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The Analysis of Antimicrobial Testing Vincetoxicum stocksii and Isolation of a Highly Active Compound Against Candida albicans by Using Various Different Techniques.

Vasim Rasul Momin
vmomin1@student.gsu.edu

The analysis of antimicrobial testing *Vincetoxicum stocksii* and isolation of a highly active compound against *Candida albicans* by using various different techniques

by

Vasim Momin

Under the Direction of Dr. Keith O. Pascoe

ABSTRACT

The purpose is to isolate a natural compound, which shows a high activity against *Candida albicans*, from plant, *Vincetoxicum stocksii*. Bio-Assay, Thin layer Chromatography, Column Chromatography, TLC bio Assay, and other extraction techniques are used in order to isolate the active compound. First, bio assay technique is carried out on the crude gum. Next, several flash chromatography columns are carried out in order to isolate the target compound, which has a R_f value of ~0.53 in 10:1 DCM/methanol solvent mixture. The TLC bioassay technique is also carried out in order to confirm the hypothesis that the target compound is indeed active.

The analysis of antimicrobial testing *Vincetoxicum stocksii* and isolation of a highly active compound against *Candida albicans* by using various different techniques

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by

VASIM MOMIN

Committee Chair: Keith Pascoe
Co-Chair: Alfons Baumstark

Committee: Davon Kennedy
Jerry Smith

Electronic Version Approved:

Office of Graduate Studies
College of Arts & Sciences
Georgia State University
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LIST OF ABBREVIATIONS

˚C	Celsius degree
Bio Assay	Biological Assay
Da	Dalton
DCM	Dichloromethane
DIBAH	Diisobutylaluminum hydride
EtOH	Ethanol
^{1}HNMR	Proton nuclear magnetic resonance
H_2O	Water
HCl	Hydrochloric acid
M	Molar
MeOH	Methanol
mL	Milliliter
Mm	Millimeter
MW	Molecular Weight
Neg.	Negative
NMR	Nuclear Magnetic Resonance
Pos.	Positive
P-TLC	Preparative Thin Layer Chromatography
R_f	Retention factor
TLC	Thin Layer Chromatography
µL	Microliter
%	Percent

INTRODUCTION

The use of natural products for healing is an ancient and universal as modern medicine itself. The therapeutic use of plants certainly goes back to Sumerian civilization, and over 400 years before the Common Era, it has been recorded that Hippocrates used approximately 400 different plant species for medicinal purposes.[17] The table below shows a historical timeline of natural product medicine.

Table 1: History of Natural Product Medicine.[17]

History of Natural Product Medicine

Period	Type	Description
Before 3000 BC	Ayurveda (knowledge of life) Chinese traditional medicine	Introduced medicinal properties of plants and other natural products
1550 BC	*Ebers Papyrus*	Presented a large number of crude drugs from natural sources (e.g., castor seeds and gum arabic)
460–377 BC	Hippocrates, "The Father of Medicine"	Described several plants and animals that could be sources of medicine
370–287 BC	Theophrastus	Described several plants and animals that could be sources of medicine
23–79 AD	Pliny the Elder	Described several plants and animals that could be sources of medicine
60–80 AD	Dioscorides	Wrote *De Materia Medica*, which described more than 600 medicinal plants
131–200 AD	Galen	Practiced botanical medicines (Galenicals) and made them popular in the West
15th century	Kräuterbuch (herbals)	Presented information and pictures of medicinal plants

Only a small fraction of the world's biodiversity has been explored for bioactivity to date. For example, there are at least 200,000 species of higher plants that exist on this planet, but merely 5-10% of these have been investigated so far.[17] Even investigation of the previously studied plants have brought forward more bioactive compound that have drug potential.

Nature has been the source of therapeutics for over a millennia, and an impressive number of modern drugs have been derived from natural sources, many based on their use of traditional medicine. Over a last century, a number of top selling drugs have been developed from natural products.[17] According to Cragg et. al, 39% of the 520 approved drugs between 1983 to 1994 were natural products or their derivatives, and 60-80% of antibacterial and

anticancer drugs were from natural origin. Apart from natural product-derived modern medicine, natural products are also used directly in the "natural" pharmaceutical industry, which is growing rapidly in Europe and North America.[17]

Natural products can contribute to the search for new drugs in many different ways. They can contribute by providing a new drug that can be used in an unmodified state; it can also provide building blocks to synthesize more complex molecules; or it could help indicate new modes of pharmacological action that allow complete synthesis of novel drug.[17] Natural products will always be considered as one of the major sources of new drugs in the future because it offers structural diversity, and most are small in size (<2000 Da).

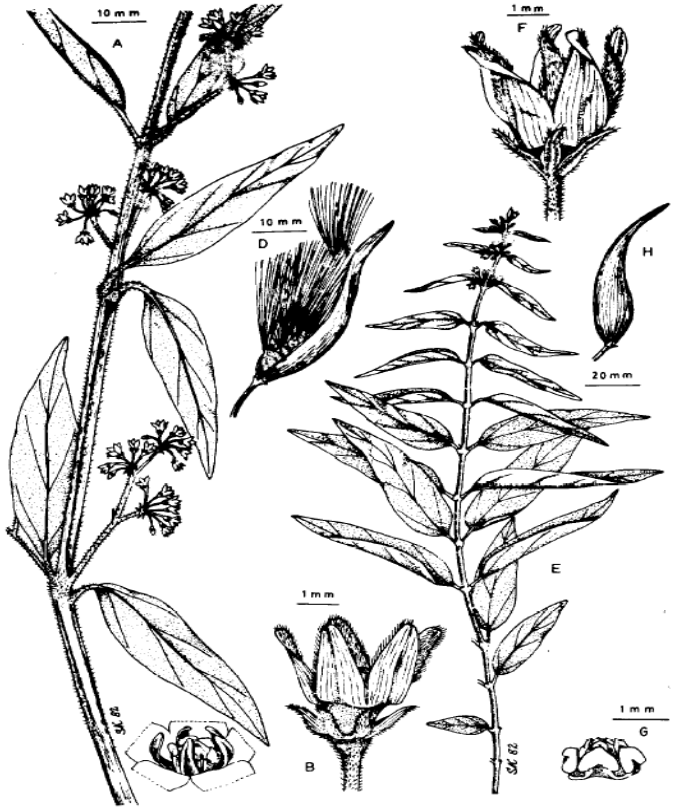


Fig. 1 *Vincetoxicum stocksii:* A. flowering branch; B. flower; C. centre of flower with corona and gynostegium (hairs of corolla omitted); D. dehisced fruit. *Vincetoxicum sakesarense:* E. flowering branch; F. flower; G. corona and gynostegium; H. fruit.

Fig. 1 *Vincetoxicum stocksii:* A. flowering branch; B. flower; C. centre of flower with corona and gynostegium (hairs of corolla omitted); D. dehisced fruit. *Vincetoxicum sakesarense:* E. flowering branch; F. flower; G. corona and gynostegium; H. fruit.

Vincetoxicum stocksii is a wild perennial shrub that is approximately 50-100cm tall Leaves are opposite; (2.5)-5-7 cm x 8-27 mm ovate to ovate.[1] Flowers are in pedunculate branched cymes, pedicel 2-4 mm long, and Calyx 1.5 mm long, divided almost to the base.[1] Corona lobes are linear to ovate, incurved, longer than the column and the anther tips are broad and membraneous.[1] This plant is manly found in south-east Asia, specifically in Pakistan. Pakistan has many medicinally important plants which are being used by many for treatment of various different diseases. *Vincetoxicum stocksii*, also known as Asclepiadaceae[1], is one of the plants that is found in Balochistan, Pakistan. It is traditionally used for the treatment of cancers, wounds, and injuries in humans as well as certain animals[1].

