ANTIFUNGAL ACTIVITY OF THE n-HEXANE FRACTION OF *EMILIA COCCINEA* WHOLE PLANT

BY

Ugwu Franklin Chisolum

PHA1406017

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

FACULTY OF PHARMACY UNIVERSITY OF BENIN

BENIN CITY

NIGERIA

December, 2022

ANTIFUNGAL ACTIVITY OF THE n-HEXANE FRACTION OF *EMILIA COCCINEA* WHOLE PLANT

BY

Ugwu Franklin Chisolum

PHA1406017

A PROJECT WRITTEN IN THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY AND SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHARMACY IN THE FACULTY OF PHARMACY, UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

December, 2022

CERTIFICATION

We certify that this work was done by **Ugwu Franklin Chisolum**, in the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Dr. E.E. Odion	**Date**
(Supervisor)	
Dr. E.E. Odion	**Date**
(Acting Head of Department)	
Franklin C. Ugwu	**Date**
(Student)	

DEDICATION

This work is dedicated to my beloved parents Mr. and Mrs. Stanley Ugwu for their endless Prayer, Love and Support.

ACKNOWNLEDGEMENT

I owe a huge debt of gratitude to the Almighty God, who made it all possible.

I would like to Sincerely thank My supervisor, Dr. Emmanuel E. Odion, for the patient guidance, encouragement, and advice he has provided throughout the course of this work. I am really happy to have a supervisor who cared so much about my work, and who answered to my inquiries and issues so promptly.

I would also like to thank all the members of staff Both academic and Non-academic Staff at Department of Pharmaceutical Chemistry, University Of Benin, Benin City who helped me during this work. Completing this work would have been all the more difficult were it not for the support and friendship provided by the members of the Laboratory staff of the Department of Pharmaceutical Chemistry. I am indebted to them for their help.

I appreciate the members of the staff of Department of Pharmaceutical Microbiology, Faculty of Pharmacy, for their valuable supervision for the antifungal property of the n-Hexane fractions.

I must express my gratitude to Dr. Ugwu Jerome Odo, Mr. Ugwu Demian, Mr and Mrs Fedrick Asogwa, Mrs. Theresa Eze and Pharm. Izuchukwu Francis Okpalanwaka, for their continued support and encouragement.

Finally, I would like to thank my course mates in the Faculty of Pharmacy, not only for providing the enabling environment which allowed me to undertake this research, but also for giving me the opportunity to attend and meet so many interesting people.

.

TABLE OF CONTENTS

Abstract

In folk medicine, *Emilia coccinea,* is used to treat microbial infections and wounds. Thus the aim of this study was to Investigate the Antifungal Activity of the n-hexane fraction of *Emilia coccinea* (Sims) G. Don. The Assessment of the n-hexane fraction of *Emilia coccinea* whole plant was investigated in order to verify its claimed folkloric usage in treatment of microbial infections. Sensitivity tests for anti-fungi activities of the whole plant n-hexane fraction were determined using Agar well diffusion method. Minimum Inhibitory Concentrations (MICs) of fractions were also determined using Agar dilution. Commercial antifungal agent (Ketoconazole) were used as positive reference standard to determine sensitivity of tested organisms. The test organisms (*Candidia albicans, Trichophyton rubrum, and Rhodotorula glutinis*) were found to be sensitive to the n-hexane fraction while no sensitive on *Aspergillus fumigatus, and Microsporum audounii*. The n-hexane fraction recorded highest mean inhibition diameter of 15.50±1.06 mm against *Candidia albican*. The results demonstrate that the n-hexane fraction of *E. coccinea* plants has antifungal activity and can be a potential source of antifungal agents. Thus, the folkloric usage of this plant for the treatment of microbial diseases is justified.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

Medicinal plants are currently significant due to their special attributes as a large source of therapeutic phytochemicals that may lead to the development of novel drugs (Sandberg and Corrigan , 2001). Phytochemicals isolated from natural products have been reported to play a significant role in the treatment and prevention of diseases (Naczk and Shahidi., 2006). Almost all nations place a high value on the use of plants in their medical systems, and many modern medicines now contain components from medicinal plants. Numerous phytochemicals and metabolites, such as steroids, terpenoids, carotenoids, flavonoids, alkaloids, tannins, and cardiac glycosides, have been identified in plants (Ajibesin., 2011).

Due to a lack of information and methods, the use of several secondary metabolites from plants is not well established. Plants contain a variety of antibiotic compounds, including alkaloids, anthraquinones, and others. The use of medicinal plants in herbal, allopathic, and many other medical systems is now widespread. The majority of medicinal plants are also utilized as food plants and spices (Akinpelu and Onakoya., 2006). One of the plants that have shown significant biological activities is *Emilia coccinea*, which is traditionally used for the treatment of stomach upset, pain, infection, constipation, cough, convulsion, swelling, and reduction in blood glucose level (Edeoga *et al*., 2005).

Emilia coccinea is a member of the Asteraceae family, with the common name Scarlet tassel flower and is native to Africa, Asia tropical and Oceania (Jeffrey., 1986). It is an annual herbaceous plant with a weak-stemmed of about 1 m tall. It may be found throughout eastern and western Africa as well as in tropical Asia. It is an attractive plant and easy to culture (Jeffrey., 1997). This species of *Emilia* is a highly invasive weed that grows in gardens, waste ground, fields, forest edges, pastures, active and abandoned cultivated areas, roadsides, dry thickets and river banks (Davidse *et al*., 2008). This species has wind-

dispersed seeds and can thrive in a wide range of environmental conditions, suggesting it may spread to new locations effectively (Davidse *et al*., 2008).

1.1 Ethnomedicinal Uses

Emilia coccinea, being a folkloric medicine is traditionally used as remedy for eye and ear ailments, for fever, convulsion in children, ulcer, craw-craw ringworm (Edeoga *et al*., 2005), rashes, measles, and other forms of inflammatory diseases (Sofawara., 1996). Also the plant *Emilia coccinea* is used to treat a range of illnesses, including tumor, inflammation, cough, rheumatism, fever, dysentery, wounds, and pregnancy prevention (Teke *et al*., 2007; Ojiako *et al*., 2015; Nwachukwu *et al*., 2017). The juice of the edible leaves is said to be used to cure eye inflammations, night blindness, and earaches. Several research projects have been done on the chemical components of *Emilia coccinea* as well as the plant's antimicrobial properties (Kamboj and Sulaj., 2011).

In Tanzania, the leaves are combined with those of Ipomoea eriocarps, pounded, soaked in water, and the liquid used as an eye drop for eye infections. The crushed green leaves can be utilized to treat wounds, sores, and sinusitis when applied topically (Bosch., 2004). East Africa (Kenya, Tanzania, and Malawi) has observed *E. coccinea* consumption as a vegetable (Burkill., 1985; Bosch., 2004), while the West tropical zone (Nigeria, Ivory Coast, and Mali) has reported usage of dried powered leaves via topical application of poultices of powdered roots or shoots beneath bandages over specific sites on the body. Traditional healers and natives of a particular culture are often the origin for plant utilization. Given the wide range of plants in existence, as well as the different locations of plants across geographical regions, it's not surprising that plants are used differently from one society to another.

1.2 Pharmacological Properties of *Emilia Coccinea*

Pharmacological effects such as Antioxidant, antidiarrheal, antimicrobial, and neuroprotective effects have been reported (Teke *et al*., 2002 and Foyet *et al*., 2014).

