | F217-F241 |
| EA: ETHANOL | 30:70 | F242-F262 |
| ETHANOL | 100 | F263-F285 |

### 3.2.6 TLC preparation

The various fractions obtained from the column chromatography were subjected to thin layer chromatography following standard method(Deepika et al., 2016). The silica precoated TLC plates measuring 20 cm by 10 cm were used. The collected fraction was

first dissolved in the respective solvents in which they were eluted and an aliquot of this fraction was spotted on the base line of the TLC plates with an interval of 10 mm between the various fractions. The spotted plates were allowed few minutes to dry prior to the development in the solvent saturated chambers. The developed plates were visualized under UV-light both at short (254 nm) and long (365 nm) wavelength. The plates were then kept in Iodine chamber and the developed spots visualized. At each visualization step the color of the visualized spot was noted and the spots marked. The Rf values of each spot calculated following the formula below and the fractions with the similar RF values were pooled together and labelled.

$$Rentention\ factor = \frac{distance\ travelled\ by\ solute}{distance\ travelled\ by\ solvent}$$

**3.3 Qualitative Antioxidant screening**

The qualitative antioxidant screening of the isolated fractions was done using TLC technique. The already pre-coated TLC plate measuring 20 x 10cm was used. The dried fractions were dissolved in the respective solvents and an aliquot of the fractions spotted on the already pre-coated TLC plate at interval of 10 mm between each spot with the first spot as L-ascorbic acid (1%). TLC plates were allowed to air dry for few minutes and then developed in the Petroleum Ether -Ethanol solvent system in the ratio of 9:1. The developed plates could air dried and sprayed with 1,1-diphenyl-2-picryhydrazyl solution (6mg in 50ml of methanol). The spots with the antioxidant activity decolorized the 1,1-diphenyl-2-picryhydrazyl to form yellow spots an indication for the presence of antioxidant activity.

### 3.4 Data management and statistical analysis

The data was collected in form of pictures and the colures of the spots noted then Rf values of the observed spots calculated. The results were then presented in form of tables and figures.

# CHAPTER FOUR RESULTS AND DISCUSSION

## 4.1 Results

### 4.1.1 Column chromatography fractionation of the phytexpoent polyherbal formulation

The column chromatography fractionation of the phytexponent polyherbal formulation resulted into collection of 285 fractions. The collected fractions were then further separated on thin layer chromatography using petroleum ether and ethanol (9:1) as solvent system. Fractions with similar properties evident by spots with similar RF values and colours were pooled together in one test tube reducing the fractions to 13 (Table 4.1; appendix 1.1).

Fractions 1 to 27 upon further separation on TLC and visualization under UV long wavelength, only two bright blue spots were noted (figure 4.3) whereas under short wavelength no spot was observed. All these fractions were pooled together to form fraction F1-27 which was further separated on TLC and at this particular point only one purple spot with the RF value of 0.65 was visualized under long ultraviolet wavelength (Table 4.1; appendix 1.1). upon separation of the collected fraction on TLC and visualization using UV at both wavelengths (long and short) no spot was observed in fraction 28 (figure 4.1).

Similarly, fraction 29 - 34 upon separation on TLC and then visualized under UV long wavelength only two spots glowing with blue color and with RF values of 0.62 and 0.59 were noted (table 4.1; appendix 1.1). Fractions 35-38 revealed two spots with RF values of 0.64 and 0.57 with one spot glowing blue and the other spot had purple color under long wavelength (table 4.1; appendix 1.1). Fraction 39 – 42 combined from fractions 39-42 upon further separation on TLC revealed two spots with RF values of 0.62 and 0.56 with

the first spot glowing blue while the second spot was purple in color (table 4.1; appendix 1.1). Fraction 43 showed on only one blue glowing spot under long wavelength.