Mudassir A. Zaidi, who is a post-doctoral student in Dept. of Chemistry at Georgia State University, is one of the first people to find this plant to be high active against *Candida albicans*. Her researched paved the way for isolating the active compound from *Vincetoxicum stocksii*.[2]

There are various types of drugs out on the market that are used to treat fungal disease. The two main type of commercial drugs are Lamisil and Amphotericin B. Lamisil, contains an anti-fungal drug that can be dispensed either in pill form or cream form. As a pill, it is commonly prescribed to treat fungal infections of the toe nails or finger nails. As a cream it is commonly prescribed to treat fungal infections of the skin or hair such as Barber's itch, jock itch, or athlete's foot.[3] The active compound in lamisil is N,6,6-trimethyl-N-(naphthalen-1-ylmethyl)hept-2-en-4-yn-1-amine, commonly referred to as terbinafine.[4]

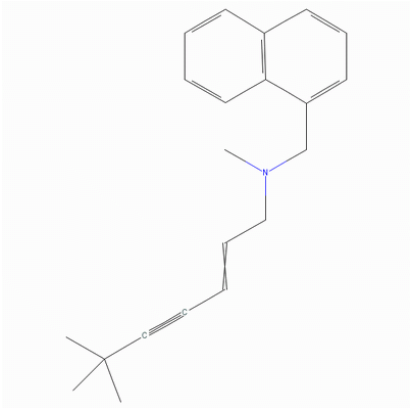
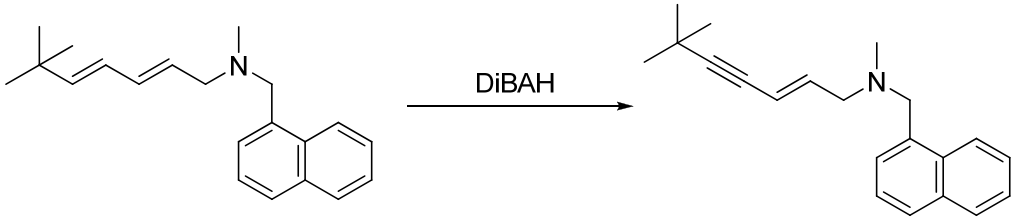


Figure 2: The molecular structure of terbinafine. The Molecular formula is $C_{21}H_{25}N$. [4]

Terbinafine is usually synthesized in 46% yield by reduction of N-(6,6-dimethyl-2,4-heptadiinyl)-N-methyl-(naphth-1-yl)methylamine by diisobutylaluminum hydride (DIBAH).[4]



Another drug that is commonly used as an anti-mycotic drug is Amphotericin B. Amphotericin B is a very potent agent, which binds to sterols, preferentially to the primary fungal cell membrane sterol, ergosterol. This binding disrupts osmotic integrity of the fungal membrane, resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites and then cellular death.[5] It is first isolated in 1955 at the Squibb Institute for Medical Research from cultures of an undescribed streptomycete isolated from the soil collected in the Orinoco River region of Venezuela.[6] Its name originates from the chemical's amphoteric properties.[5] It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other as shown in figure 3.[5] The structure and absolute configuration of Amphotericin B is determined by X-ray crystallography and is reported in 1970 by Mechlinski et al., 1970, Tetrahedron Lett. 44:3873-3876.

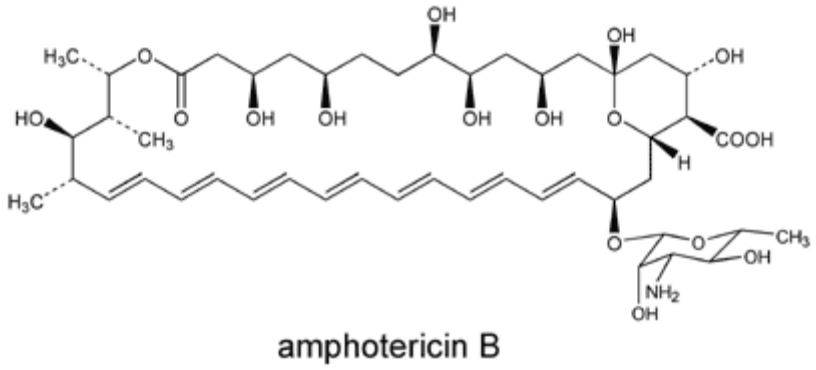


Figure 3: The structure of Amphotericin B.

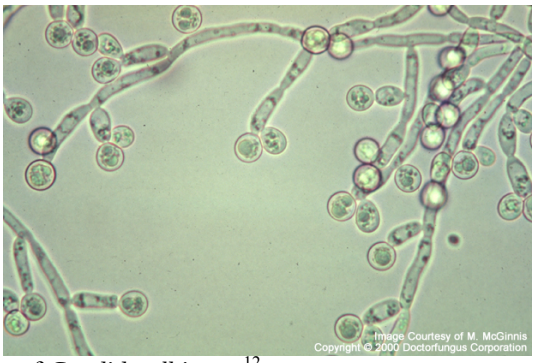


Figure 4: The microscopic image of *Candida albicans*.[12]

The fungal organisms, Candida albicans, are the organisms that the activity of plant, *Vincetoxicum stocksii* is tested on. *Candida albicans* are is a dimorphic fungus, which are normally present in mouth, vagina, and rectum; it can take two forms. Most of the time it exists as oval, single yeast cells, which reproduce by budding. Most yeasts do not produce mycelia (a mass of branching, threadlike hyphal filaments), but *Candida* has a trick up its sleeve.[13] Normal room temperatures favor the yeast form of the organism, but under physiological conditions (body temperature, pH, and the presence of serum) it may develop into a hyphal form.[13] *Candida albicans* are primary cause of athlete's foot. A medical history, physical exam, and laboratory tests, including blood tests, blood cultures, and wound cultures may be done in order to diagnose the disease. Tissue biopsy may be necessary to diagnose invasive systemic disease.[13]

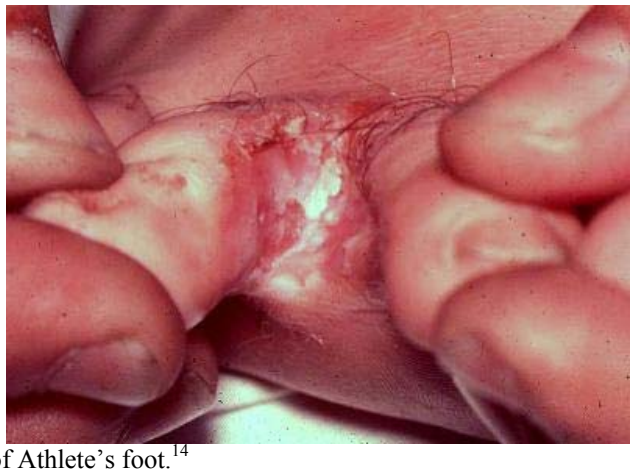


Figure 5: The picture of Athlete's foot.[14]

The most important factor that has to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. The general features of molecule that are helpful to ascertain the isolation process include solubility, acid-base properties, charge, stability, and size. A good flow chart shown in Figure 7 is a good summary of the isolation protocol.