(Shetonde *et al.*, 2015) isolated 24 compounds from the leaf extracts (19 and 7 compounds from the Hexane and DCM extracts, respectively; 2 being common to both extracts).The most abundant components in the hexane extract were caryophyllene (22.07%), 1-octadecanol (19.34%), caryophyllene oxide (17.74%), 1-tridecene (7.70%), geranylgeraniol (7.46%), tetracosane (5.50%), and ethyl hexadecanoic, whereas for the dichloromethane extract, they were pentadecanal (40.03%), 1,E-11,Z-13-octadecatriene (11.35%), 1-octadecanol (7.31%), 1-tridecene (6.39%) and 4,8,12,16-tetramethylheptadecan-4-olide (2.59%)

On collection of plants, the extracts from the plants are prepared by phytochemists (Ingle *et al.,* 2017) who assess them for biological activity using pharmacologically relevant assays before going on to isolate and characterize the active component(s) via bioassay-guided fractionation. Several naturally occurring plants or treatments have been discovered in ethnobotanical research. Many traditionally used plants and remedies have been identified in various native communities across the world. Natural medicines, as well as their knowledge and appropriate use, have been developed and enhanced over many generations.

Traditional medicine knowledge of medicinal plants is essential since it allows chemists and pharmacologists to begin their work with targeted study, the isolation of new treatments, and natural drugs for treating a variety of diseases.

1.2. 0 The neuroprotective effect

Using behavioral assay techniques such as open-field, elevated plus maze, forced swimming, and Y-maze, a study evaluating the impact of methanolic *Emilia coccinea* leaf extract on the central nervous system, including anxiety, depression-like behavior, and memory in Wistar rats, revealed that it significantly increased the number of open arm entries and time spent in the open arms of the elevated plus maze test. Both the raising time and the amount of time spent in the open field's center were greatly extended. The time spent climbing and the

number of lines crossed on this exercise were both dramatically reduced. After three days of therapy, the extract was just as efficient as imipramine in shortening the time spent immobile during the forced swimming test. In the Y-maze exercise, it dramatically enhances spatial memory (Foyet., 2014).

1.2.1 The Antibacterial Effect

E. coccinea (Sims) G. Don is said to have a variety of therapeutic qualities, including include antibacterial activity. According to the experiment, the methanol extract was more effective against Escherichia coli whereas the aqueous extract was inactive at the measured dose (5 mg/ml). These findings may support the historic usage of this herb to treat a variety of infectious disorders, including diarrhea (Teke *et al.*, 2007).

1.2.2 The Antioxidants Effect

Because *Emilia coccinea* leaves contain flavonoids and phenolic substances, it is possible that this plant has neuroprotective potential and has antioxidant characteristics. This was demonstrated in an experiment employing two model systems to test the antioxidant activities of the extract: ferric iron reduction activity assay and 2, 4-dinitrophenyl-1-picryl hydrazyl (DPPH) radical scavenging activity. It has a significant concentration of polyphenolic substance, a strong indicator of antioxidant action.

1.2.3 The Anti-diarrheal Effect

In rats exposed to castor oil-induced diarrhea, the antidiarrheal effects of methanol and aqueous extracts of *Emilia coccinea* leaves were examined at dosages of 200, 400, and 600 mg/kg body weight. The methanol extract and, to a lesser extent, the aqueous extract, reduced the frequency of diarrhea episodes and the propulsion of charcoal meal through the gastrointestinal system in a dose-dependent way. They also dramatically lengthened the time it took to induce diarrhea (Teke *et al.*, 2007).

1.3 Fungi as a human pathogen

Fungal infections are a primary cause of morbidity and death in immunocompromised people. Fungi that are pathogens are generally plant-pathogenic Fungi. There are comparably few organisms that are pathogenic to animals, particularly mammals. There are about a little 1.5 million described species of fungus (Hawksworth., 1992),. A little more than 400 of these species are known to cause illness in animals, and considerably fewer of these species will particularly cause sickness in humans.

Some of these pathogens are widely recognized, example *Aspergillus fumigatus*. *Aspergillus fumigatus* is the leading cause of nosocomial fungal pneumonia and is the source of the second most prevalent fungal infections. The antifungal medicine Azole was successful against this pathogen but recently it has begun developing a resistance to it (Moye-Rowley., 2015). Fungal keratitis, an infection of the cornea, is caused by a rare *Trichophyton* spp. (Sharma et al., 2014). *Candida* spp. has been identified to be the fourth largest cause of nosocomial infections (Pfaller and Diekema., 2007). They caused roughly 88% of infections in the United States between 1980 and 1990. *Candida albicans* is the species most usually isolated from clinical material and accounts for 40-70 % of cases of candidiasis. The epidemiology data reveals that 5 to 10 of every 1000 risk individuals will contract *Candida* bloodstream infection and roughly 35 % of these patients will die as a consequence of infection, while 30 % will die as a result of a underlying condition (Lockhart *et al*., 2009).

Fungal infections of the oral cavity are caused by a group of saprophytic fungi that comprises eight species of the genus *Candida*. *Candida albicans* are the most frequent species that live in the oral cavity in humans, accounting for 70–80% of oral isolates. *Candida albicans* is also the principal cause of vaginal candidiasis, a common mucosal infection that occurs with the greatest incidence in women aged 20–30 years (Sojakova *et al.,* 2003). Because of the resilience of yeast infections to pharmacotherapeutics, candidiasis treatment usually fails. The

resistance of *Candida* species to azole antifungals is the most widespread kind of resistance to antifungals (Cross *et al.,* 2000). Researchers have revealed that 3.6% of *C. Albicans* vaginal isolates were found to be resistant to fluconazole, while 16.2% were resistant to itraconazole (Sobel *et. al.,* 2003 and Richter *et al.,* 2005). In addition to resistance to antifungals, fungal infections caused by opportunistic pathogens have grown increasingly prevalent, partly as a consequence of extended antibiotic treatment and the rising number of immunocompromised individuals (Castón-Osorio *et al.,* 2008). Therefore, creating therapeutic techniques for the treatment of candidiasis in immunocompromised individuals is especially crucial, with the objective of generating medications with improved efficacy, fewer side effects, and cheaper costs.

Aspergillus species are widespread, a saprophytic fungus that plays a vital role in global carbon and nitrogen recycling. Although their major biological habitat is soil or decaying plants, aspergilli generate microscopic, hydrophobic conidia that spread readily into the air and may withstand a wide variety of climatic conditions. The genus *Aspergillus*, which encompasses approximately 200 species, has a major influence on public health both beneficially as the workhorse of industrial uses and badly as plant and human diseases (Gugnani., 2023).

Among the human pathogenic species of *Aspergillus, A. fumigatus* is the predominant causative agent of human infections, followed by *A. flavus, A. terreus, A. niger,* and the model organism, *A. nidulans* (Denning., 1998). *Aspergilli* induce a broad spectrum of human illnesses depending on the immunological condition of the host (Large, 1999). In those with impaired lung function such as asthma and cystic fibrosis patients, aspergilli may develop allergic bronchopulmonary aspergillosis, a hypersensitivity reaction to fungal components. Noninvasive aspergillomas may grow with repeated exposure to conidia and target preexisting lung cavities such as the healed lesions in TB patients. Invasive aspergillosis (IA)

is possibly the most destructive of Aspergillus-related illnesses, targeting highly immunocompromised people. Those most at risk for this life-threatening disease are individuals with hematological malignancies such as leukemia; solid-organ and hematopoietic stem cell transplant patients; patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients; individuals with genetic immunodeficiencies such as chronic granulomatous disease (CGD); and individuals infected with human immunodeficiency virus (Wiederhold., 2003 and Post., 2007).