Fraction 44 and 45 a pool from fractions 44 and 45 upon separation on TLC and visualization, six different spots were noted. The first three spots were bright blue (Spots A, B and C) and with RF values of 0.86, 0.77 and 0.66 under long UV. However, spots A, B and F with the RF values of 0.77, 0.66 and 0.53 were as well visible as a dark blue spot under short UV wavelength (table 4.1; appendix 1.3). Spots D and E with RF values of 0.66 and 0.57 were visible under long UV as red and purple spot respectively. Similarly, fraction 46 – 51 that was achieved by combining the fractions 46 to 51, six different spots were noted. Spots A, C and D with RF values of 0.86, 0.73 and 0.67 were observed as bright blue spots under long UV while spot E with RF value of 0.62 was observed as a purple spot under long UV (table 4.1; appendix 1.1). Similarly, spots B, C and F with RF values of 0.78, 0.73 and 0.55 were observed as dark blue spots under short UV (table 4.2; appendix 1.3).

In fraction 52, four different spots (A, B, C and D) were separated on TLC with spots B and C with RF values of 0.67 and 0.59 and bright blue in color being noted upon visualization under long UV. Spots A and D with RF values of 0.73 and 0.50 were observed as dark blue spots when visualized under short UV (table 4.1; appendix 1.3). Fraction 54 -56 on the TLC showed separation of three spots (A, B and C) with RF values of 0.64, 0.62 and 0.53. Spots B and C were observed as bright blue and purple spots under long UV respectively (table 4.1; appendix 1.1). Similarly, spot A and C were observed as dark blue spots under short UV (table 4.1; appendix 1.2).

Fraction 58 - 60 revealed the presence of four spots. In this fraction spots A and C were bright blue in color while spot D was purple in colour under long UV. Under short UV,

spots A, B and D were dark blue in colour (table 4.1; appendix 1.3). The combined fraction of 61 and 62 revealed no visible spot at both long and short UV (table 4.1; appendix 1.1,1.2). In fraction 63 f our spots were identified, A, B, C and D with RF values of 0.84, 0.77, 0.66 and 0.47. under long UV, spots A, B were observed as bright blue spots while spot D showed a purple colour (table 4.1; appendix 1.3). Spots A, C and D were observed to be dark blue in colour under short UV wavelength (table 4.1; appendix 1.3).

**Table 4. 1: Table of the isolated fractions and the retention factors**

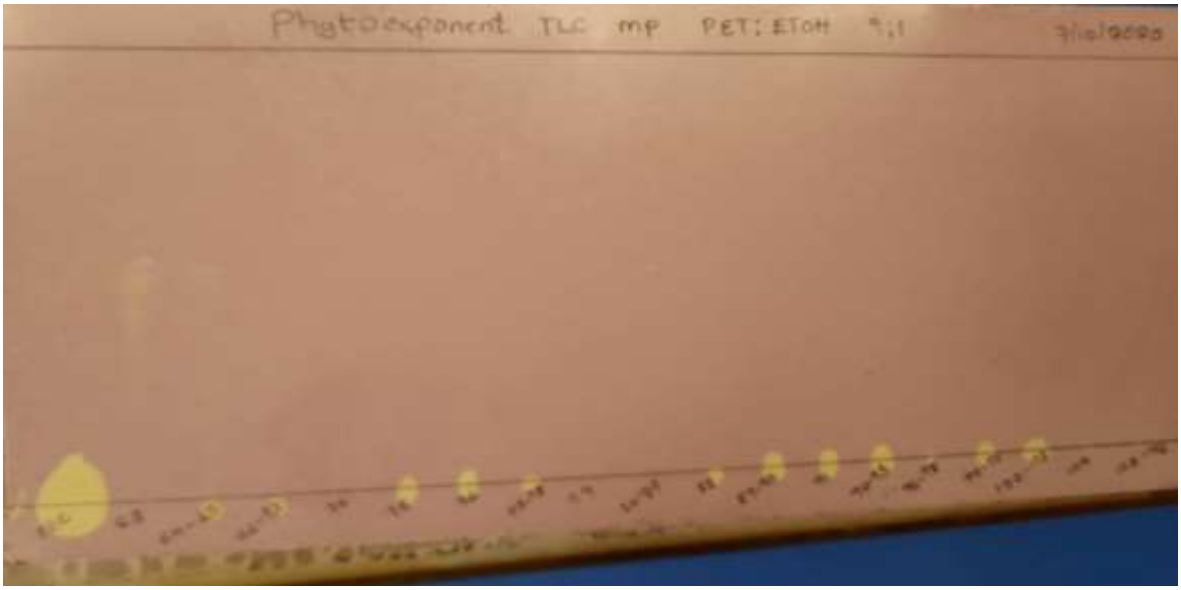
| Fraction(s) | Spot(s) | Rf Values | Short wavelength (254nm) | Long wavelength (365nm) |
|---|---|---|---|---|
| F1-27 | Spot A | 0.65 | No observable changes | A purple spot was observed |
| F28 | No observable change | No change | No change | No observable changes |
| F29-34 | Spot A | 0.62 | No observable changes | A blue glowing spot was observed |
| | Spot B | 0.59 | No observable changes | A blue glowing spot was observed |
| F35-38 | Spot A | 0.64 | No change | Blue glowing spot was observed |
| | Spot B | 0.57 | No change | Purple spot was observed |
| F39-42 | Spot A | 0.62 | No change | Blue glowing spot was observed |
| | Spot B | 0.56 | No change | A purple spot was observed |
| F43 | Spot A | 0.62 | No change | A blue glowing spot was observed |
| F44-45 | Spot A | 0.86 | No change | A bright blue spot was observed |
| | Spot B | 0.77 | A dark blue spot was observed | A bright blue spot was observed |
| | Spot C | 0.72 | A dark blue spot was observed | A bright blue spot was observed |
| | Spot D | 0.66 | No change | A red spot was observed |
| | Spot E | 0.57 | No change | A purple spot was observed |
| | Spot F | 0.53 | A dark blue spot was observed | No change |
| F46-51 | Spot A | 0.86 | No change | A bright blue spot was observed |
| | Spot B | 0.78 | A dark blue spot was | No change |