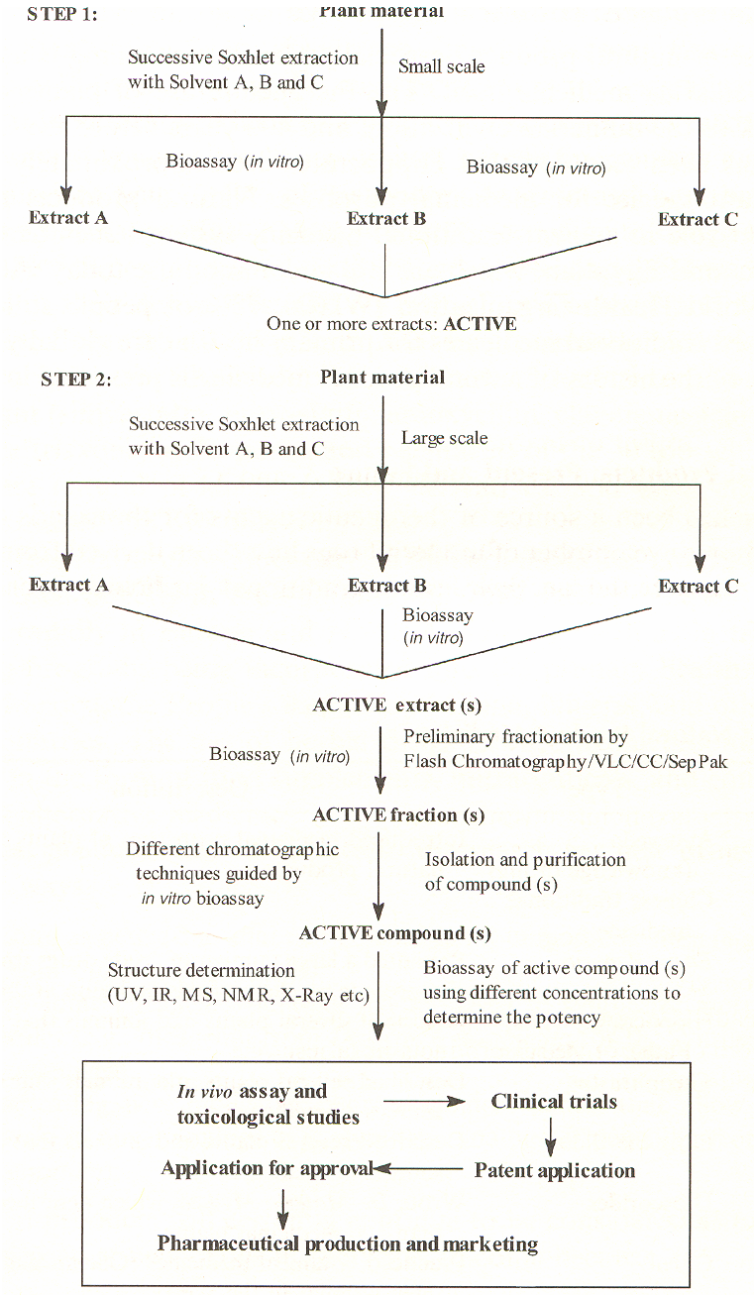


Figure 6: An example of natural product drug discovery process (bioassay guided approach).[17]

The compound of interest is very active against this particular organism, *Candida albicans*. In order to isolate the active compound from *Vincetoxicum stocksii*, various different techniques were adapted in order to collect the pure compound. Some procedures include Thin Layer Chromatography, Bio Assay, TLC Bio Assay, and Flash Chromatography.

Thin Layer Chromatography is a chromatography technique used to separate chemical compounds. It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the experimental solution based on the polarity of the components of the compound in question.[7] In performing this technique, correct solvent mixture is crucial. Separation of compounds is mainly based on the interaction of the solute and the mobile phase for binding places on the stationary phase. For example, if normal phase silica gel is used as the stationary phase (SiO_2) it can be considered polar. Given two compounds which differ in polarity, the most polar compound has a stronger interaction with the silica and therefore it does not move up the silica plate. Consequently, the less polar compound moves higher up the plate.

Table 2: Chemical properties of common solvents used in natural product isolation.[17]

Solvent	Polarity index	Boiling point (°C)	Viscosity (cPoise)	Solubility in water (% w/w)
n-Hexane	0.0	69	0.33	0.001
Dichloromethane	3.1	41	0.44	1.6
n-Butanol	3.9	118	2.98	7.81
iso-propanol	3.9	82	2.30	100
n-Propanol	4.0	92	2.27	100
Chloroform	4.1	61	0.57	0.815
Ethyl acetate	4.4	77	0.45	8.7
Acetone	5.1	56	0.32	100
Methanol	5.1	65	0.60	100
Ethanol	5.2	78	1.20	100
Water	9.0	100	1.00	100

If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding location and all compounds on the TLC plate will move higher up the plate as shown in figure 7.[7] The most important information collected from this technique is the retention factor value (R_f value). This will give the exact location of the compound of interest. It can be calculated by the formula given below.

$$R_f = \frac{\text{Distance from origin to spot}}{\text{Distance from origin to solvent front}} \qquad (1)$$

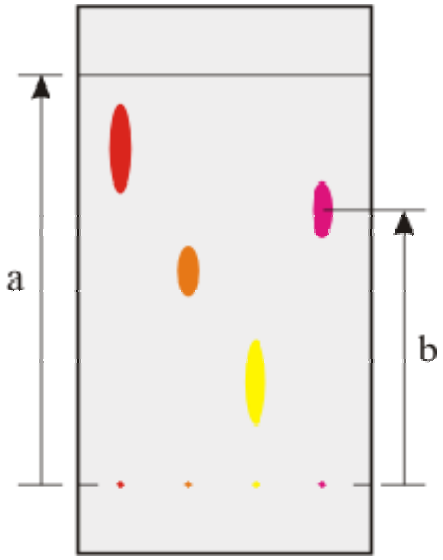


Figure 7: An Illustration of the thin layer chromatography. Note that the R_f value is calculated using (b/a).[8]

Another well known technique, which accompanies the thin layer chromatography technique, is preparative thin layer chromatography (P-TLC). In P-TLC, the compound is separated based on the hydrogen bonding capabilities to the silica gel just as TLC technique.[7] The more polar the compound, the less it will travel up on the plate and vice versa for non-polar compounds. A good solvent system is a crucial part of the P-TLC, since it will decide the resolution of the bands. The mobile phase, which is also the solvent system, will travel up the plate via capillary action. For this reason, it is crucial that silica gel is laid out in a uniform patter across the P-TLC plate.[7] The main difference between TLC and P-TLC is that the latter one can hold more crude compound and after the separation, the different R_f bands can be scrapped off and collected in order to test for activity. The different R_f bands are viewable under U.V. light (254nm and 366nm wavelength).[7]

Figure 8: A P-TLC plate soaking in solvent mixture for separation.[8]

One of the most important techniques that are practiced during natural product isolation is column chromatography. It is a method which is use to purify compound based on their polarity. It is often used for preparative applications on a scale from grams to kilograms. Column can be packed two ways, dry packing column and wet packing column. A wet packing column also known as flash column is nothing but a glass tube from 5 to 50mm wide and height from 50cm to 1m with a stop cock at the bottom.[11]

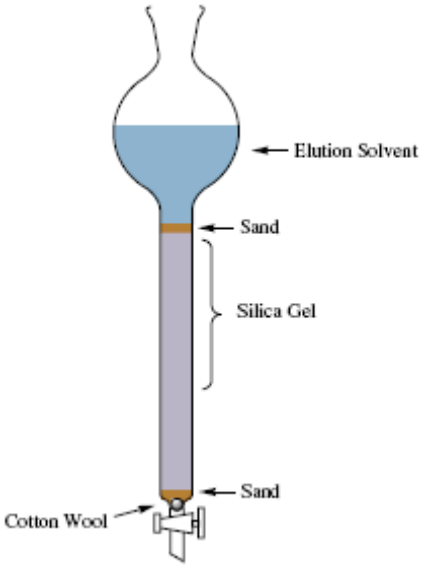


Figure 9: The illustration of the flash column.[9]

