2015

GARCINIA KOLA: PHYTOCHEMICAL, BIOLOGICAL AND FORMULATION STUDIES

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BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACEUTICAL SCIENCE

UNIVERSITY OF RHODE ISLAND

2015

MASTER OF SCIENCE

OF

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2015

ABSTRACT

The objective of this research was to search for an antiviral compound, garcinol (or guttiferone F) from the *Garcinia kola* nuts using two different extraction techniques and formulate a suitable liposome for control release using an antiviral compound. The two extraction techniques used were: liquid-liquid extraction (for simplicity purposes, the term solvent-solvent, S-S, will be used interchangeable) and Supercritical Fluid Extraction (SFE). Both techniques were used to profile compounds found in *Garcinia kola* and for statistical comparison purposes. Extracts from both techniques were collected and analyzed for separation using Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using Ultraviolet Photodiode Array (UV-PDA) detection, determined physical and chemical properties by proton and phosphorus Nuclear Magnetic Resonance (^{1H}NMR and ^{31}P-NMR), and quantify by Ultra Performance Convergence Chromatography (UPC^2) coupled to Quadrupole Time of Flight Mass Spectroscopy (Q-ToF/MS). The UPC^2 Q-ToF/MS data was processed using TransOmics Informatics for Metabolomics and Lipidomics (TOIML) and statistical analysis was performed interrogating similarities and dissimilarities of each extraction techniques. Biological assay were conducted on three of the S-S extracts and compared to know antiviral inhibiting drugs as controls. For the formulation study, Dynamic Light Scattering (DLS), Differential Scanning Calorimetry (DSC) and release studies were conducted in order to characterize the garcinol liposomes. Initial RP-HPLC results showed no compounds with photodiode array (PDA) fingerprints characteristic of garcinol were found in either of the extracts from both extraction techniques. NMR confirmed what was already observed:

garcinol has the same molecular formula, weight and chemical structure as the previously reported guttiferone F and both names (garcinol and guttiferone F) are used interchangeable. Comparison of the different extraction techniques by UPC^2 Q-ToF/MS resulted in the identification of 1291 chemical features and the features were grouped together statistically based on their relative extraction solvents and techniques. Of the 1291 detectable mass constituents, 43.81% can be extracted by S-S technique and 40.09% can be extracted by SFE technique, relatively similar extraction profile. Approximately 20.99% of the mass constituents were detected using both extraction techniques. Overall, results from the SFE showed the comprehensive profile of all mass constituents found within this natural product and each mass grouped statistically based on their relative extraction solvents to determine extraction coverage. In addition, these statistical analysis and extraction techniques have never been performed simultaneously and or compared on *Garcinia kola* seeds. Biological data revealed that extracts from the S-S technique was negative for the inhabitation of antiviral activities as compare to the controls. The results of the liposome release studies indicated initial rapid release of less than 1% of garcinol from the liposomes, followed by a sharp drop in concentration at 4 hours, suggesting precipitation in the dissolution medium with no further release as the concentration leveled off to 140 hours. DSC results indicated that the surface charge of the liposome was -10.5 mV and remained relatively constant for 7 days suggesting stability issues. DLS results indicated an increase in particle size and Polydispersity index over a 7 day period which also suggests stability issues. Overall, the formulation study showed garcinol released less than 1% and may interact significantly with the DPPC phosphate head

groups, as indicated by the large increase in net negative surface charge. Although no antiviral activities were active in *Garcinia kola*, this edible plant could serve as an abundant source of naturally-occurring, bioactive compounds for further pharmaceutical development for other biological activities.

ACKNOWLEDGEMENTS

I'd like to thank my Lord and Savior Jesus Christ for being the head of my life and providing the necessary resources to complete this research project. I'd like to thank my major advisor Dr. David Worthen for going above and beyond in believing in and supporting me throughout graduate school. I cannot thank him enough! Next my aunt, Elizabeth "Poor Girl" Duncan for her words of wisdom about traditional Liberian plants and igniting the fire that gave birth to this research project. I could not have successfully finished this research project without the Natural Products team at Waters Corporation in Milford, Massachusetts especially Michael Jones, Dr. Giorgis Isaac and Dr. Dustin Yaworsky who took the time out of their busy schedules to training, mentoring, and provide access to their analytical laboratory. Working with them truly helped take the research project to another level. I'd also like to express my deepest gratitude to Dr. Henry Genieva for all of her guidance on the isolation portion of this research. Many thanks to the Raga Institute for Medical Research especially Dr. Christophe Pannecouque and Dr. Pieter Leyssen for the biological assay work. I'd also like to thank my mentor Charles "Chuck" Watson in the College of Engineering Dean's Office for all of his support and encouragement throughout graduate school. And lastly I'd like to thank my natural and spiritual family and friends for their unconditional love, support, encouragement and prayers: truly without all of the names mentioned above, my project wouldn't have been a success. Ma God bless everyone of you!