| | | | | |
|---|---|---|---|---|
| | | | observed | |
| | Spot C | 0.73 | A dark blue spot was observed | A bright blue spot was observed. |
| | Spot D | 0.67 | No change | A bright blue spot was observed. |
| | Spot E | 0.62 | No change | A purple spot was observed. |
| | Spot F | 0.55 | A dark blue spot was observed. | No change |
| F52 | Spot A | 0.73 | A dark blue spot was observed. | No change |
| | Spot B | 0.67 | No change | A bright blue spot was observed |
| | Spot C | 0.59 | No change | A bright blue spot was observed |
| | Spot D | 0.5 | A dark blue spot was observed | No change |
| F54-56 | Spot A | 0.64 | A dark blue spot was observed | No change |
| | Spot B | 0.62 | No observable change | A bright blue spot was observed |
| | Spot C | 0.53 | A dark blue spot was observed | A purple spot was observed |
| F58-60 | Spot A | 0.80 | A dark blue spot was observed | A bright blue spot was observed |
| | Spot B | 0.75 | A dark blue spot was observed | No observable change |
| | Spot C | 0.62 | No observable change | A bright blue spot was observed |
| | Spot D | 0.53 | A dark blue spot was observed | A purple spot was observed |
| F61-62 | No spots | No change | No observable change | No change |
| F63 | Spot A | 0.84 | A dark blue spot was observed | A bright blue spot was observed |
| | Spot B | 0.77 | No observable change | A bright blue spot was observed |
| | Spot C | 0.66 | A dark blue spot was observed | No observable change |
| | Spot D | 0.47 | A dark blue spot was observed | A purple spot was observed |

### 4.1.2 The qualitative antioxidant screening of the isolated fractions of the phytexponent polyherbal formulation

The elucidated fractions of the phytexponent polyherbal formulation and positive control (L-ascorbic acid) were tested for their antioxidant activity using rapid TLC screen. The results for the qualitative antioxidant activity screening phytexponent polyherbal formulation fractions are in figure 4.1. The results revealed only a single yellow spot on the purple background in fractions 64-65, 66-73, 75, 76, 77-78, 88, 89-90, 91, 92-95, 96-98, 99-101 and 102- 103 upon spraying with the DPPH reagent (figure 4.2). All the yellow spots observed in the fractions were on the baseline indicating that most of the antioxidant compounds in the isolated fraction are more polar.