Flash chromatography, also known as medium pressure chromatography, was popularized in 1978 by Clark Still of Columbia University, as an alternative to slow and often

inefficient gravity-fed chromatography.[10] Flash chromatography differs from the classical technique in two ways. First, slightly smaller silica gel particles (250-400 mesh) are used, and second, due to restricted flow of solvent caused by the small gel particles, pressurized gas (10-15 psi) is used to drive the solvent through the column of stationary phase. The net result is a rapid and high resolution chromatography.[10]

In order to perform flash chromatography, choosing a good solvent system is really crucial; without the right solvent mixture, the separation will be poor. The compound of interest should have a TLC R_f of 0.15 to 0.20 in the solvent system chosen.[10] The ratio of solvents determines the polarity of the solvent system, and hence the rates of elution of the compounds to be separated.[10] Common binary solvent systems in order of increasing polarity are hexane/ethyl acetate dichloromethane/hexane, ether/hexane, hexane/ethyl acetate, and dichloromethane/methanol.[11] If the R_f is a 0.2, one will need a volume of solvent approximately 5X the volume of the dry silica gel in order to run the column.[10] Another important aspect for achieving a good separation is the amount of silica gel used. It depends on the *R*f difference of the compounds and on the amount of sample being used for separation. Usually, the ratio is approximately 1:30 compound to silica gel.[11]

After coming up with solvent mixture and the right proportion of crude gum to silica gel, the column must be packed very tightly in order to achieve uniform separation as the compound moves down the column. First, obtain a glass column and make sure that it has a plug of cotton wool directly above the stopcock to prevent the silica gel from escaping from the column through the stopcock.[11] Next, put a ~1/2 in. layer of clean sand above the plug of glass wool. Use only as much as is necessary to obtain a flat surface, with the same diameter as that of the body of the column. Make sure the surface is flat. Then pour in the silica gel using a funnel.

After pouring the silica into the column, use air pressure to squeeze all of the silica together. This ensures uniform packing of silica.[11]

The dry packing column, which is usually used for Vacuum liquid chromatography (VLC) is an efficient way to pack a column and is commonly used for regular or bonded silica gel. The dry stationary phase is poured into the column. The stationary phase is then wetted using appropriate solvent. In VLC, vacuum is often used for a compact packing of the column.[17]

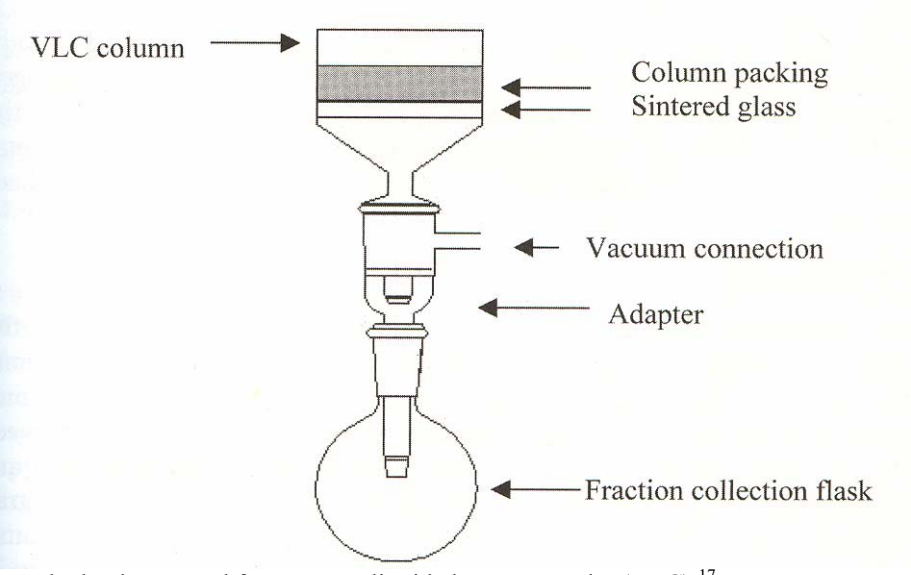


Figure 10: Dry packed column used for Vacuum liquid chromatography (VLC).[17]

Another technique that is used during this experiment is the Biological Assay technique. Bio Assay involves the use different types of media; for example, Trypsin Soy Agar, SDA agar, etc. Bioassays are typically conducted to measure the effects of a substance on living organisms. There are two different types of bioassay; qualitative and quantitative. For this paper, quantative bioassay, which is a measure of potency of certain compound on a living organism, is mainly used.

Final technique, which is not well known is TLC Bioassay technique. It is a combination of thin layer chromatography and bioassay technique. In order to carry out this procedure, understanding of two dimensional TLC plate is crucial. Two dimensional plate is often used for

screening of complex mixtures. The extract is spotted onto the plate in normal fashion, then the plate is developed and dried. Next, the plate is turned 90˚ and developed a second time as shown in figure 11.

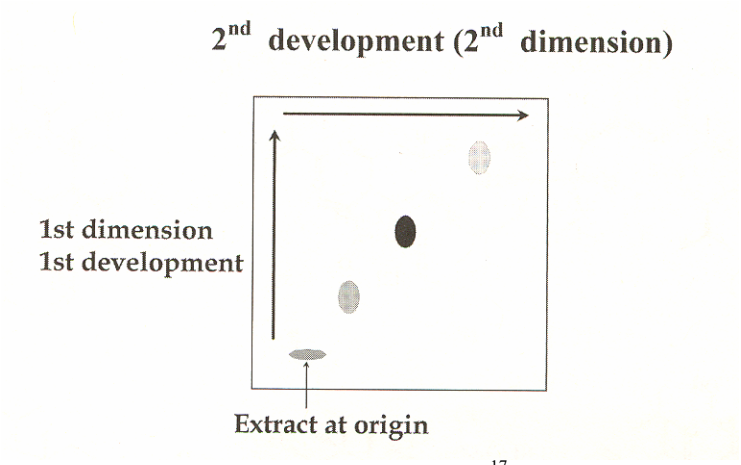


Figure 11: Two dimensional TLC plate after two developments.[17]

The advantage of this process is better resolution of the compound of interest. Usually a different solvent system is used in the second dimension, which further enhances the resolving power.[17] After two dimensional plate is formed, the bioassay technique is performed on top of the TLC plate. Ultimately, this helps in identification of the active compound.

Experimental Procedure

A crude natural compound is literally a cocktail of compounds. It is difficult to apply a single separation technique to isolate one compound from this crude mixture. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes.[17]

Extraction of Crude

The extraction technique was performed by Mudassir A. Zaidi. First, the leaves from a *Vincetoxicum stocksii* tree were cut into approximately 1 cm in size to ensure the evaporation of

all the water in the plant. After cutting the leaves, they were spread out on a bench and left to be air dried (approx. 24-48 hours). The dried chopped leaves were placed in a large Erlenmeyer flask and methanol is poured over it as a solvent and left for 48 hours. Next day, the solvent in which the leaves were soaking, was poured into a 1000 ml round bottom flask with the aid funnel and a cotton ball, producing a dark green filtrate. The methanol was then evaporated with the aid of roto-vap until crude gum is the only material left in the flask. The above steps were repeated several times over several weeks until the plant produced no more green filtrate. All crude plant gum is finally combined into a single flask.

Bio Assay 1

After collecting the crude gum, it was tested against *Candida albicans* for antifungal properties of the plant. In order to prove that the compound is indeed active, bio assay is performed.[15] First, several 6mm paper disc (Becton & Dickinson Co., Sparks, MD) were placed in three different containers labeled, positive, negative, and crude gum. As a negative control, 20µL of methanol is pipetted on three discs. For positive control, 10µL of 100 mg *Amphotericin B* (Sigma Aldrich, Lot # 5178X) is pipetted on another 3 discs. Then, 20µL of plant extract (4:1 dilution with methanol and crude gum from *Vincetoxicum stocksii*) is poured on separate discs. The organism of interest is: *Candida albicans.* Fresh 24hour cultures were grown on TSA plates and incubated at 37ºC for 24 hours. First, pure organism is scrapped off the agar plate with the aid of cotton swab and the turbidity is matched to the saline solution (pH of 9.9) with organism to 0.5 McFarland standard. The dissolved bacteria solution is spread in a checker pattern to ensure the even spread of bacteria on the Mueller Hinton II plates. Then, the 6mm discs made earlier were placed on the plate. They were left in a 37ºC incubator for approximately 24 hours. This experiment was performed in triplicates in order to verify the results.