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CHAPTER 1

INTRODUCTION

Garcinia kola is a species of flowering plants which grows in tropical climates across Western Africa, Asia, and Australia. *Garcinia kola* belongs to the *Guttiferae* (formally *Clusiaceae*) family. There are more than thirty genera of *Guttiferae,* including *Calophyllum, Hypericum, Allanblackia*, and *Garcinia*. (Whitmore et al., 1973). *Guttiferae* is divided into three subfamilies: *Kielmeyeroideae*; *Hypericoideae*; and *Clusioideae* (Gustafsson et al., 2002). *Garcinia* belongs to the subfamily *Clusioideae*. There are well over fifty species of evergreen trees and shrubs belonging to the *Garcinia* genus, including *Garcinia cambogia, Garcinia indica, Garcinia mangostana, Garcinia livingstonei, Garcinia candelliana* and *Garcinia kola*. (Smith *et. al*., 2004). Known products containing *Garcinia mangostana* are commonly sold as dietary supplements in the United States under the trade name Hydroxycut ™.

There are four main classes of secondary metabolites with biological activities found in the *Guttiferae* family: benzophenones; bioflavonoids; coumarins; and xanthones. The chemical structures of several of the benzophenones contain the polyisoprenylated benzophenone core bicyclo [3.3.1]-nonane-2, 4, 9-trione linked to a 13, 14-dihydroxybenzoyl phenyl ring is found in Figure 1 (McCandish et al., 1976). This core structure is a common structural motif of the benzophenones which have been isolated from plants. The benzophenones are increased in hydrophobicity per number of prenyl functional groups attached.

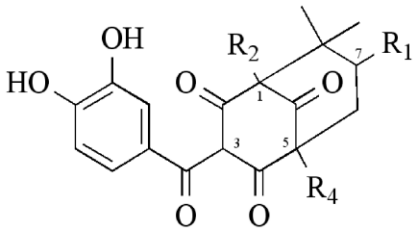


Figure 1: General structure of polyisoprenylated benzophenones (Acuna *et. at.* 2009)

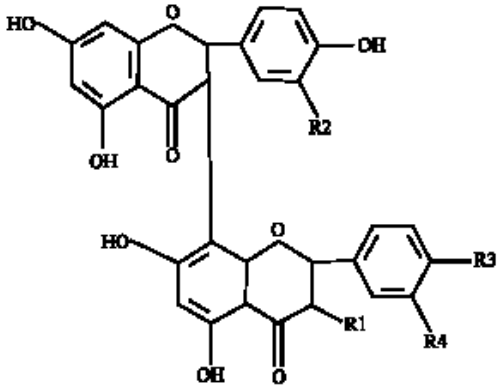
Garcinia kola has a distinct bitter taste-hence its common name "bitter kola" and also its common name "male kola" because of its claimed aphrodisiac activity. There is an average of four seeds contained in a fruit (Figure 2). The seeds are extracted by breaking open the fruit. The seed contains fat, proteins, carbohydrates, xanthones, bioflavonoids, benzophenones, and other compounds. They are chewed for the relief of cough, colds, colic, hoarseness of voice, and throat infection (Ofokansi et al., 2008). The fruit pulp is used to treat jaundice (Uko et al., 2001). The sap is used for the treatment of parasitic skin diseases, while the latex is orally ingested for the treatment of gonorrhea (Ofokansi et al., 2008) and applied to wounds to assist in healing (Uko et al., 2001). The roots and stems are eaten as a snack food known as a chewing stick for dental care, to suppress appetite and to aid in the digestion process.

Compounds isolated from *Garcinia kola* and other members of the *Garcinia* genus have been shown to have useful therapeutic, bio-protective, and other biological activities. *Garcinia kola* is used for the treatment of liver disorders (Adaramoye et al., 2006). More recently, *Garcinia kola* has been assessed for its potential utility for fighting infectious viral diseases, such as Ebola, by preventing viral replication (BBC Health News, 1999). Despite its bitter taste, *Garcinia kola* has been traditionally consumed for cultural, social, and traditional ceremonies, and for the worship of

ancestral gods. *Garcinia kola* has also been employed as a substitute for hops in tropical beer (Ajebesone et al, 2004). Paradoxically, it has been claimed to cause both nervous alertness and to induce insomnia (Uko et al., 2001). Thus, there is significant ethnobotanical and epidemiological evidence available suggesting that *Garcinia kola* is relatively non-toxic to humans when ingested or applied to the body.