Rhodotorula is a widespread environmental yeast that is found in air, soil, lakes, ocean water, milk, and fruit juice. *Rhodotorula* species, members of the Basidiomycota phylum, colonize plants, humans, and other animals. The genus *Rhodotorula* comprises eight species, of which *R. mucilaginosa, R. glutinis, and R. minuta* are known to cause illness in people (Larone., 1995). *Rhodotorula* generates pink to red colonies and blastoconidia that are unicellular missing pseudohyphae and hyphae. Several writers have isolated *Rhodotorula* in diverse habitats and situations as well as characterized infections in animals. *Rhodotorula* spp. has been acknowledged as an emerging yeast infection in humans in the past two decades. While no instances of *Rhodotorula* infection were described in the medical literature before 1985, the number of infections rose after that time, most likely because of the broader use of intensive therapies and central venous catheters (CVCs) (Miceli *et al.,* 2011).

Microsporum audouinii is an anthropophilic dermatophyte prevalent in Africa. It commonly causes tinea capitis and tinea corporis in children. While *Microsporum canis*, a zoophilic dermatophyte, is still the most prevalent cause of tinea capitis in Europe, a rise in anthropophilic tinea capitis has been noticed, notably in metropolitan settings (Ginter-Hanselmayer., 2007). The anthropophilic *Trichophyton tonsurans* is the most commonly reported etiologic agent in the UK, whilst *Trichophyton soudanense and M. audouinii* are

most prevalent in France. *M. audouinii* instances have also been recorded in Italy (Panasiti., *et al.,* 2007), Spain (Rezusta *et al.,* 2009), and Portugal (Roque *et al.,* 2006). These anthropophilic fungi are especially widespread in immigrant groups from Africa and Asia. They induce fewer inflammatory responses and have a greater potential to be connected with chronic illness as compared to infections caused to *M. canis.* The latter may be the consequence of late identification owing to the lack of subjective symptoms, even though kerion may exist.

Dermatophytes are agents of often benign superficial infections, among which the non-inflammatory, scaly lesions of toe webs owing to Trichophyton rubrum are recognized as the most prevalent example (Seebacher *et al.,* 2008). However, there are several cases of severe and even life-threatening dermatophytic infections among the expanding number of immunocompromised individuals, demonstrating that dermatophytoses may offer a more significant hazard to these patients (Marconi *et al.,* 2010)

1.4 Extraction of Medicinal Plants

Extraction, as the term is used pharmaceutically, is the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by utilizing selected solvents in typical extraction processes. The products thus produced from plants are rather impure liquids, semisolids or powders designed solely for oral or topical application.

These include groups of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts, and powdered extracts. Such medicines generally have been dubbed galenicals, named after Galen, the second-century Greek physician.

Medicinal plants are extracted and processed for direct use as herbal or traditional medicine or made for experimental reasons. The notion of preparation of medicinal plants for experimental purposes requires the correct and timely gathering of the plant, verification by an expert, suitable drying, and grinding. This is followed by extraction, fractionation, and

isolation of the bioactive component if appropriate. In addition, it involves the determination of the amount and quality of bioactive chemicals

Recently, plants as a source of medication are gaining worldwide appeal because of their natural origin, availability in local communities, cheaper to acquire, convenience of administration, and potentially less problematic. Also, herbal medicine may be a valuable alternative therapy in case of multiple adverse effects and medication resistance (Azwanida., 2015).

Extraction of medicinal plants is a technique of separating active plant elements or secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, steroids, and glycosides from inert or inactive material using an appropriate solvent and standard extraction procedure. Plant materials with high concentration of phenolic compounds and flavonoids were shown to contain antioxidant qualities and thus are utilized to treat age-related disorders such as Alzheimer's disease, Parkinsonism, anxiety, and depression (Azwanida., 2015, Sasidharan *et al*., 2011). Several procedures were utilized in the extraction of medicinal plants such as maceration, infusion, decoction, percolation, digestion, Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extraction. The choice of an acceptable extraction technique relies on the type of the plant material, the solvent employed, pH of the solvent, temperature, and solvent to sample ratio. It also relies on the planned application of the final goods (Doughari., 2012).

1.4 METHODS IN THE EXTRACTION OF MEDICINAL PLANTS

1.4.1 Decoction

This is a method that includes continuous hot extraction utilizing a specific amount of water as a solvent. A dried, ground and powdered plant material are put into a clean container. Water is then added and mixed. Heat is then provided throughout the procedure to expedite the extraction (Pandey *et al*., 2014). The procedure has lasted for a brief length generally

around 15min. The ratio of solvent to a crude drug is commonly 4:1 or 16:1. It is utilized for the extraction of water-soluble and heat-stable plant material (Azwanida., 2015).

1.4.2 Maceration

This is an extraction process in which coarsely powdered drug material, either leaves or stem bark, or root bark, is put within a container; the menstruum is poured on top until thoroughly coated with the drug material. The container is then closed and preserved for at least three days (Majekodunmi., 2015). The content is swirled occasionally, and if put within the container it should be shaken from time to time to ensure full extraction. At the completion of extraction, the micelle is removed from the marc by filtering or decantation. Subsequently, the micelle is removed from the menstruum by evaporation in an oven or on top of a water bath (Azwanida., 2015). This approach is easy and extremely good for thermolabile plant material.

1.4.3 Infusion

This is an extraction method such as maceration. The medication ingredient is pounded into a fine powder and then put into a clean container. The extraction solvent hot or cold is then put on top of the drug material, soaked, and stored for a brief length of time (Majekodunmi., 2015). This approach is useful for extracting bioactive components that are easily soluble. In addition, it is a good procedure for the production of the fresh extract before usage. The solvent-to-sample ratio is commonly 4:1 or 16:1 depending on the intended purpose (Pandey *et al*., 2014).

1.4.4 Digestion

This is an extraction technique that requires the application of mild heat during the extraction process. The solvent of extraction is put into a clean container followed by a powdered drug substance. The mixture is put over a water bath or in an oven at a temperature of around 50°C (Pandey *et al*., 2014). The heat was administered throughout the extraction process to lower

the viscosity of the extraction solvent and promote the removal of secondary metabolites. This approach is ideal for plant materials that are easily soluble (Pandey *et al*., 2014).

1.4.5 Percolation

The apparatus used in this procedure is called a percolator. It is a narrow-cone-shaped glass vessel with an aperture at both ends. A dried, ground and finely powdered plant material are moistened with the solvent of extraction in a clean container. More amount of solvent is added, and the mixture is maintained for a period of 4h. Subsequently, the material is then put into the percolator with the bottom end closed and left to stand for a period of 24h (Majekodunmi., 2015). The solvent of extraction is then poured from the top until the drug material is thoroughly saturated. The bottom half of the percolator is then opened, and the liquid is allowed to trickle gently. Some quantity of solvent was supplied continuously, and the extraction took place by gravitational force, forcing the solvent through the drug material downhill (Pandey *et al*., 2014). The addition of solvent ceased when the volume of solvent added reached 75% of the planned amount of the complete preparation. The extract is separated by filtering followed by decantation. The marc is then expressed and a final quantity of solvent is added to achieve the desired volume (Azwanida., 2015).

1.5 Chromatography

Chromatography is a process for separating the components, or solutes, of a mixture on the basis of the relative quantities of each solute divided between a moving fluid stream, termed the mobile phase, and a contiguous stationary phase. The mobile phase may be either a liquid or a gas, whereas the stationary phase is either a solid or a liquid.