Thin Layer Chromatography 1

After making sure that the crude gum is indeed showed activity against *Candida albicans*, Thin Layer Chromatography (TLC) was performed on the plant extract to determine the solvent system best suited for the plant. This is done by placing 3 to 4 drops of the diluted plant extract (in methanol) on a small TLC plate. The plate was then placed in a mixture containing Hexane and Ethyl Acetate (Fisher Scientific, Fairlawn, NJ). After many trials, the best ratio for separating the crude gum initially was a 30:20 solution by volume containing Hexane and Ethyl Acetate solution respectively. After separation, the plates were analyzed underneath two different wavelengths (254nm and 365nm).

After analyzing the plates under the U.V detector, the bands were marked and a retention factor (R_f) is calculated by using the formula given below:

$$R_f = \frac{\text{Distance from origin to spot}}{\text{Distance from origin to solvent front}} \qquad (1)$$

Vacuum Liquid Chromatography

Vacuum liquid chromatography is used to separate a large amount of crude gum based on its polarity. The stationary phase is silica gel with a mesh size of 70Å (Sigma Aldrich, St.louis, MO) and the mobile phase varied throughout the experiment in order to separate the compounds based on their respective polarity. For mobile phase, the solvents were chosen from very nonpolar solvents such as Hexane to a very polar solvent such as Methanol (see table 2), in order to give a good separation. The solvents used were Hexane, Dichloromethane, Acetone, and Methanol in that order.

First, 230 grams of silica gel is added into a 500ml of hexane solution in order to prevent the inhalation of silica particles, which can lead to cancer. It is crucial that silica gel be handled with great care since it is a carcinogen.[11] The sludge is then slowly poured into the column in order to prevent trapping of air bubbles, which can lead to uneven separation. The volume ratio of crude gum to silica gel is approximately 1:30, therefore, 7.6 grams of crude gum is added into the column to match the 230 grams of silica used. Then, 500ml of hexane is poured into the column and the eluant is collected in multiple test-tubes (approximately 50ml in each tube). Following hexane, 500ml of dichloromethane, 600ml of acetone, and 750ml of methanol is used to elute the compound from the column. The tubes were labeled according to the solvent collected.

Thin layer chromatography is performed on all of the test tubes in order to see which tubes have similar compounds in order for the like compounds to be grouped together. Then the like mixtures were combined and dried using roto-vap (Brinkman RE 111) so bio assay could be performed to see which group is active.

Bio Assay 2 (without 6mm paper discs)

The purpose of this procedure is to show that the test compound can be placed straight onto the agar plate and a zone of inhibition will still form only if the compound is active. This procedure can be done even if there are no 6mm paper discs.

After collecting different fractions from the latter column, it is crucial to see which fraction indeed is active against *Candida albicans*. The procedure is very similar to the Bio-Assay procedure described previously. The bacteria are still mixed with pH of 9.9 saline solution and the turbidity is matched to 0.5 McFarland standard. The organisms were then streaked on to the plate as stated previously. After streaking the organism, a spatula is used to

carve out a round well from the agar plate, which will resemble a 6mm disc. Now, the test compound can be poured inside the well with the aid of a pipetter. Then, it is placed into the 37ºC incubator for 24 hours.

The zones may not be as perfect as it would be if 6mm paper discs were used, however, the zones are good enough to crudely tell whether or not the compound of interest is active. The advantage of this method over a traditional bio assay technique is that no 6mm paper discs are needed, while the disadvantage is that the results are not official and it requires a great deal of care and precision. This experiment is performed in triplicates in order to verify the results.

Flash Column Chromatography 1

After testing different fractions for activity against *Candida albicans* using bio assay, the most active is the methanol fraction. The second column consisted of the crude that was extracted with methanol solution from previous column (4.4118 grams from G4). However, finer particles (mesh size of 250-360Å) silica is instead of the larger particles used in the previous column (mesh size 70). This will ensure a more tightly packed column, which will separate the compound more precisely. After carefully packing the column, approximately 500ml of the 6:1 ethyl acetate/methanol mixture is used in order to remove all the unwanted compounds from the column. After eluting the column with 6:1 ethyl acetate/methanol, 500ml of 12:1 DCM/methanol is used to move the target compound down the column, while eluting off all the unwanted material. The target compound can be seen if a UV light is held against the column. The target compound is the only one that fluoresces green under the light. After eluting it with 12:1 DCM/methanol, the polarity is slowly increased to 10:1 DCM/methanol (400ml) to move the target molecule even further down the column. Then the polarity is increased again to 8:1 DCM/methanol (400ml), then 6:1 DCM/methanol (400ml). When eluting with 6:1

DCM/methanol, the target compound starts to elute off the column. The fraction is collected in an Erlenmeyer flask in 50ml portion.

After collecting the targeted compound in different flasks, thin layer chromatography is performed in order to see if only the compound of interest is present in the flask. TLC showed two bands.

Bio Assay 3

The different fractions were collected and TLC method is performed on each fraction to see which fractions are identical or different, and from the TLC analysis like fractions were mixed together.

Fractions collected in previous column are need to be tested in order to see which fractions indeed are active. This experiment is carried exactly as Bio Assay 1, but instead of using the crude gum as the test subject, different fractions collected in flash column 1 were used. This experiment is performed in triplicates in order to verify the results.

TLC Bio Assay[16]

Since the last fraction collected has two bands present and it also shows high active again *Candida albicans*, TLC bio assay is performed as an alternate to see if it is possible to tell which band is indeed active. First, the TLC silica plate is cut into a square to fit the holder. Then, drops of the diluted crude extract were placed on the bottom left hand corner of the plate. After placing the plate in a 10:1 solution (DCM/Methanol) to separate out the compound, it is then placed into a 9:1 solution (DCM/Methanol) sideways, to even further separate the compounds that might have the same R_f values. After running the plates on both sides with respective solution, a drop of 100mg *Amphotericin B* is placed on the top right hand corner as a positive control.

After having the TLC plate ready for further experiments, the agar is prepared next. For this experiment, Sabouraud Dextrose Agar (SDA) (Becton & Dickinson Co., Sparks, MD) is used. The agar is prepared as instructed on the label. *Candida albicans* were then mixed into saline solution and turbidity is matched to 0.5 McFarland standard. Then, 6.5ml of the saline solution (mixed with *Candida albicans*) is added into 500ml of SDA agar solution (make sure that the agar is not too hot otherwise, it would instantly kill the bacteria. 2,3,5-Triphenyl tetrazolium chloride (Fisher Scientific, Fairlawn, NJ) is used a marker in order to see the activity space. Approximately 22ml of agar solution is poured onto previously made TLC plates. This step is done with a great care, since it is crucial to spread the agar solution evenly across the plate. After, solidification of the agar on-top of the TLC plate, it is placed in a 31ºC incubator for 24 hours.

Flash Column Chromatography 2

After knowing the exact R_f band that is active against the organisms from the TLC Bio assay method, it is crucial to collect some pure compound containing only the target band. First, a small column is packed using silica (mesh size of 250-360Å) and air pressure. Next, 100mg of the mixture dissolved in methanol, containing the target band is added to round bottom flask with tiny amount of silica. Then, the mixture is roto-vap so the crude mixture would stick to the silica making it easier to add it to the column. Next, some sand is added on top in order to keep the flow of the solvent even.