**Figure 4. 1: TLC plate depicting the qualitative DPPH free radical scavenging activity of isolated phytexponent fractions**



# CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATION

## 5.1 Discussion

Plants have been in use for many years and their increased demand has been linked to their bioactive compounds that are synthesized by many medicinal plants. The lower expenses incurred and lower side effects associated with them have contributed to their demand. The therapeutic value of medicinal plants has been of great impact in managing and treating many chronic diseases such as cancer. Many of the natural products from plants such as penicillin, morphine and paclitaxel are characterized by complex structures that make it difficult to replicate in the laboratory via various synthesis methods due to the high cost involved. The side effects and high cost associated with most of the conventional medicines has as well been of great concern. This has raised the urgent need for evaluation of the alternative remedies from natural sources such as plants. Various secondary metabolites from plants are of great interest among many researchers. The isolation and purification of these bioactive compounds is therefore very paramount. The isolation and purification of the extracted bioactive constituents involves the use of various chromatographic techniques such as thin layer chromatography and column-chromatography(Bajpai et al., 2016). These chromatographic techniques have been successfully used in the fractionation and purification of the biologically active compounds from variety of natural sources such as plants. This study visualized successful application of column chromatography in fractionation of the active compounds in the phytexponent polyherbal formulation and identification of the fractionated compounds on the thin layer chromatography. The collected and combined fractions were elucidated by the non-polar solvents. These non-polar combined could be comprised of the aromatic compounds in mojority. The fractionation of the phytexponent polyherbal formulation revealed most of the compounds with bright blue, purple and red colours when visualized under long UV wavelength (366 nm). The fractions showed spots that were dark blue in

colour when visualized under short UV wavelength (254 nm). These could be minerals that fluorescents with different colours at both short and long UV wavelength. The minerals such as barite, calcites, corundum and halite fluorescents red, pectolite and margarite fluorescents blue and apatite that fluorescents purple. Under short UV albite, bentonite and calcite that fluorescents blue was were observed.

Many of the active constituents of natural products from plants have been reported to possess strong antioxidant properties. The phenolics are one of those compounds that have been reported to account for most of the strong antioxidant activity witnessed in plants and natural products(Dela Torre et al., 2017). These compounds scavenge free radicals by donating electrons to them. The evaluation of the antioxidant properties of natural products has been done using various methods. DPPH free radical assay is one of the methods and this assay has been widely used to investigate the scavenging activities of natural compounds such as phenols or crude plant extracts. DPPH is a stable radical at room temperature which forms a purple methanolic or ethanolic solution and accepts an electron or hydrogen radical to become stable diamagnetic molecule. Test principle is based on the ability of the extract or the standard to donate electrons to the free radical which are responsible of the reduction of the DPPH free radical (Amzad Hossain & Shah, 2015). The DPPH free radical is reduced in the presence of an antioxidant to form a yellow solution or spot on the TLC. The yellow spots on the violet/ purple background depicts the antioxidant activity. In this study the antioxidant activity of the isolated fractions of the phytexponent polyherbal formulation was qualitatively investigated using the DPPH free radical assay. The various isolated spots were spotted on the TLC and then separated with petroleum ether: ethanol solvent system. The developed plates were then sprayed with DPPH methanolic solution (6mg in 50 ml) using L-ascorbic acid as the standard. The presence of the yellow spots in some of the fractions indicated that they

possessed the antioxidant properties. The antioxidant properties were comparable to that of the standard (L-ascorbic acid) as the intensity of the yellow spots of the fractions was similar to that of the L-ascorbic acid (0.1%). This activity can be attributed to the presence of the phenolic compounds such as flavonoids that have been reported to be the most antioxidant compounds in natural products of plant.

### 5.1 Conclusion

In conclusion based on the findings from the current study its evident that the phytexponent polyherbal formulation has various compounds. The majority of the compounds are non-polar as seen from the various TLC screening of the isolated fractions. These compounds consist of the aromatic compounds and minerals that were able to florescence in various colors under the long UV (365 nm). The various compounds isolated from the phytexponent polyherbal formulation as well showed to be potential antioxidant.

### 5.2 Recommendation

From the current study various recommendations can be made:

I. The invitro antioxidant activity of the isolated fractions should be done
II. The various fractions isolated should be screened for the phytochemicals by spraying with respective chemicals
III. The isolated fractions of the phytexponent polyherbal formulation to be characterized and their structure elucidated.