Chromatography is based on the idea that molecules in a mixture are placed onto the surface or into the solid, and the fluid stationary phase (stable phase) is separated from each other while moving with the help of a mobile phase. The elements efficient in this separation process include molecular properties linked to adsorption (liquid-solid), partition (liquid-

solid), and affinity or variations among their molecular weights (Cuatrecasas *et al.,* 1996). Because of these variances, certain components of the mixture remain longer in the stationary phase, and move slowly through the chromatographic system, while others flow swiftly into the mobile phase, and exit the system quicker (Harris., 2004).

Based on this method three components constitute the foundation of the chromatography process. Stationary phase: This phase is always made of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support". Mobile phase: This phase is always made of "liquid" or a "gaseous component."

Separated molecules

The kind of interaction between the stationary phase, mobile phase, and substances present in the mixture is the fundamental component effective in the separation of molecules from each other. Chromatography techniques based on partition are particularly successful in the separation, and identification of tiny molecules such as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (i.e ion-exchange chromatography) are more successful in the separation of macromolecules such as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, Esther, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is utilized for the purification of RNA, DNA particles, and viruses (Gerberding *et al*., 1998).

The stationary phase in chromatography is a solid phase or a liquid phase deposited on the surface of a solid phase. The mobile phase flowing over the stationary phase is a gaseous or liquid phase. If the mobile phase is liquid it is termed liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is employed for gases,

mixtures of volatile liquids, and solid materials. Liquid chromatography is employed primarily for heat unstable, and non-volatile substances (Donald *et al.,* 2006). The goal of employing chromatography which is utilized as a technique of quantitative analysis aside from its separation is to obtain a sufficient separation within an acceptable time period.

Various chromatographic techniques

1.5.0 Column chromatography

Since proteins have distinct characteristic qualities such as size, shape, net charge, stationary phase utilized, and binding ability, each one of these characteristic components may be purified using chromatographic procedures. Among these approaches, column chromatography is utilized. This approach is utilized for the purification of biomolecules. On a column (stationary phase) initially the sample is separated, then a wash buffer (mobile phase) is added. Their passage via interior column material put on fiberglass support is assured. The samples are gathered in the bottom of the device in a time-, and volume-dependent manner (Das *et al*., 1998).

1.5.1 Ion-exchange chromatography

Ion-exchange chromatography is based on electrostatic interactions between charged protein groups, and solid support material (matrix). The matrix contains an ion load opposite to that of the protein to be separated, and the affinity of the protein to the column is obtained through ionic ties. Proteins are separated from the column either by modifying pH, the concentration of ion salts, or the ionic strength of the buffer solution (Karlsson and Ryden., 1998). Positively charged ion-exchange matrices are termed anion-exchange matrices and adsorb negatively charged proteins. While matrices associated with negatively charged groups are known as cation-exchange matrices and absorb positively charged proteins.

1.5.2 Gel- permeation (molecular sieve) chromatography

The primary premise of this technology is to employ dextran-containing materials to separate macromolecules based on their variations in molecular sizes. This approach is essentially used to estimate the molecular weights of proteins and to reduce salt concentrations of protein solutions (Walls and Loughran., 2011). In a gel- permeation column stationary phase comprises inert molecules with tiny pores. The solution comprising molecules of varying diameters is fed continuously with a steady flow rate through the column. Molecules bigger than pores cannot infiltrate into gel particles, and they are held between particles within a confined space. Larger molecules penetrate through crevices between porous particles and travel swiftly within the column. Molecules smaller than the pores are diffused into pores, and as molecules grow smaller, they exit the column with correspondingly longer retention durations (Helmut.,1969). Sephadex G type is the most often utilized column material. Besides, dextran, agarose, and polyacrylamide are also employed as column materials (Determann., 2012).

1.5.3 Affinity chromatography

This chromatography approach is employed for the purification of enzymes, hormones, antibodies, nucleic acids, and particular proteins (Wilchek and Chaiken., 2000). A ligand that can build a compound with a certain protein (dextran, polyacrylamide, cellulose, etc) attaches to the filling material of the column. The particular protein which builds a complex with the ligand is connected to the solid support (matrix), and kept in the column, whereas free proteins depart the column. Then the bound protein departs the column by means of modifying its ionic strength by the adjustment of pH or the addition of a salt solution (Firer., 2001).

1.5.4 Paper chromatography

In the paper, chromatography support material consists of a layer of cellulose heavily saturated with water. In this procedure, a thick filter paper composed the support, and water

droplets deposited in its pores made up the stationary “liquid phase.” The mobile phase consists of a suitable fluid put in a growing tank. Paper chromatography is a “liquid-liquid” chromatography (Stoddard., 2007).

1.5.5 Thin-layer chromatography

Thin-layer chromatography is a “solid-liquid adsorption” chromatography. In this approach, the stationary phase is a solid adsorbent material placed on glass plates. As adsorbent material, any solid substances are employed. in column chromatography (alumina, silica gel, cellulose) may be employed. In this procedure, the mobile phase goes upward via the stationary phase The solvent goes up the thin plate saturated with the solvent by means of capillary action. This technique, also propels the mixture priorly dropped on the bottom sections of the plate using a pipette upwards with varying flow rates. Thus the separation of analytes is accomplished. This upward moving rate relies on the polarity of the substance, solid phase, and solvent (Sherman., 1991).

In circumstances when molecules of the sample are colorless, fluorescence, radioactivity, or a particular chemical reagent may be utilized to generate a visible colored reaction result so as to identify their locations on the chromatogram. The creation of a visible color may be noticed under room light or UV light. The location of each molecule in the mixture may be estimated by measuring the ratio between the lengths traveled by the molecule and the solvent. This measurement value is termed relative mobility and is indicated with the sign Rf. Rf. value is employed for the qualitative description of the molecules (Donald., 2006).

1.5.6 Gas chromatography

In this approach, the stationary phase is a column that is inserted in the device and includes a liquid stationary phase that is adsorbed onto the surface of an inert solid. Gas chromatography is a “gas-liquid” chromatography. Its carrier phase includes of gases such as He or N2. The mobile phase which is an inert gas is passed via a column at high pressure.

The material to be studied is vaporized and goes into a gaseous mobile phase. The components present in the sample are disseminated between the mobile phase and stationary phase on the solid support. Gas chromatography is a simple, versatile, very sensitive, and swiftly applied technology for the exceedingly good separation of very minute molecules. It is utilized in the separation of extremely tiny quantities of analytes

1.5.7 Dye-ligand chromatography

The design of this approach was based on the showing of the capacity of several enzymes to bind purine nucleotides for Cibacron Blue F3GA dye (Amicon., 1989). The planar ring structure with negatively charged groups is comparable to the structure of NAD. This comparison has been established by showing the binding of Cibacron Blue F3GA dye to adenine, ribose binding sites of NAD. The dye acts as an analog of ADP-ribose. The binding capacity of this kind of adsorbent is 10–20-fold greater than that of the affinity of other adsorbents. Under suitable pH settings, elution with high-ionic strength solutions, and exploiting the ion-exchange property of adsorbent, the adsorbed proteins are removed from the column (Scopes., 1984).

1.5.8 Hydrophobic interaction chromatography (HIC)

In this procedure, the adsorbents manufactured as column material for the ligand binding in affinity chromatography are utilized. The HIC approach is based on hydrophobic interactions between side chains linked to the chromatographic matrix (Mahn., 2005).