After packing the column, 300ml 6:1 ethyl acetate/methanol is added in order to remove the unwanted bands. It can be seen under the U.V. light. The compound of interest fluoresces green. After eluting all the waste bands, 300ml 8:1 DCM/methanol is added in order to elute out the target band. The latter solvent mixture moves the band half way down the column. Next,

500ml 6:1 DCM/methanol is added in order to completely elute the target band. The band of interest is collected and rotovaped.

Thin Layer Chromatography is performed in order to make sure that only the target band is present and not any other unwanted bands.

Bio Assay 4

Finally, after isolating the target compound from the mixture, the last step is to make sure the compound isolated is indeed the active compound. Bio Assay is performed in order to verify our hypothesis. This technique is performed similar to Bio Assay 1, but instead of using crude gum as the test subject the isolated band is used. This experiment is only done one time instead of triplicates because not enough isolated material is obtained.

Results

Crude gum Testing

Table 3: Bio assay analysis of the crude gum. This was performed to show that the crude gum is indeed active. 10µL of *Amphotericin B* is used as positive control and 20µL of methanol is used as negative control. Also, 20µL of the crude gum on each 6mm paper disc is used for testing.

Trial	Positive	Negative	Crude gum
A	14mm	6mm	35mm
B	14mm	6mm	33mm
C	14mm	6mm	33mm

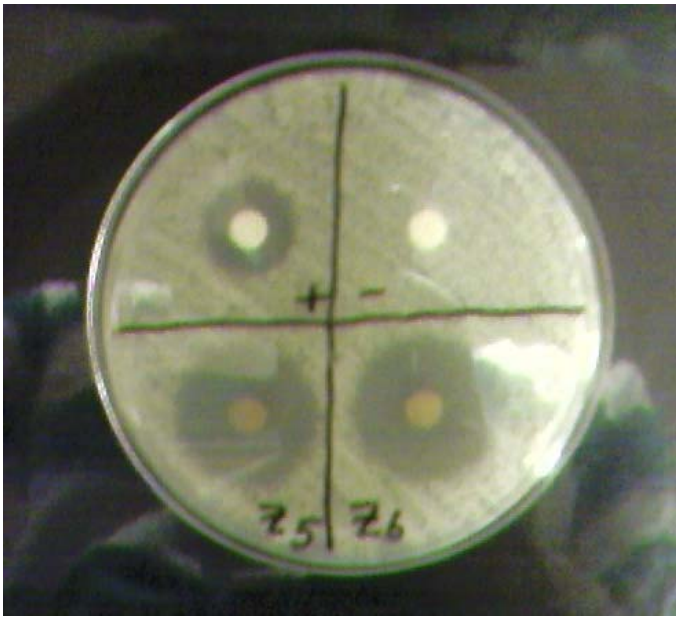


Figure 12: The Bio Assay image done on the crude gum. The positive control is Amphotericin B and the negative control is methanol.

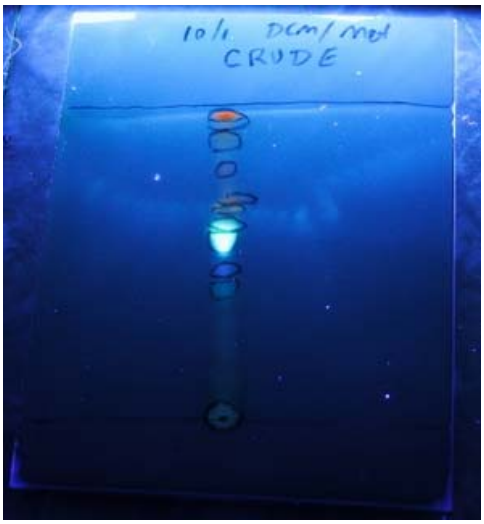


Figure 13: TLC plate of the crude gum. The solvent used to develop the plate is 10:1 DCM/methanol.

Table 4: R_f values for the fractions crude gum shown above. The stationary phase is silica gel and the mobile phase is 10:1 solution of DCM/methanol.

	Crude
R_f 1	0.98
R_f 2	0.93
R_f 3	0.89
R_f 4	0.78
R_f 5	0.69
R_f 6	0.64
R_f 7	0.60
R_f 8	0.56
R_f 9	0.47
R_f 10	0.40
R_f 11	0.00

Vacuum Liquid Chromatography

Table 5: Fractions collected with the VLC. The VLC is performed with silica gel as a stationary phase and mobile phases varied from 500ml of Hexane, 500ml of Ethyl Acetate, 600ml of Dichloromethane, and 750ml of Methanol in that specific order.

Crude gum used	7.6015g
Silica gel used	230g

Fractions	Amt. of compound collected (grams)
F-1 in Hexane	0.2598
F-2 in $MeCl_2$	0.0709
F-3 in Acetone	0.5119
F-4 in Methanol	4.4118
F-5 in Methanol	0.1766

Total active compound mixture	4.5884
% yield of active compound	60.36%

Table 6: Bio assay analysis of the fractions. 20µL of crude gum is used as a positive control, and 20µL methanol is used as a negative control. Also, 20µL of the fraction (1:4 dilution of fraction and methanol, respectively) on each 6mm paper disc is used for testing.

Fractions	Positive	Negative	Trial 1	Trial 2
F-1	33mm	6mm	6mm	6mm
F-2	33mm	6mm	6mm	6mm
F-3	33mm	6mm	6mm	8mm
F-4	33mm	6mm	32mm	33mm
F-5	33mm	6mm	32mm	31mm

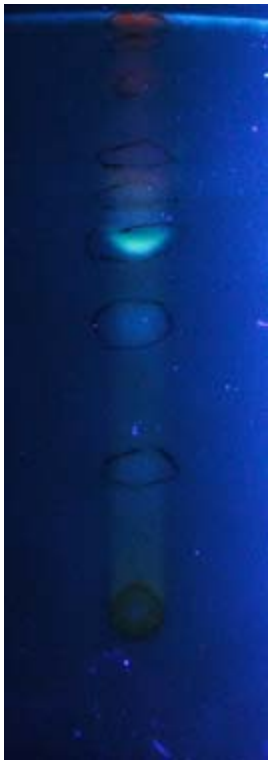


Figure 14: TLC plate of F-4. The solvent used to develop the plate is 10:1DCM/methanol.

Table 7: R_f values for the fractions F-4 fraction shown above. The stationary phase is silica gel and the mobile phase is 10:1 solution of DCM/methanol.

	F-4
R_f 1	0.95
R_f 2	0.93
R_f 3	0.85
R_f 4	0.73
R_f 5	0.65
R_f 6	0.58
R_f 7	0.47
R_f 8	0.20
R_f 9	0.00

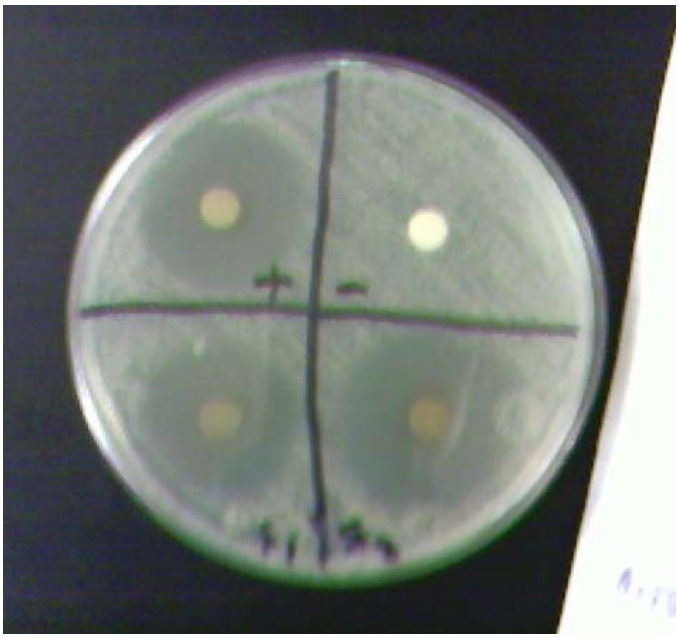


Figure 15: The Bio Assay image done on F-4 and F-5 fraction. The positive control is crude gum and the negative control is methanol. The bottom left is F-5 fraction and the bottom right is F-4 fraction.