Figure 2: *Garcinia kola* seeds (courtesy of Google image)

The most abundant biologically active constituent in the seed of *Garcinia kola* is known as kolaviron (Figure 3). Kolaviron is a mixed biflavoniod complex derived from the defatted alcoholic extract of the plant. The complex consists of GB-1, GB-2, and kolaflavanone) shown below in Figure 3 (Cotterill et al., 1978). The primary difference between GB-1 and GB-2 is the replacement of hydrogen (H) with a hydroxyl group (OH).



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Figure 3: The structure of bioflavonoids: Kolaviron (courtesy of Google image)

Earlier work on the phytochemistry of *Garcinia* species resulted in the isolation of pharmacological compounds from several chemical classes including xanthones, bioflavonoids, triterpenes and benzophenones. Xanthones are polyphenols that arise from the biosynthetic pathways of shikimate and acetate. A number of xanthones are known for their anti-cancer and anti-obesity properties (Iwu et al., 1987). The fruit hull of *Garcinia mangostana* possesses anti-cancer activity and this biological effect has been attributed to xanthones. Xanthones are believed to act by blocking inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression (Wan et al., 2004). Xanthones also possess anti-inflammatory activities (Chen et al., 2008) by the reduction of iNOS in murine macrophages. Bioflavoniods are phenolics, a class of phytochemicals which primarily act as plant pigments and possess antioxidant activities. Bioflavonoids have been isolated from the *Garcinia kola* and are abundant in the seeds of the plant (Faromi et al., 2011). Triterpenes are compounds comprising six isoprene units which are also precursors of steroids. Triterpenes are found in the root bark of *Garcinia polyantha* and they possess antimalarial properties (Lannang et al., 2008). Benzophenones are ketone molecules used in perfume and medicine. Kolanone is a prenylated (addition of a hydrophobic prenyl moiety) benzophenone isolated from *Garcinia kola* possessing antimicrobial properties (Hussain et al., 1982). These chemical classes are important and they may play a role in modulating various molecular metabolic pathways essential to human life. They also show the diverse functions that this natural product possesses, suggesting many potential therapeutic and biological applications for *Garcinia kola*.

The central objective of this study is to compare two different extraction techniques for the isolation, identification and conduct a pharmaceutical formulation of a potential antiviral compound responsible for inhibiting viral replication from *Garcinia kola*. Although there is evidence that such isolation methods can be applied to isolate potential benzophenone antiviral compounds from related plant genera, these particular methods have not been applied to *Garcinia kola*. In order to accomplish this, the following hypotheses are made:

1. Utilize liquid-liquid extraction technique of Fuller *et al.*1999 for the isolation of antiviral compound Guttiferone F (also called Garcinol) found in the rootwood of *Allanblackia stuhlmannii* and applying the same extraction technique to *Garcinia kola* nuts could lead to the isolation of the same antiviral compound. In addition, profile the compounds found in this extraction technique.
2. Utilize a second extraction technique, Supercritical Fluid Extraction (SFE) on *Garcinia kola* nuts for the isolation of antiviral compound Guttiferone F (also called Garcinol). In addition, profile the compounds found in this extraction technique and compare both extraction techniques.
3. Formulate a representative antiviral compound into suitable liposomes for controlled drug release.

The advantage of using both extraction techniques is to obtain a wide range of extracted compounds found in *Garcinia kola* and for comparison purposes.

CHAPTER 2

REVIEW OF THE LITERATURE

This chapter will focus on the literature related to the published biological activities, formulation studies conducted on *Garcinia kola* and other *Garcinia spp* as well as two separation techniques used in the pharmaceutical industry: liquid-liquid extraction and Supercritical Fluid Extraction (SFE).

2.1 Antiviral properties found in the *Garcinia* family

There are various species in the *Garcinia* genus which are reported to possess antiviral properties. These species include *Garcinia livingstonei, Garcinia ovalifolia* (Gustafson et al., 1992), and *Garcinia mangosteen* (Chen et al., 1996), among others. The compounds responsible for the antiviral properties have been identified as guttiferones. Guttiferones are polyisoprenylated benzophenone derivatives that inhibit by cytopathic effects the activity of the virus responsible for HIV infection (Gustafson et al., 1992). Most polyprenylated benzophenones isolated from these plants also contain carbonyl, hydroxyl and other polar groups.