1.5.9 Pseudo Affinity chromatography

Some compounds such as anthraquinone dyes and azo-dyes may be employed as ligands because of their affinity, notably for dehydrogenases, kinases, transferases, and reductases The generally recognized kind of this sort of chromatography is immobilized metal affinity chromatography (Queiroz., 2001).

1.5.10 High-pressure liquid chromatography (HPLC)

Using this chromatography technology it is feasible to do structurally, and functional analysis, and purification of numerous compounds within a short time, This approach offers flawless results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other physiologically active substances, In HPLC, mobile phase travels throuıgh columns under 10–400 atmospheric pressure, and with a high (0.1–5 cm/sec) flow rate. In this approach, the employment of tiny particles, and the application of high pressure on the rate of solvent flow enhances the separation power, of HPLC and the analysis is done within a short time.

Essential components of an HPLC apparatus include a solvent store, high-pressure pump, commercially manufactured column, detector, and recorder. Duration of separation is managed with the use of a computerized system, and material is accumulated (Regnier., 1983).

1.6 OBJECTIVES OF STUDY

The objectives of the study include;

- To obtain the n-hexane fraction of Emilia coccinea whole plant
- To obtain a crude extract of Emilia coccinea whole plant
- To evaluate antifungal activities of the n-hexane fraction on fungal pathogens

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Apparatus and equipment

Beaker, Rotatory Evaporator (ST150SA), Separating funnel, Whatman's filter paper (Number 1), Glass jar, Milling Machine (Galiham), Distilled water, Capillary tube, Glass column, Test tube, Measuring cylinder, Beaker, Silica gel (LOBA CHEMIE PVT LTD), Bijou and Universal bottle, Mccartney, porcelain dish, Sterile swab sticks and Portable autoclave (Gallenkamp UK)

2.2 Solvent Sources

Analytical grade reagent used in this studies was 99.8% Methanol (LOBA CHEMIE PVT LTD), 96% n-Hexane (Merck), 99.8% Dichloromethane (Central Drug House), 99.5% Ethyl-acetate (LOBA CHEMIE PVT in LTD), 1% Sulphuric acid, 1% Barium chloride, 10% Tween 80, and 10µg/ml Ketoconazole

2.3 Plant collection and preparation

Emilia coccinea plants were collected within the premises of the University of Benin, Benin City, Nigeria in August between 10 to 11 am in the year 2021 by uprooting the plant material from the soil. The plants were carefully selected. The roots of the plant's material were washed in the tap running water. It was identified by Prof Akinnibosun of the Department of Plant and Plant Biotechnology, Faculty of Life Science, University of Benin. Herbarium specimen number (E443) was issued and a specimen sample was kept in the herbarium.

The fresh whole plants of *Emilia cocinnea* were air-dried for 16 days (two weeks and two days) at room temperature and then put in an oven at 60^{o}C for 4 hours before being ground to a fine powder with the aid of a mechanical grinder (British milling machine) and kept in a close tight container until used.

2.4 Extraction and Fractionation

To the fine powdered plant material of *Emilia coccinea.* The methanolic extraction was carried out via a glass jar by maceration. The 561.80 g of the powdered plant material was added into 4.5 L of methanol in an extractive Jar with intermittent agitation and allowed to stand at room temperature for 3 days. Pressure was applied to the mixture and filtered using a Whatman filter after 3 days. The filtrate was concentrated via *in vacuo* using Rotatory Evaporator at 40^{o}C. It was kept in a refrigerator at 4^{o}C. The crude extract was suspended inside the mixture of a 1:4 ratio of methanol to water in the separating funnel. Exactly 300ml of n-hexane was added and shaken carefully, to mix very well and allowed to stand until two layers until two clear layers are formed. Then decanted into a separate container. Another 300ml of n-hexane was added to the content in the separating funnel and the process of swirling and decantation was repeated. The process was repeated until the n-hexane fraction collected becomes colourless (ten times). The n-hexane fraction collected was put together, concentrated *in vacuo* using a Rotary evaporator, and kept in Refrigerator at 4^{o}C.

2.5 Source of Test Microorganisms

The microbial cultures used were obtained from stock cultures of clinical isolates cases of *Tinea capitis* (ringworm of the scalp) maintained in Sabouraud Dextrose Agar (SDA) slant from State University Teaching Hospital in Oghara, Delta State, Nigeria. The reference fungal cultures include *Aspergillus fumigatus, Trichophyton rubrum, Candida albicans, Microsporum audounii* and *Rhodotorula glutinis.*

2.6 Antifungal Assay of the Extract and Fractions

The modified agar well diffusion method described by Cheesbrough, (2006) and Dowey *et al.* (2016) was used to determine the antifungal sensitivity potency of the methanol crude extract and fractions (n-hexane) of the *Emilia coccinea* whole plant. Wells of 6 mm in diameter were

made into previously seeded Potato Dextrose Agar (PDA) plates using a flamed (sterilized) cork borer. Prior to seeding, isolated colonies/spores stored in SDA slants were sub-cultured in Sabouraud dextrose broth, vigorously shaken and diluted (1:100) to achieve 0.5 Macfarland tubidity standard (containing approximately 10^6 spores/mL when counted using a cytometer). Sterile swab sticks was then dipped into the standardized microbial suspension and gently spread over (seeding) the surface of the agar plates in even strokes to obtain a uniform growth pattern across the entire surface of the plate. This was achieved by rotating the plate 90 degrees followed by 45 degrees with continuous streaking, and finally by streaking round the diameter of the agar. The 6 mm wells were filled with equal volumes (100 µL) of the crude extract and fractions corresponding to 100, 80, 60 and 40 mg/mL concentrations. The same quantity of 10 % Tween-80 and 10 µg/mL of Ketoconazole served as negative and positive controls respectively. The plates were incubated at ambient temperature (27±2^{o}C) for 48-72 hours in an upright position to allow proper diffusion of extracts and the experiments were done in triplicates. After incubation, the absence or presence of growth was observed on the plates and the diameter of clear zones were measured using a millimetre (mm) calibrated ruler and the mean Inhibition Zones Diameters (IZDs) calculated and recorded.

2.7 Minimum Inhibitory Concentration Determination of the Extract and Fractions

Agar dilution technique was used to decide the MICs of the whole plants extracts and n-hexane fractions of *Emilia coccinea* . Stock concentrations of 200 mg/ml of extract and fractions were prepared and two-fold serial dilutions were made to get concentrations of extract and fraction solutions ranging from 10.00 mg/ml to 36.00 mg/ml. Isolates of the fungal that grew on the plates were diluted to 10^6 CFU/ml and 0.025 ml volume was placed on marked areas that contained different concentrations of the extract tested. Incubation of the plates was at a temperature of 25^{o}C for a period of 48 hours for species of *Candida* and a

period of 72 hours for the isolates of the other fungal. The lowest concentration at which there was no noticeable fungal growth was recorded as the MICs.

2.8 Statistical Analysis

The mean (±) standard error of the mean are used to represent the results of the sensitivity tests in tables. Using GraphPad Prism software, a one-way ANOVA was performed on the mean of the results obtained for the n-Hexane fraction at a 95% confidence level. Dunnett's multiple comparison test between the mean of each result obtained and the mean of the Positive control was also carried out at a confidence interval of 95% and the results were found to be statistically significant. The sensitivity test was plotted in a graph using Excel software packages.