Flash Chromatography 1

Table 8: Fractions collected with the column. The column chromatography is performed with silica gel as a stationary phase and mobile phase is varied. G-1 contain 6:1 ethyl acetate/methanol fraction; G-2 contain 12:1 DCM/methanol fraction; G-3 contain 10:1 DCM/methanol fraction; G-4 contain 8:1 DCM/methanol fraction; G-5 contain 6:1 DCM/methanol fraction; G-6 contain all methanol fraction.

Crude gum used	4.4118g
Silica gel used	133g

Fractions	Amt. of compound collected (grams)
G-1	2.237
G-2	0.0582
G-3	0.0914
G-4	0.1152
G-5	0.148
G-6	1.363

Total active compound mixture	0.2632
% yield of active compound	5.966%

Table 9: Bio assay analysis of the fractions for column 2. 20µL of crude gum is used as a positive control, and 20µL methanol is used as a negative control. Also, 20µL of the fraction (1:4 dilution of fraction and methanol, respectively) on each 6mm paper disc is used for testing.

Fractions	Positive	Negative	Trial 1	Trial 2
G-1	14mm	6mm	6mm	6mm
G-2	14mm	6mm	6mm	6mm
G-3	14mm	6mm	6mm	6mm
G-4	14mm	6mm	31mm	31mm
G-5	14mm	6mm	36mm	37mm
G-6	14mm	6mm	9mm	8mm

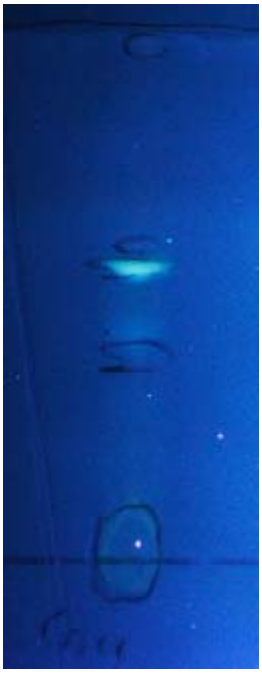


Figure 16: TLC plate of G-4 and G-5 combined. The solvent used to develop the plate is 8:1DCM/methanol.

Table 10: R_f values for the fractions G-4 and G-4 combined shown above. The stationary phase is silica gel and the mobile phase is 8:1 solution of DCM/methanol.

	G-4 + G-5 combined
R_f 1	0.97
R_f 2	0.55
R_f 3	0.53
R_f 4	0.37
R_f 5	0.00

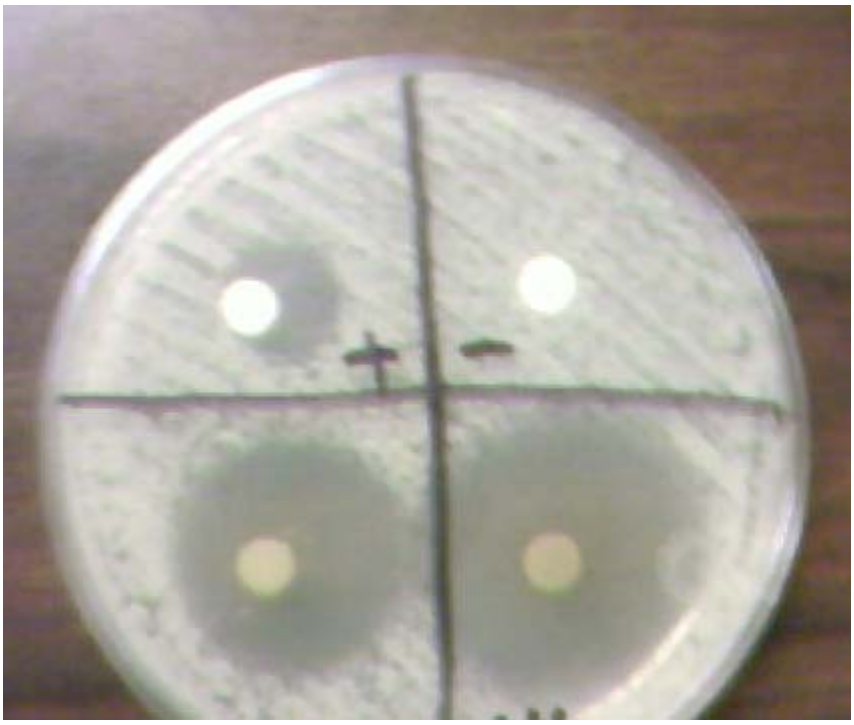


Figure17: G-4 fraction and G-5 fraction activity against *Candida albicans*. The bottom left is G-4 and the bottom right is G-5. The positive control used is Amphotericin B and the negative control is methanol.

TLC Bioassay

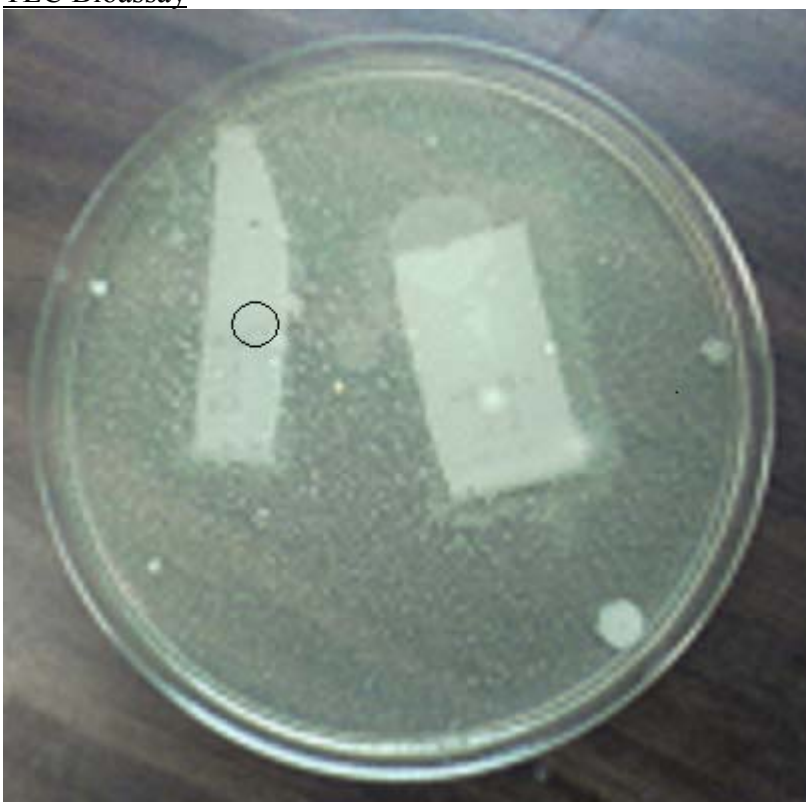


Figure 18: TLC Bioassay on G-4 and G-5 fraction combined. The circled portion is the compound of interest.

Flash Chromatography 2

Table 11: Fractions collected with the column. The column chromatography is performed with silica gel as a stationary phase and mobile phase is varied. V-1 contain 6:1 ethyl acetate/methanol fraction; V-2 contain 8:1 DCM/methanol fraction; V-3 contain 6:1 DCM/methanol fraction.