Perhaps the most cited naturally-occurring, *Garcinia*-derived benzophenone in the literature is camboginol, also a synonym for garcinol. Garcinol is a polyisoprenylated benzophenone which has been recovered from *Garcinia assigu,* (Ito et al., 2003) *Garcinia indica* (Hong et al., 2007), *Garcinia purpurea* (Matsumoto et al., 2003), *Garcinia banacana* (Rukachaisirikul et al., 2005), *Moronobea coccinea* (Marti et al., 2008), and *Allenblackia monticola* (Lenta et al., 2007) and assayed for

cytotoxicity as well as anti-bacterial, anti-viral, and anti-parasitic activities. Garcinol has the same or even very similar structural to that of Guttiferone F (Gustafson et al., 1992) and the names are said to be used interchangeable (Fuller et. al., 1999). Guttiferone and related benzophenone antiviral compounds have been reported to be isolated from *Garcinia kola* and other *Garcinia spp*. and they contain a number of valuable biological active compounds. However, relatively few studies have been reported describing the design and performance of *Garcinia* derived formulations and drug delivery systems on its proposed antiviral properties. Accordingly, another goal of this work is to develop a pharmaceutical formulation for the controlled delivery of a benzophenone antiviral compound.

2.2 Natural Product Development

According to Newman *et al*. in "Natural Products as Sources of New Drugs over the Period 1981-2002", over 70% of small molecule drugs on the market are derived from or are based on natural product sources (Figure 4). Natural product sources listed includes: Unmodified Natural Products (N), Natural Product Derived (ND), Natural Product Mimic (NM), Biological (B), and Total Synthesis (S*). Though there are numerous compounds isolated from natural products, formulating them into effective dosage form is a challenging process due to the lack of standardization, as well as poor solubility and limited chemical and metabolic stability. An increase in the study of components in the *Garcinia* genus has risen within the past 20 years, but there have only been a few investigations conducted in the United States and few-if any- on the isolation and formulation of compounds purported to be responsible for their antiviral properties. Thus far, the only

formulation studies performed on *Garcinia kola* focus on the formulation of the main constituent, kolaviron. To date, there are only two FDA approved drugs derived from botanical preparation: Veregen which is a topical green tea extract approved in 2006 for the treatment of genital warts, and Fulyzaq (Crofelemer) an orally administered drug approved in December 2012 for the treatment of HIV-associated diarrhea. This implies that out of the 39 FDA approved new molecular entities in 2012, only 1 of which is for HIV related diseases (Figure 5). Exploring this novel area of the pharmaceutical potential of *Garcinia kola* may result in additional approved products for the palliation and treatment of various pathologies.