CHAPTER THREE

3.0 RESULTS

Extract/Fractions	Weight(g)
Methanol	170.98
n-hexane	21.55

Table 2: Zones of inhibition in mm of different concentrations of the n-hexane fraction of EC

Fungi	40 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml	ketoconazole	Tween-80
Candida albicans	2.25±0.18	6.50±1.06	11.50±0.35	15.50±1.06	20.50±0.35	0.00±0.00
Aspergillus fumigatus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	17.50±1.06	0.00±0.00
Trichophyton rubrum	1.00±0.00	6.50±1.76	8.00±2.12	12.00±3.54	22.0±0.18	0.00±0.00
Rhodotorula glutinis	1.50±0.35	4.50±0.35	6.00±0.71	7.00±0.71	21.50±0.35	0.00±0.00
Microsporum audounii	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	24.50±0.35	0.00±0.00

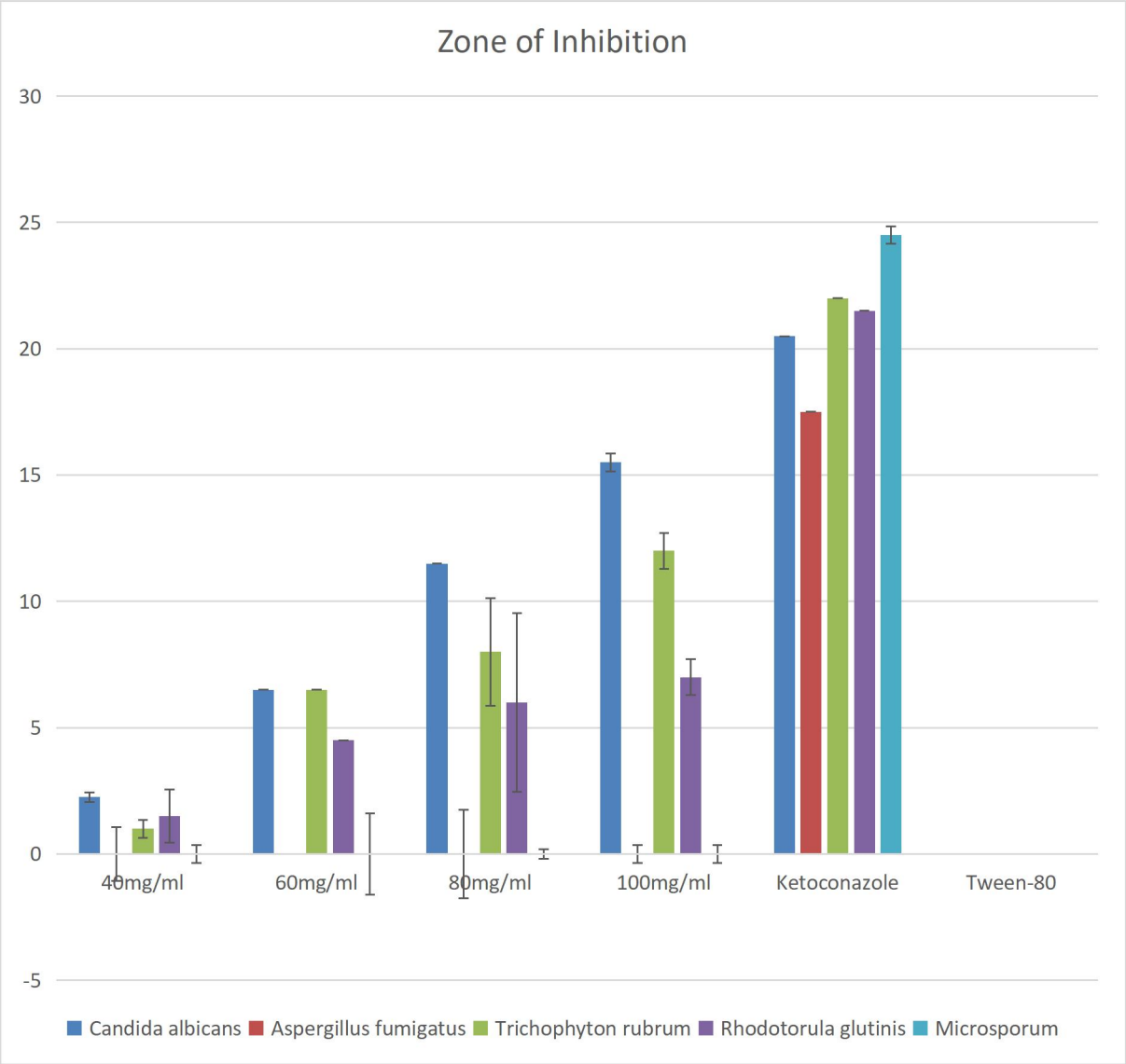
Zone of Inhibition
30
25
20
15
10
5
0
-5
40mg/ml
60mg/ml
80mg/ml
100mg/ml
Ketoconazole
Tween-80
Candida albicans
Aspergillus fumigatus
Trichophyton rubrum
Rhodotorula glutinis
Microsporum

Table 3: MIC of n-hexane fraction against selected fungi species

Concentration (mg/ml)														
Fungi	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Candida albicans	+	+	+	+	+	+	+	+	+	+	+	–	–	–
Aspergillus fumigates	+	+	+	+	+	+	+	+	+	+	+	+	+	>36
Trichophyton rubrum	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Rhodotorula glutinis	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Microsporum audounii	+	+	+	+	+	+	+	+	+	+	+	+	+	>36

Key: +: Growth**,** –: No Growth

CHAPTER FOUR

DISCUSSION

Emilia coccinea (Sims) G. Don is reported to possess a number of medicinal properties including antimicrobial activities. The antifungal effects of n-Hexane fraction *Emilia coccinea* whole plant were studied on different species of fungi Candida albicans, *Aspergillus fumigatus, Trichophyton rubrum, Rhodotorula glutinis, and Microsporum audounii* at the doses of 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36 Mg/ml. The n-Hexane fraction, significantly inhibit the growth of *Candidia albicans* at tested concentration of 32, 34 and 36 mg/m,l and *Trichophyton rubrum, and Rhodotorula glutinis* at concentration of 34, and 36 mg/ml. It was observed that susceptibility increased with increased concentration of the fraction. The n-Hexane fraction did not have any antifungal activity at the tested concentrations (10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36 Mg/ml), on *Aspergillus fumigatus, and Microsporum audounii.*

These results may support the fact that this plant is used traditionally to treat microbial infections.

n-Hexane fraction recorded the highest antifungal activity against *C. albicans* at the highest concentration of 100 mg/ml with an inhibition diameter of 15.50+1.06 mm. *Rhodotorula glutinis* was the least sensitive fungus and test organism, while no sensitive on *Aspergillus fumigatus, and Microsporum audounii.*

This is supported by the fact that the fraction contains both pharmacological and non pharmacological active substances as against pure active substances contained in the antibiotics. The effect of the commercial antifungal drug (Ketoconazole) tested at the concentration of 200mg/ml against the test fungi (Table 2) can be considered not better in activity when compared with the fraction, particularly at the highest tested concentration of 100 mg/ml which was 2 times lower in concentration than that of the fungal antibiotics. This

probably implies that if the concentrations of the fractions are increased, it can lead to increased activity. It can be observed from the zone of inhibition values that the n-hexane fraction was obtained in table 2.