## REFERENCES


Ahmad Dar, A., Sangwan, P. L., & Kumar, A. (2020). Chromatography: An important tool for drug discovery. *Journal of Separation Science*, *43*(1), 105–119. https://doi.org/10.1002/jssc.201900656

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D. G., & Lightfoot, D. A. (2017). Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. *Plants*, *6*(4). https://doi.org/10.3390/plants6040042

Amzad Hossain, M., & Shah, M. D. (2015). A study on the total phenols content and antioxidant activity of essential oil and different solvent extracts of endemic plant Merremia borneensis. *Arabian Journal of Chemistry*. https://doi.org/10.1016/j.arabjc.2011.01.007

Arika, W., Kibiti, C. M., Njagi, J. M., & Ngugi, M. P. (2019). In Vitro Antioxidant Properties of Dichloromethanolic Leaf Extract of Gnidia glauca (Fresen) as a Promising Antiobesity Drug. *Journal of Evidence-Based Integrative Medicine*, *24*, 1–17. https://doi.org/10.1177/2515690X19883258

Arreola, R., Quintero-Fabián, S., López-Roa, R., Flores-Gutiérrez, E., Reyes-Grajeda, J., Carrera-Quintanar, L., & Ortuño-Sahagún, D. (2015). Immunomodulation and Anti-Inflammatory Effects of Garlic Compounds.: Discovery Service for Endeavour College of Natural Health Library. *Journal of Immunology Research*, *2015*, 1–13. https://doi.org/10.1186/1471-244X-12-121

Azwanida Nn. (2015). A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Medicinal & Aromatic Plants*, *04*(03), 3–8. https://doi.org/10.4172/2167-0412.1000196

Bajpai, V. K., Majumder, R., & Park, J. G. (2016). Isolation and purification of plant

secondary metabolites using column-chromatographic technique. *Bangladesh Journal of Pharmacology*, *11*(4), 844–848. https://doi.org/10.3329/bjp.v11i4.28185

Dela Torre, G. L. T., Arollado, E. C., Atienza, A. A., & Manalo, R. A. M. (2017). Evaluation of antioxidant capacity and identification of bioactive compounds of crude methanol extracts of Caesalpinia pulcherrima (L.) Swartz. *Indian Journal of Pharmaceutical Sciences*, *79*(1), 113–123. https://doi.org/10.4172/pharmaceutical-sciences.1000207

Deshmukh Krishi Vidyapeeth, P., Mangesh Moharil Biotechnology Centre, I. P., Vaibhav Khelurkar Biotechnology Centre, I. C., Ingle, K. P., Deshmukh, A. G., Padole, D. A., Dudhare, M. S., Moharil, M. P., & Khelurkar, V. C. (2017). Phytochemicals: Extraction methods, identification and detection of bioactive compounds from plant extracts. *Journal of Pharmacognosy and Phytochemistry*, *6*(1), 32–36.

Hellinger, R., Koehbach, J., Fedchuk, H., Sauer, B., Huber, R., Gruber, C. W., & Gründemann, C. (2014). Immunosuppressive activity of an aqueous Viola tricolor herbal extract. *Journal of Ethnopharmacology*, *151*(1), 299–306. https://doi.org/10.1016/j.jep.2013.10.044

Hossain, M. A., Al-Hdhrami, S. S., Weli, A. M., Al-Riyami, Q., & Al-Sabahi, J. N. (2014). Isolation, fractionation and identification of chemical constituents from the leaves crude extracts of Mentha piperita L grown in Sultanate of Oman. *Asian Pacific Journal of Tropical Biomedicine*, *4*(Suppl 1), S368–S372. https://doi.org/10.12980/APJTB.4.2014C1051

K. Deepika , Mahesh Kumar M. V. S., T. S. D. R. and S. V. R. (2016). *Studies on Isolation, Purification and Structure Elucidation of Chemical Constituents From Methanolic Flower Extract of Blepharis Molluginifolia Pers. and Their Biological*

*Activities*. *7*(12), 4893–4904. https://doi.org/10.13040/IJPSR.0975-8232.7(12).4893-04

Kothari, V., & Gupta, A. (2014). *Extraction methods for preparation of bioactive plant extracts : A comparative study* (Issue June).