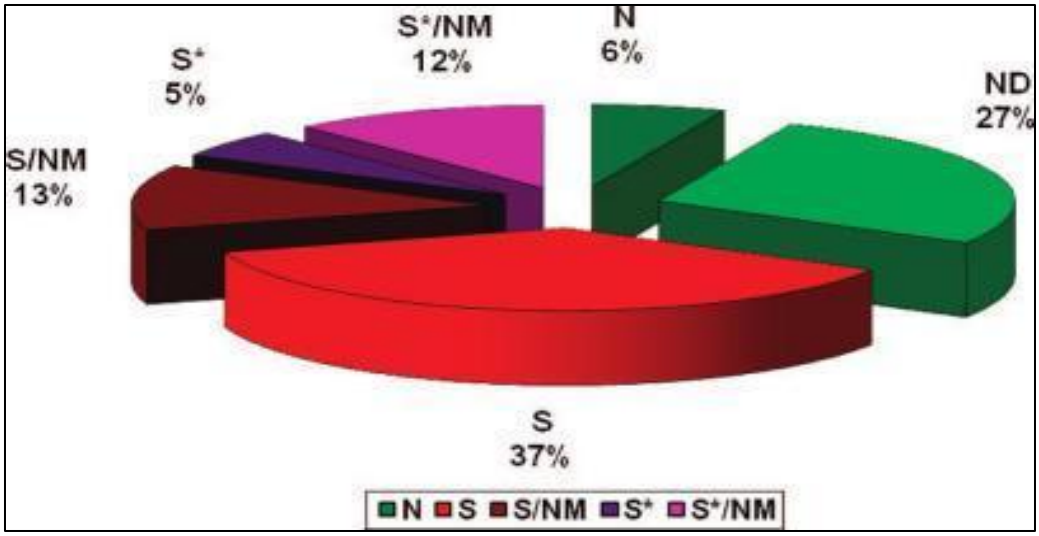


Figure 4: Small molecule new chemical entities 1981-2008 by Newman *et al.* 2003

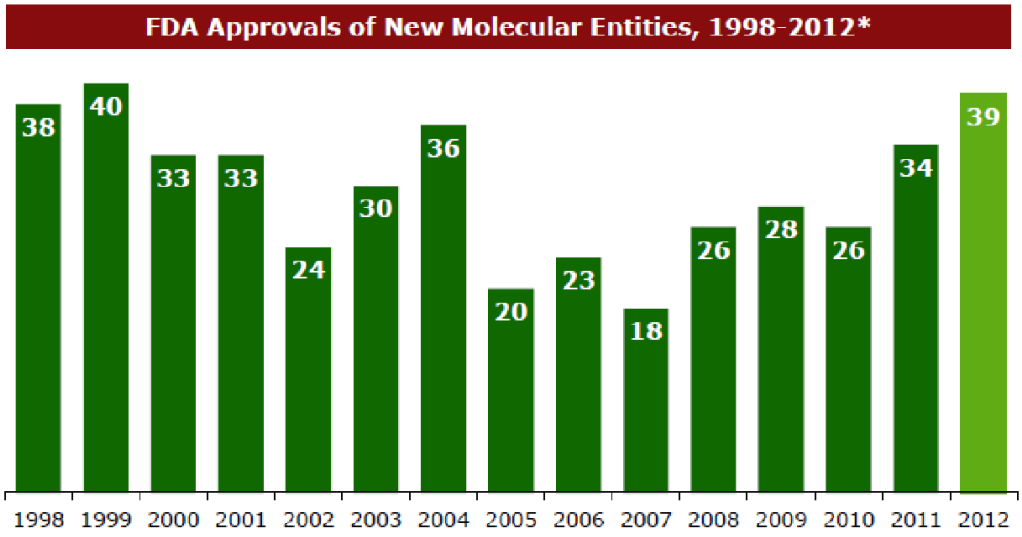


Figure 5: FDA approved new molecular entities 1998:2012 (courtesy of FDA.gov)

2.3 Pharmaceutical formulation in *Garcinia kola* and *Garcinia spp*.

The development of effective and acceptable dosage forms for biologically active natural product delivery, both locally and systemically, has been investigated but it remains an important challenge due to the need to measure desirable biological activities, the diverse physical-chemical properties of individual molecules, as well as the lack of standardization and performance in dosage forms and delivery systems. There are only a few cited research studies conducted proposing various formulations of *Garcinia kola* constituents such as a tablet, eye drops, and a toothpaste. One of the formulation studies reported was a study in 2004 by Onunkwo et al. describing a compressed tablet formulation intended for use as an oral dosage form using a wet granulation method. Dosage form parameters were evaluated (tablet weight variation, thickness and diameter; hardness; content uniformity; friability; disintegration time and dissolution profile) in order to measure and qualify the physical properties of the

tablets (Onunkwo et al., 2004). Briefly, in their method, a chemical assay was conducted on the dry, powdered seeds as well as the crude aqueous extract of the seeds. Next, the powdered material (50 mg) and an aliquot part of the crude extract (10 mg) were formulated into tablets using the wet granulation method. Extraction of the powdered seeds was performed using ethanol as a solvent and concentrated using a rotary evaporator, resulting in a dry mass. After evaluating those parameters listed above, the results indicated that the tablets had acceptable disintegration time, dissolution, hardness and friability profiles.

A more recent study conducted in January 2010 at the Lagos University Teaching Hospital in Nigeria emphasized that untreated increases in pressure due to glaucoma in the eye can eventually lead to blindness. Accordingly, a new treatment based upon *Garcinia kola* was developed and tested for effectiveness in glaucoma patients. The study showed that a topical eye drop solution containing *Garcinia kola* (0.5% aqueous) extract significantly reduced eye pressure in patients with glaucoma using an intraocular dose twice a day. The design of the study used commercial timolol 0.5% eye drops as a comparative control. Patient results showed that 47.8% ± 0.8% of test subjects showed a significant reduction in eye pressure when using *Garcinia kola* as compared with a 48.2% ± 1.03% reduction in pressure in patients using the control. It was concluded that *Garcinia kola* eye solution significantly reduced eye pressure and this effect was comparable to the effect of the commercial beta-blocking control, timolol (Adebukunola et al., 2010). While these formulation studies underscore the utility and formulatability of certain crude *Garcinia kola* preparations, a formulation study of *Garcinia kola* comprising one of its purported

anti-viral benzophenones has yet to be reported.