Crude gum used	0.2631g
Silica gel used	13g

Fractions	Amt. of compound collected (grams)
V-1	0.1783
V-2	0.0036
V-3	0.0120

Total active compound pure	0.0120
% yield of active compound	4.56%

Figure 19: TLC plate of V-3. The solvent used to develop the plate is 8:1 DCM/methanol.

Table 12: R_f values for the fractions V-3 shown above. The stationary phase is silica gel and the mobile phase is 8:1 solution of DCM/methanol.

	V-3
R_f 1	0.59

Discussion

Vincetoxicum stocksii is known to have many herbal qualities, specifically; it shows high activity towards *Candida albicans*. In order to show that it is indeed very active, the crude gum of the plant is collected and tested by using a bio assay procedure. As Table 3 shows, the crude showed a 35mm zone of inhibition for trial A and 33mm for trial B and C. One can see in figure 12, the 35mm zone of inhibition. This proves that the crude gum is indeed active. After proving the activity, a good solvent system is needed in order to separate the compound in order to test each band, which will help narrow down to the final compound that shows activity against *Candida albicans*.

After trying many different types of solvent mixtures, 10:1 DCM/methanol is the best solvent mixture in order to separate the bands as it can be seen in figure 13. Table 4 shows the R_f values of the crude mixtures. There are eleven different bands separated with 10:1 DCM/methanol solvent mixture. The R_f values for the bands are 0.98, 0.93, 0.80, 0.78, 0.69, 0.64, 0.60, 0.56, 0.47, 0.40, and 0.00. The band of interest is at R_f value 0.56, which is proven via various different separation and analyzing techniques performed. It is important to note in figure 12 that the positive control shows a zone of inhibition of 14mm, while the active crude compound shows 35mm zone of inhibition. It can be assumed that the unknown compound is more potent than the positive control, Amphotercin B.

After checking for bioactivity and determining the correct solvent mixture, a Vacuum Liquid Chromatography (VLC) is carried out in order further separate the bands into fractions. As shown in Table 5, approximately 7.6 grams of crude gum, previously tested, is used. In hexane layer, 0.2598 grams of the crude is eluted. Next, dichloromethane is used to elute, but only 0.0709 grams of compound is eluted out. The polarity is gradually increased by using Acetone as the eluant solvent; 0.5119 grams of compound is extracted out. Finally, methanol, which is one of the most polar solvent, eluted out most of the crude gum, specifically, 4.4118 grams of it.

After running VLC, the different fractions collected using different solvents are tested in order to see which fraction is indeed active. As shown in table 6, fractions F-1 and F-2 shows no activity against *Candida albicans* (6mm zone of inhibition). Fraction F-3 produces 8mm zone of inhibition, this may be due to the increase in polarity which may have eluted some of the active compound. The F-4 fraction, which is methanol fraction produces 33mm zone of inhibition as shown in figure 15. The percent yield of activity compound collected is 71.46%. Since F-4 showed the most activity, a TLC is done with 10:1 DCM/methanol in order to see which bands have been eluted out. As seen in figure 14, there are at least nine compounds present in F-4. The R_f values are 0.95, 0.93, 0.85, 0.73, 0.65, 0.58, 0.47, 0.20, and 0.00. The band of interest has R_f value of 0.58. However, the VLC technique is not very accurate; rather it is a crude way to separate the fractions. It can be seen when comparing the crude TLC to F-4 fraction TLC that they both are identical and no good separation is performed via VLC method. More separation technique must be implemented in order to isolate the compound of interest (R_f 0.58).

After carrying out the VLC technique, more precise, Flash Chromatography is needed in order to accurately separate and isolate the pure compound. 4.4118 grams of F-4 fraction is used

in this technique. The solvent mixture used initially to separate is 6:1 ethyl acetate/methanol. Most of the bands were accumulated into one big band due to high polarity of solvent. Approximately 2.237 grams is collected initially with 6:1 ethyl acetate/methanol solvent mixture. Next, the polarity is gradually increased to 12:1 DCM/methanol, which elutes out 0.0582 grams. Again, the polarity is increased to 10:1 DCM/methanol, which elutes 0.0914 grams. Then 8:1 DCM/methanol elutes 0.1152 grams; 6:1 DCM/methanol elutes 0.148. Finally pure methanol is used as solvent to elute the rest of the compound left in the column.

The different fractions collected using flash chromatography are tested using bioassay technique in order to see which fraction contains the active product. As seen in table 9, G-1 to G-3 showed 6mm zone of inhibition, which is equivalent to negative control; therefore, it can be assumed that G-1 to G-3 showed no activity. However, G-4 and G-5 showed higher activity than positive control (Amphotericin B), with 31mm and 37mm zone of inhibition respectively as shown in figure 17. Since both G-4 and G-5 contains activity, they are combined in order to give more active sample so further tests can be performed. The percent yield of active compound from flash chromatography is 5.96%.

Next thing to figure in the quest of isolation is the number of different bands present in the active mixture. G-4 + G-5 mixture is separated on TLC plate using 10:1 DCM/methanol in order to see what bands are present. As shown in figure 16, there are five different bands present. They R_f values for the bands are 0.97, 0.55, 0.53, 0.37, and 0.00. Since the compound of interest with R_f value 0.53 is present then it confirms the fact that the mixture is active. It can also be seen from figure 16 that the compound is interest is really concentrated when compared to the other bands, which means that concentration ratio of target compound to the rest of the compounds is high.

Even though flash chromatography eliminated some bands, it doesn't provide an isolated pure compound or anyway to know which compound out of the five collected band is active. Another less common technique, TLC bioassay, is carried out in order to give a hard evidence on which compound is target compound. Figure 18, shows the positive identification of the target compound. Even though it is a little difficulthe band with R_f value 0.53 showed a 12mm zone of inhibition around it. One may wonder why such a small zone? This may be due to the fact that the concentration of compound on the TLC is not high. Therefore, since the concentration is less, the zone of inhibition is smaller. The main purpose of this procedure is to confirm the hypothesis that the band with R_f value of ~0.53 is indeed active.

After the confirmation, more separation is needed in order to collect some pure compound (R_f value ~0.53). Another flash chromatography is carried out with the G4 + G-5 mixture. Approximately 0.2631 grams of the G-4 + G-5 mixture is used along with 13 grams of silica. In Table 11, three different fractions were collected. First eluant solvent mixture is 6:1 ethyl acetate/methanol mixture, which eluted 0.1783 grams of compound. Next, 8:1 DCM/methanol mixture is poured, which eluted 0.0036 grams. Finally, 6:1 DCM/methanol solvent mixture is used to elute the target compound. The target compound is eluted in fraction V-3. The percent yield of the active pure compound is 4.56%.

TLC is carried out on V-3 fraction as shown in figure 19. It can be seen that the target compound (R_f value ~0.53) is indeed isolated and the V-3 fraction collected is pure. In this case, the target compound R_f value is a little higher (R_f value 0.59 as shown in table 12) since the solvent mixture used is 8:1 DCM/methanol rather than 10:1 DCM/methanol like others. Bio Assay is performed on the isolated fraction, which gave a 42mm zone of inhibition.

The isolated compound is then used to perform various NMR techniques in order to determine the structure. ^{1}HNMR spectrum is taken of the isolated compound; however, the spectrum is to complex to figure out the structure.

Conclusion

All in all, the main purpose of this experiment is to isolate a natural compound, which shows a high activity against *Candida albicans*, from plant, *Vincetoxicum stocksii*. The compound of interest has the R_f value of ~0.53 in 10:1 DCM/methanol solvent mixture. ^{1}HNMR spectrum is taken in order to figure out the structure of the active compound, however, the structure is too complex to figure out in given amount of time. Further research is needed in order to analyze various different spectrum in order to propose a structure.

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