Majekodunmi, S. O. (2015). Review of extraction of medicinal plants for pharmaceutical research. *Merit Res. J. Med. Med. Sci*, *3*(11), 521–527. http://www.meritresearchjournals.org/mms/index.htm Copyright

Majewski, M. (2014). Allium sativum: facts and myths regarding human health. *Roczniki Państwowego Zakładu Higieny*, *65*(1), 1–8.

Manayi, A., Vazirian, M., & Saeidnia, S. (2015). Echinacea purpurea: Pharmacology, phytochemistry and analysis methods. *Pharmacognosy Reviews*, *9*(17), 63–72. https://doi.org/10.4103/0973-7847.156353

Moutia, M., Habti, N., & Badou, A. (2018). In Vitro and In Vivo Immunomodulator Activities of Allium sativum L. *Evidence-Based Complementary and Alternative Medicine*, *2018*. https://doi.org/10.1155/2018/4984659

Nigussie, G. (2020). *Isolation , Characterization and Structural Elucidation of the Roots of Rumex Organic Chemistry : Current Research*. *09*(200), 1–7. https://doi.org/10.35248/2161-0401.20.9.200

Pandey, A., Tripathi, S., & Pandey, C. A. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *Journal of Pharmacognosy and Phytochemistry JPP*, *115*(25), 115–119.

Sanguigno, L., Casamassa, A., Funel, N., Minale, M., Riccio, R., Riccio, S., Boscia, F., Brancaccio, P., Pollina, L. E., Anzilotti, S., Renzo, G. Di, & Cuomo, O. (2018).

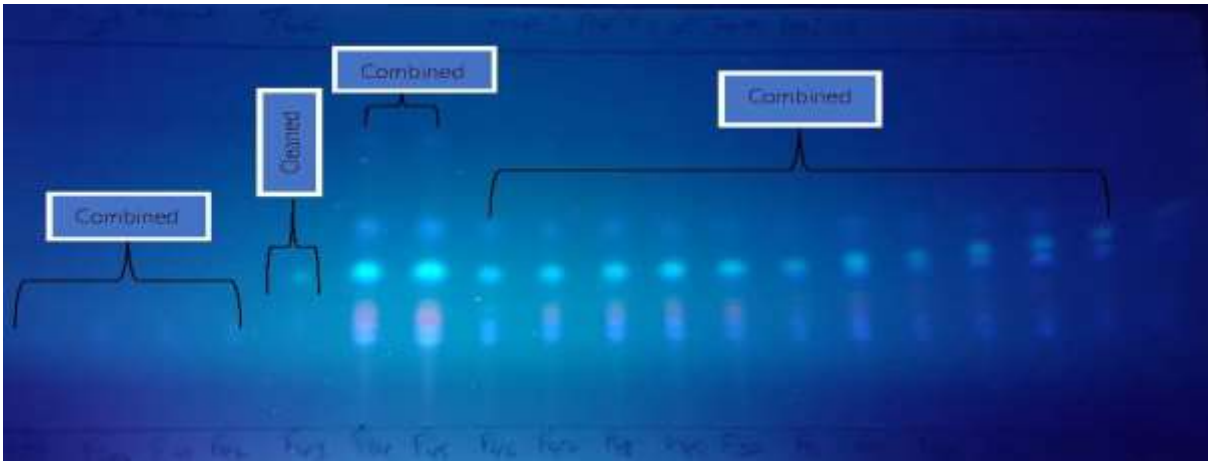
Triticum vulgare extract exerts an anti-inflammatory action in two in vitro models of inflammation in microglial cells. *PLoS ONE, 13*(6), 1–14. https://doi.org/10.1371/journal.pone.0197493

Tradit, A. J., Altern, C., Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., Latha, L. Y., Bedong-semeling, J., & Nasi, B. A. (2007). EXTRACTION, ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM PLANTS' EXTRACTS. *Lecture Notes in Mathematics*, *1902*, 121–130. https://doi.org/10.1007/978-3-540-69153-2_6

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine (United Kingdom)*, *13*(1), 1–26. https://doi.org/10.1186/s13020-018-0177-x