A published research study on the isolation and identification of antiviral compounds was conducted by in 1999 by Fuller *et al*. In short, a series of HIV-inhibitory prenylated benzophenones known as guttiferones A-E was previously reported from extracts of three different genera (*Garcinia, Clusia,* and *Symphonia*) from the large plant family Guttiferae or Clusiaceae (Gustafson et al., 1992). Fuller *et al*. isolated the first member of the guttiferone F (also called garcinol) class of polyprenylated benzophenones from the genus *Allanblackia* (also in the *Clusiaceae* family). In this study it is hypothesized that the same or similar antiviral compounds occur in, and may be isolated from, *Garcinia kola* since it is in the same genus. If the *Garcinia* genus contains guttiferones A-E, there is a possibility that *Garcinia kola* might also contain guttiferone F; which has yet to be isolated and identified in *Garcinia kola,* which is said to have antiviral properties. It is hypothesized that, by using the isolation methods of Fuller *et al*., the same or similar compounds may be isolated from *Garcinia kola*. These are core to be answered by this research.

One of the most important issues that arise when formulating natural products is poor aqueous solubility and stability. Many new chemical entities display poor aqueous solubility. However, there are many techniques used to possibly overcome solubility, such as nanosupensions, drug dispersion, and chemical modification. Nanosuspensions are colloidal dispersion of particles of a drug that are stabilized by surfactants. Nanosupensions also increases dissolution rates due to a large surface area to unit volume ratio. Drug dispersion in carriers which include solid solutions (solid solute molecularly dispersed in solid solvent), eutectic mixtures (fusion melt of

solute and solvent showing complete miscibility at or above all constituent melting points) and solid dispersion (dispersion of one or more compounds in an inert carrier in the solid state) (Gowthamarajan et al., 2010). To be more specific, solid dispersion is used to improve the solubility of poorly soluble compounds, as well as mask the taste of a drug substance, improve the disintegration of oral tablets, and reduce the formulation particle size and thus increase the dissolution rate. This technique is widely applied in the pharmaceutical industry. The last technique that will be discussed for the use to overcome poor solubility is chemical modification. Some examples of chemical modification are the formation of a salt complex and the use of a prodrug (Mohanachandran et al., 2010).

As written in the previous paragraphs, solubility is a challenge in the successful formulation of compounds such as the benzophenones found in the *Garcinia* family. An example of this is found in the work described by Bhaskar *et al*. According to Bhaskar *et al*., *Garcinia mangosatana* is proven to be active against *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium* acne, which are responsible for the outbreak of acne. Preformulation studies using a mixture of the aqueous extract of *Garcinia mangosatana* and with the excipient *Aloe vera* proved to overcome poor solubility and results shows *Garcinia mangosatana* to be a potential for drug delivery as a topical agent for the treatment of acne, which was the goal of the study. Another study of *Garcinia mangostana* (specifically α-Mangostin) sought to improve the solubility and bioavailability of poorly-soluble α-mangostin using solid dispersion (Aisha et al., 2011). The poor solubility of α-mangostin hinders its therapeutic application by limiting its oral absorption. The study

showed an enhancement in the solubility of α-mangostin using solid dispersion systems. Also, further research suggests the need for a new therapeutic strategy of HIV therapy due to the poor solubility of such compounds (Gupta et al., 2010). Overall, the formulation of *Garcinia mangostana* and HIV therapies in general are often shown to have issues of poor solubility and the utilization of a dispersion system maybe a useful technique for enhancing solubility. Thus, it is hypothesized that if anti-viral *Garcinia mangostana* constituents derived from a plant from the same family as *Garcinia kola*, have poor solubility, than analogous materials derived from *Garcinia kola* could also display poor solubility and be amenable to solid dispersion or another solubility type enhancing formulation. A carrier formulation that may protect the plant constituents from oxidation or hydrolysis, but that may also be useful for oral or topical delivery, such as a physiologically-compatible liposome, may be a viable formulation alternative.

In summary, there are antiviral compounds found in the *Garcinia spp*. There is a need on the market for more antiviral therapeutic drugs. Research suggests that although there are many studies of *Garcinia spp*. which focus on formulation, the formulation of antiviral compound(s) from *Garcinia kola* is lacking. Now that we know what types of compounds are found within the *Garcinia spp*., it's important to also look at different extraction techniques used to extract these compounds from their natural source.