Conclusion

The outcomes of these studies offer proof for the ethnomedical use of the investigated plant. Secondary metabolites can be found in the *E. coccinea* whole plants and are regarded to be primarily responsible for its therapeutic effects. The n-hexane fraction of *E. coccinea* showed activity against some of the test fungi (*Candida albicans, Trichophyton rubrum, and Rhodotorula glutinis*). It can therefore probably serve as a potential source of antifungal agents. However further studies are recommended on the chemical characterization and biological availability of the fraction

Reference

1. Ajibesin K. K. (2011). Dacryodes edulis (G. Don) H. J. Lam: a review on its medicinal, phytochemical and economic properties. Research Journal of Medicinal Plant 5 (1): 32 - 41.
2. Akinpelu (2012) Effect of stem - bark of Erythrophleum suaveolens (Guill. & Perri.) saponin on fresh water snail (Lanistes lybicus) tissues. African Journal of Environmental Science and Technology 6(11):446–451.
3. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA (2017) Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts, 6:42.
4. Amicon, (1989) Dye-ligand chromatography. Applications method. Theory of matrix gel media, Amicon Division. Cherry Hill Drive, MA 01923: N.R. Grace&Company-Conn. 24.
5. Azwanida NN (2015). A review on the extraction methods use in medicinal plants, principle, strength, and limitation. Med Aromat Plants, 4:196.
6. Bhan M (2017) Ionic liquids as green solvents in herbal extraction. Int J Adv Res Dev.x, 2:10–12.
7. Beena P, Rajesh KJ, Arul B (2016). Preliminary phytochemical screening of Cicer arietinum in folklore medicine for hepatoprotection. J Innov Pharm Biol Sci, 3:153–9.
8. Bosch, C.H. (2004). Emilia coccinea (Sims) G. Don. [Internet] Record from PROTA4U. Grubben, G.J.H. & Denton, O.A. (Editors). PROTA (Plant Resources of Tropical Africa / Resources végétales de l'Afrique tropicale), Wageningen, Netherlands.
9. Burkill HM (1985). The useful plants of West Africa. Kew: Royal Botanical Gardens.

10. Chikezie, P.C., Ojiako, O.A. and Nwufo K.C. (2015) Overview of Anti-Diabetic Medicinal Plants: The Nigerian Research Experience. Journal of Diabetes and Metabolism, 6, 546.
11. Cuatrecasas P, Wilchek M, Anfinsen CB. (1968) Selective enzyme purification by affinity chromatography. Proc Natl Acad Sci U S A. 61:636–643.
12. Cutler P. (2004) Methods in molecular biology, Dye-ligand affinity chromatography. 2nd ed. Totowa, NJ: Humana Press.
13. Das K, Tiwari RK, Shrivastava DK. (2010) Techniques for evaluation of medicinal plant products as antimicrobial agents: Current methods and future trends. J Med Plants Res. 2010;4:104–111.
14. Das M, Dasgupta D.(1998) Pseudo-affinity column chromatography based rapid purification procedure for T7 RNA polymerase. Prep Biochem Biotechnol. 1998;28:339–348.
15. Davidse G, Sousa-Sánchez M, Knapp S, Chiang F, UUoa Ulloa C, Pruski J F, (2018). Flora Mesoamericana, Volumen 5, Parte 2: Asteraceae. [ed. by Davidse G, Sousa-Sánchez M, Knapp S, Chiang F, UUoa Ulloa C, Pruski J F]. St. Louis, USA: Missouri Botanical Garden Press. xix + 608.
16. Dhawan D, Gupta J. (2017). Comparison of different solvents for phytochemical extraction potential from Datura metel plant leaves. Int J Biol Chem. 11:17–22.
17. Banu KS, Cathrine L. (2015) General techniques involved in phytochemical analysis. Int J Adv Res Chem Sci. 2:25–32.
18. Denning, D. W. (1998). Invasive aspergillosis. Clinical. Infectious. Disease. 26:781-803
19. D. H. Larone (1995), Medically Important Fungi—A Guide to Identification, American Society for Microbiology, Washington, DC, USA, 3rd edition.

20. Donald PL, Lampman GM, Kritz GS, Engel RG. (2006) Introduction to organic laboratory techniques. 4th ed. Thomson Brooks/Cole; 2006. pp. 797–817.
21. Doughari JH. (2012). Phytochemicals: Extraction methods, basic structures, and mode of action as potential chemotherapeutic agents, phytochemicals—a global perspective of their role in nutrition and health. In: Venketeshwer R, editor. A Global Perspective of Their Role in Nutrition and Health.
22. Edeoga, H. O., Okwu, D. E., Mbaebie, B. O. (2005). Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology. 4(7): 685-688.
23. Eloff JN. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol. 60:1–8.
24. E. W. Cross, S. Park, and D. S. Perlin (2000). "Cross-resistance of clinical isolates of Candida albicans and Candida glabrata to over-the-counter azoles used in the treatment of vaginitis," Microbial Drug Resistance, vol. 6, no. 2, pp. 155–216.
25. Foyet HS, Abdou BA, Ngatanko AHH, Manyi FL, Manyo NA, ShuNyenti PN. (2014). Neuroprotective and memory improvement effects of a standardized extract of Emilia coccinea SSon animal models of anxiety and depression. J. Pharmacog. Phytochem. 3(3):146-154.
26. Firer MA. (2001) Efficient elution of functional proteins in affinity chromatography. J Biochem Biophys Methods. 49:433–442.
27. Gerberding SJ, Byers CH. (1998). Preparative ion-exchange chromatography of proteins from dairy whey. J Chromatogr A. 808:141–151.
28. Ginter-Hanselmayer G, Weger W, Ilkit M, Smolle J.(2007). Epidemiology of tinea capitis in Europe: current state and changing patterns, Mycoses, vol. 50 6-13
29. Gugnani, H. C. (2003). Ecology and taxonomy of pathogenic aspergilli. Front. Biosci. 8:346-357.

30. Harborne JB.(1998) Phytochemical methods: A guide to modern techniques of plant analysis. 3rd ed. New York, NY: London, UKThomson Science; 219.
31. Harris DC.(2004) Exploring chemical analysis. 3rd ed. WH. Freeman&Co.
32. Harwood LM, Moody CJ. (1989). Experimental organic chemistry: Principles and Practice. Oxford: Blackwell Science; 180–185.
33. Hawksworth, D. L. (1992). Fungi: A neglected component of biodiversity crucial to ecosystem function and maintenance. Canadian Biodiversity 1: 4-10.
34. Heftmann F. (1992). Chromatography: Fundamentals and application of chromatographic and electrophoretic techniques. 5th ed. Amsterdam, The Netherlands: Elsevier; 281–285.
35. Helmut D .(1969) Gel Chromatography, gel filtration, gel permeation, molecular sieves:a laboratory handbook. Springer-Verlag.
36. Hossain MA, Al-Hdhrami SS, Weli AM, Al-Riyami Q, Al-Sabahi JN. (2014) Isolation, fractionation and identification of chemical constituents from the leaves crude extracts of Mentha piperita L grown in sultanate of Oman. Asian Pac J Trop Biomed 4:S368–S372.
37. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurkar VC. (2017) Phytochemicals: Extraction methods, identification, and detection of bioactive compounds from plant extracts. J Pharmacogn Phytochem 6:32–36.
38. J. A. Vazquez and J. D. Sobel, (2002). “Mucosal candidiasis,” Infectious Disease Clinics of North America 16: 793–820.
39. J. D. Sobel, M. Zervos, B. D. Reed.,(2003). “Fluconazole susceptibility of vaginal isolates obtained from women with complicated Candida vaginitis: clinical implications,” Antimicrobial Agents and Chemotherapy, vol. 47, no. 1, pp. 34–38