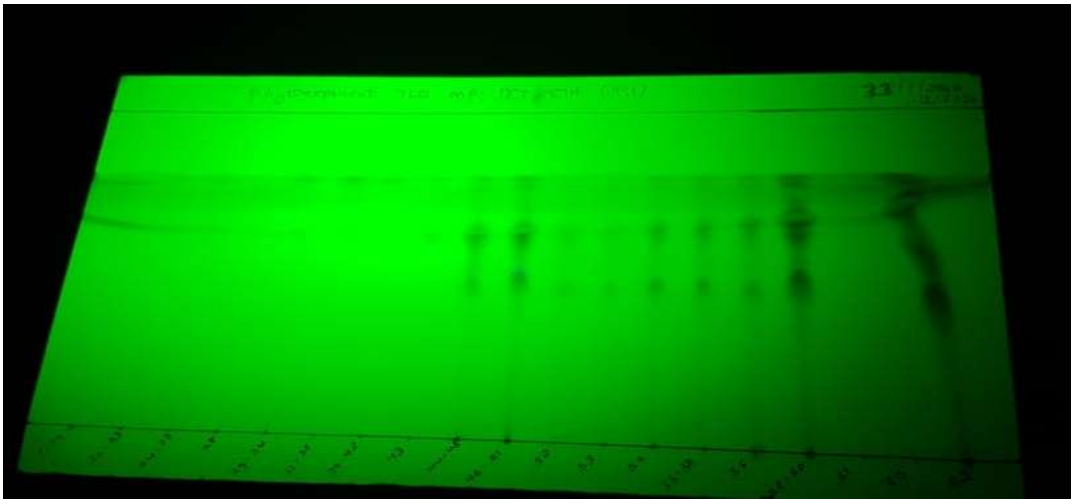
## APPENDICES

*Appendix 1. 1 TLC plate for the combined fractions visualized at long UV (365 nm).*

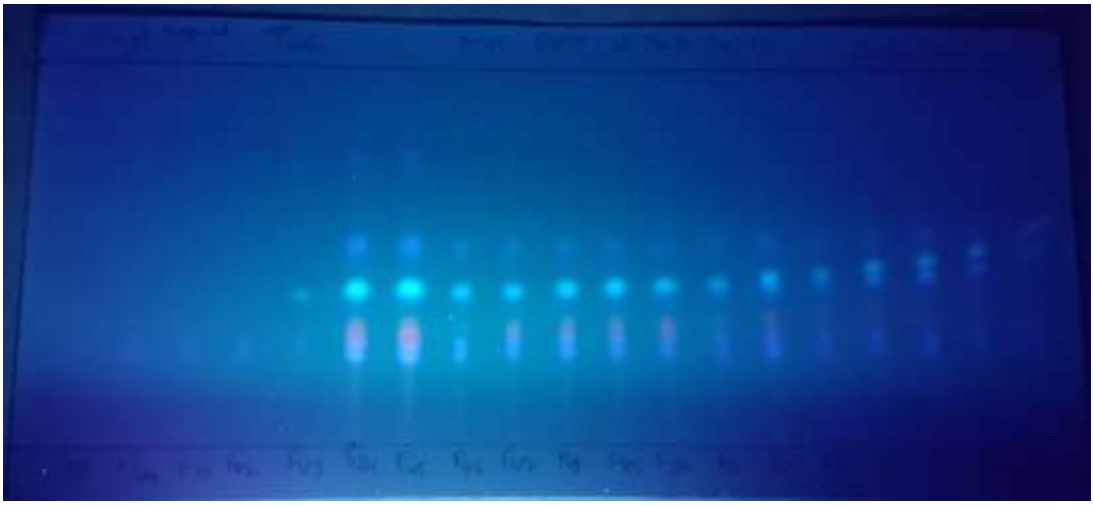



***Appendix 1. 2 packed column***

***Appendix 1. 3 TLC plates for the combined fractions visualized at short UV (245).***



***Appendix 1. 4 TLC plate for the un combined fractions visualized at long UV wavelength (366).***



**Appendix 1. 5 TLC plates for the isolated fractions visualized at long UV wavelength (366).**