2.4 Extraction technique overview

Extraction is a broad term used in the pharmaceutical industry and it involves the separation of medicinally active compound(s) of plants and or animals from the

inactive components by using solvents from extraction procedures. One of the most universal forms of extraction is brewing coffee: low molecular weight/concentration molecule (caffeine) is removed from the high molecular weight/concentration material (coffee bean). Another example is brewing tea which uses the same principle. This is an example of solid/liquid extraction. On the other hand, liquid-liquid extraction is commonly used in organic laboratory to separate and purify the desire product from a mixture leaving behind a starting material and by-products. For example, the extracts from plants are fairly impure therefore standardized extraction procedures is needed for the final quality of the medicinal compound which can lead to a potential drug. Both techniques are governed by distribution coefficient, a measure of how an organic compound distribute between aqueous and organic phase. Distribution coefficient is very important in drug delivery because a drug must be carried throughout the body to its targeted site. A drug must have enough water solubility to dissolve in the blood and organic solubility to get through the cell. Below are two extraction techniques used in the pharmaceutical industry as well as some advantages and disadvantages for their use.

2.4.1 Liquid-Liquid extraction

Liquid-liquid extraction/partitioning (the term solvent-solvent (S-S) extraction will be used interchangeable) is a method in which a desire compound is pulled from one solvent to another solvent only if the two solvents are immiscible (example oil and water, Figure 6). The most common method of liquid-liquid extraction is using a separatory funnel. Separatory funnels are used to extract a compound either from or into an aqueous layer. Depending on the density of the solvent(s) repetitive use of the

separatory funnel or another type of extractor is needed. Some advantage of using liquid-liquid extraction includes it being the simplest form of extraction every used for wide range of application in both chemical laboratories and pharmaceutical industries. Other advantages include the ability to operate in a continuous mode, and the use of two solvent phases. One major disadvantage is the use and disposal of disposal of solvents used. In addition, this technique can be performed with simple equipment, and its selective separation is usually highly efficient.

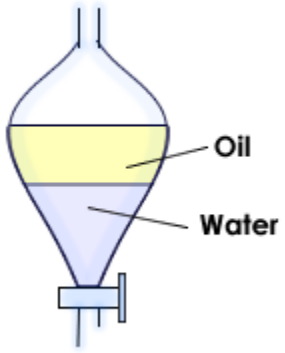


Figure 6: Separatory funnel (courtesy of Google image)

2.4.2 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is a technique used to separate one component from another component using supercritical fluids as the extracting solvent. SFE is also an alternative sample preparation method generally used to reduce the use of organic solvents and increase sample throughput. SFEs are produced by heating a gas above its critical temperature or compressing a liquid above its critical pressure (Figure 7). Because of its low critical parameters (31.1°C, 73.8 bar), carbon dioxide (CO_2) is the most used supercritical fluid used and is at times modified by co-solvents such as methanol and ethanol in order to overcome polarity limitations.

Advantage of using CO_2 includes its favorable physical properties, its inexpensive, relatively safe and in abundance.

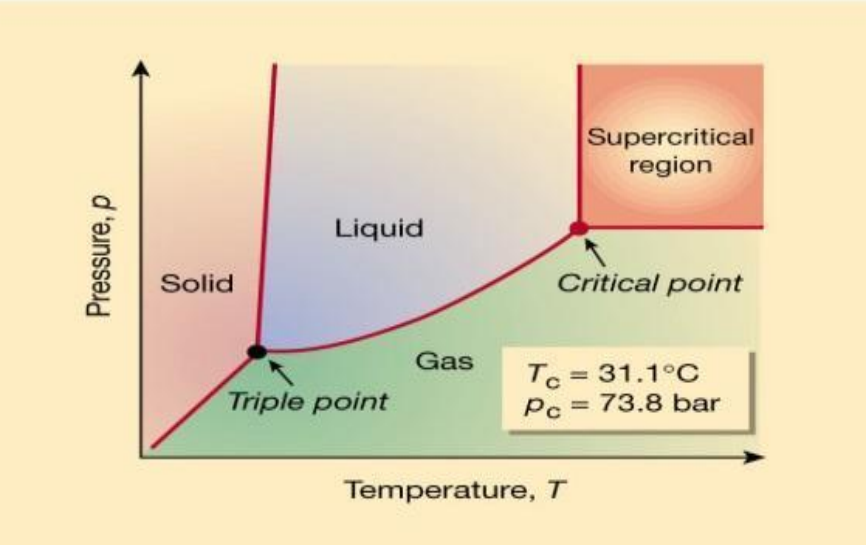


Figure 7: Phase diagram of Carbon dioxide, CO_2 (courtesy of Google image)

A typical SFE system includes a CO_2 source, a pump to pressurize the gas, an oven containing the extraction vessel, a pressure regulator, an analyte collector and a detector (Figure 8). Another advantage of using SFE is its extraction procedure having little to no solvent residue and extraction at very low temperatures. The rationale for the use of SFE was because of its shorter extraction time, and selective extraction by varying temperature, pressure and or modifier.