40. Jeffrey, C. (1986).The Senecioneae in East Tropical Africa: notes on Compositae: IV. In: Kew Bulletin,41. 873–943.
41. Jeffrey C (1997). What is Emilia coccinea (Sims) G. Don (Compositae)? A revision of the large-headed Emilia species of Africa. In: Kew Bulletin,52. 205–212.
42. J. J. Castón-Osorio, A. Rivero, and J. Torre-Cisneros, (2008). "Epidemiology of invasive fungal infection," International Journal of Antimicrobial Agents, vol. 32, no. 2, S103–S109.
43. Kamboj, A, and Saluja, A. K. (2011). Isolation of stigmasterol and β -Sitosterol from petroleum ether extract of aerial parts of Ageratum conyzoides (Asteraceae). International. Journal of Pharmaceutical Science. 1: 94-96.
44. Karlsson E, Ryden L, Brewer J. (1998) Protein purification. Principles, High-Resolution Methods, and Applications. Ion exchange chromatography. 2nd ed. New York: Wiley.
45. Latge, J. P. (1999). Aspergillus fumigatus and aspergillosis. Clinical. Microbiology. Review. 12:310-350.
46. Mahn A, Asenjo JA. (2005) Prediction of protein retention in hydrophobic interaction Chromatography. Biotechnol Adv. 2:359–368.
47. Majekodunmi SO (2015). Review of extraction of medicinal plants for pharmaceutical research. MRJMMS 3:521–527.
48. Marconi, V. C., Kradin, R., Marty, F. M., Hospenthal, D. R., and Kotton, C. N. (2010). Disseminated dermatophytosis in a patient with hereditary hemochromatosis and hepatic cirrhosis: case report and review of the literature. Med. Mycol. 48:518–527
49. M. H. Miceli, J. A. Díaz, and S. A. Lee,(2011) "Emerging opportunistic yeast infections," The Lancet Infectious Diseases, vol. 11, no. 2, 142–151.

50. M. Sojakova, D. Liptajova, M. Simonicova, M. Borovsky, and J. Subik (2003) "Vulvovaginal candidiasis and susceptibility of pathogens to antifungal agents," Ceská Gynekologie, vol. 68, 24–29.
51. MVS MK, Talluri VP, Rajagopal SV.(2015) Purification and characterization of bioactive compound from the methanolic leaf extract of Millingtonia hortensis linn. Int J Pharm Bio Sci. 6:348–358.
52. Naczk M., Shahidi F. (2006) Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. J. Pharm. Biomed. 41:1523–1542.
53. Nwachukwu, V. A., Udedi, S. C., Ezeonu, F. C., Orji, F. A., Ezeanyanaso, C. S., Bartholomew, I. C., Brai, S. F. O. and Elemo, G. N. (2017). An analysis of food value and some selected secondary metabolites of Emilia coccinea (Asteraceae) Leaf. Journal of Complementary and Alternative Medical Research. 2(2):1-11.
54. Panasiti V, Devirgiliis V, Borroni RG. (2007) Epidemiology of dermatophytic infections in Rome, Italy: a retrospective study from 2002 to 2004, Med Mycol, vol. 45:57-60
55. Pandey A, Tripathi S.(2014) Concept of standardization, extraction, and pre-phytochemical screening strategies for herbal drug. J Pharmacogn Phytochem. 2:115–119.
56. Porath J. (1992) Immobilized metal ion affinity chromatography. Protein Expr Purif. 3:263–281.
57. Post, M. J., C. Lass-Floerl, G. Gastl, and D. Nachbaur. (2007). Invasive fungal infections in allogeneic and autologous stem cell transplant recipients: a single-center study of 166 transplanted patients. Transplantation Infectious Disease. 9:189-195.
58. Queiroz JA, Tomaz CT, Cabral JM (2001). Hydrophobic interaction chromatography of proteins. J Biotechnol. 87:143–159.

59. Regnier FE. High-performance liquid chromatography of biopolimers. Science. 1983:245–252.
60. Rezusta A, Betran A, Querol I, Palacian MP, Revillo MJ.(2009). Tinea capitis caused by Trichophyton soudanense and Microsporum audouinii in an adult: a case report, Mycoses.
61. Rimando AM, Olofsdotter M, Dayan FE, Duke SO, Agron J (2001). Searching for rice allelochemicals: An example of bioassay-guided isolation, 93:16–20.
62. Roque HD, Vieira R, Rato S, Luz-Martins M. (2006). Specific primers for rapid detection of Microsporum audouinii by PCR in clinical samples, J Clin Microbiol, vol. 44, 4336-4341
63. Rungsung W, Ratha KK, Dutta S, Dixit AK, Hazra J (2015). Secondary metabolites of plants in drugs discovery. World J Pharm Res, 4:604–613.
64. Sandberg F, Corrigan D (2001) Natural Remedies. Their Origins and Uses. Abingdon: Taylor & Francis;.
65. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts 8:1–10.
66. Seebacher, C., Bouchara, J. P., and Mignon, B. (2008). Updates on the epidemiology of dermatophyte infections. Mycopathologia 166:335–352
67. Scopes RK (1984). Use of differential dye-ligand chromatography with affinity elution for enzyme purification:2-keto-3-deoxy-6-phosphogluconate aldolase from Zymomonas mobilis. Anal Biochem 136:525–9.
68. Sherman J, Fried B, Dekker M (1991). Handbook of Thin-Layer Chromatography
69. Sofawara, E. A. (1996). Research on medicinal plants and traditional medicine in Africa. Journal of Alternative and Complementary Medicine 2 (3): 365-372.

70. Sofowora A (1980) The present status of knowledge of the plants used in traditional medicine in western Africa: A medical approach and a chemical evaluation. J Ethnopharmacol 2:109–18.
71. S. S. Richter, R. P. Galask, S. A. Messer, R. J. Hollis, D. J. Diekema, and M. A. Pfaller, (2005) "Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases," Journal of Clinical Microbiology, vol. 43, no. 5, 2155–2162.
72. Stoddard JM, Nguyen L, Mata-Chavez H, Nguyen K.(2007). TLC plates as a convenient platform for solvent-free reactions. Chem Commun (Camb), 12:1240–1241.
73. Teke, G. N., Kuiate, J. R., Ngouateu, O. B. and Gatsing, D. (2007). Antidiarrhoeal and antimicrobial activities of Emilia coccinea (Sims) G. Don extracts. Journal of Ethnopharmacology. 112(2): 278-283.
74. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur (2011). Phytochemical screening and extraction: A review. Int Pharm Sci. 1:98–106.
75. Trease GE, Evans WC (1989). Textbook of pharmacognosy. 13th ed. London, UK; Toronto, Canada; Tokyo, Japan: Bailiere Tindall, 200–201.
76. Ujang ZB, Subramaniam T, Diah MM, Wahid HB, Abdullah BB, Rashid AA, Appleton D (2013). Bioguided fractionation and purification of natural bioactive obtained from Alpinia conchigera water extract with melanin inhibition activity. J Biomater Nanobiotechnol, 4:265–272.
77. Walls D (2011), Loughran ST. Protein chromatography: Methods and protocols, methods in molecular biology;681
78. Wallis TE. Text book of pharmacognosy. Delhi, India: CBS Publishers and Distributors; 1989. pp. 356–549.

79. Wiederhold, N. P., R. E. Lewis, and D. P. Kontoyiannis. (2003). Invasive aspergillosis in patients with hematologic malignancies. Pharmacotherapy 23:1592-1610.

80. Wilchek M, Chaiken I (2000) An overview of affinity chromatography in affinity chromatography–Methods and protocols. Humana Press, 1–6.