SFE has been used for various extraction and isolation of natural products including decaffeination of coffee, and extraction of hops, spices and tea because it is an eco-friendly alternative to chemicals and solvents used by chemist. This is because Chemist generate large amount of waste organic solvents which is growingly attributed in environmental pollution and hazardous to human health. However, the use of SFE in natural product extraction is limited to plant natural products.

The goal of the SFE technique was to search for garcinol by performing liquid-liquid extractions, and SFE extractions with CO_2, modifier which included methanol, ethanol, and isopropanol, and analyze via UPC^2 coupled with Q-Tof-MS. The second goal was to perform principal component analysis (PCA) of the SFE and S-S extracts in order to find correlations to streamline sample prep approach, exploit TransOmics workflows to aid visualization of data, targeted processing for garcinol, and comprehensive processing for feature differences.

Figure 8: Supercritical Fluid Extraction system (courtesy of Waters Corporation)

CHAPTER 3

METHODOLOGY

Both separation techniques, Solvent-solvent (S-S) and Supercritical Fluid Extraction (SFE) were conducted and analysis was performed by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using Ultraviolet Photodiode Array (UV-PDA) detection, Proton and Phosphate Nuclear Magnetic Resonance (^{1H}NMR and ^{31P}NMR), and Ultra Performance Convergence Chromatography (UPC^2) coupled to Quadrupole Time of Flight Mass Spectroscopy (Q-ToF/MS). Biological assay was conducted and compared to controls. Characterization of the liposome formulation was performed using Differential Scanning Calorimeter (DSC) and Dynamic Light Scattering (DLS). Lastly, the rate of release and extent of release was conducted using dissolution tests. The follow discuss the materials and methodology used.

3.1 Materials

Garcinia kola nuts (2 kg) were purchased from Gold Coast Trading Company (Bronx, New York) in January 2011. Organic solvents (hexane, methanol, acetone, dichloromethane, and methyl-*tert*-butyl ether (MTB) were purchased from Fisher Scientific (Fair Lawn, New Jersey). Sephadex LH-20 resin for size exclusion chromatography was purchased from Sigma Aldrich (St. Louis, MO). Potassium phosphate monobasic and dibasic was also purchased from Sigma-Aldrich (St. Louis, MO). The active antiviral compound Garcinol (MW 602.8) was purchased from Enzo Life Sciences (Farmingdale, New York). For the liposome formulation study, 1, 2-

Dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) was purchased from Corden Pharma Switzerland LLC (Switzerland). Cellulose membranes (Spectra/Pro ® Float-A-Lyzer® G2) with a molecular weight cutoff of 3500D used for drug release tests were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, California).

3.2 Extraction techniques

The following sections include the extraction methodology employed on *Garcinia kola* using the two extraction techniques mentioned above.

3.2.1 Liquid-liquid extraction

Two kilograms of *Garcinia kola* nuts were spread on a plastic bag and dried in a Robertshaw Drying Oven at 135°F for five days. Upon exhaustive drying, the nuts were peeled and the peelings were kept separately for future evaluation. The nuts were ground using a coffee grinder yielding a total of 1071 grams of ground material. One kilogram of the ground nuts was weighed and transferred to a large glass chromatography extraction column with a mechanical mixer. Three liters of hexane, then three liters acetone, then three liters of methanol, and then with one liter of a combined 1:1 methylene chloride-methanol was then introduced to the nuts respectfully. It is important to note that one liter of solvent was added at a time, mixed for 12 hours to 24 hours, and then removed. This entire process was monitored for roughly six days. The seeds were also subject to other solvents including ethyl acetate and chloroform (<250 mL each) solvent extraction scale. The various solvent systems served as a removal vehicle of compounds found in *Garcinia kola* nuts. After each successive extraction, the solvents were removed by rotary evaporation, and the

resulting residues were labeled, sealed, covered and stored. The hexane extract was bright yellow, and the acetone and methanol extracts reddish-brown and brownish-red, respectively.

Next, the method described by Fuller *et al*., comprising solvent-solvent extraction (hence the use of the term S-S because the rotary evaporated samples, mentioned above were re-introduced to solvent), was applied to the initial extract residues by combining a 5 g portion of the 1:1 methylene chloride-methanol and methanol extracts (2.5g each). This combination was further separated by solvent-solvent partitioning into hexane (200 mL), methyl-*tert*-butyl ether (MTB, 10 mL), ethyl acetate, and water. The purpose of partitioning was to ensure that the compounds were separated based on their solubilities into two different immiscible liquids. In short, after the portioning fraction was added to the separating funnel with the 1:1 methylene chloride-methanol and methanol extracts combined, the funnel was closed, shaken abruptly which lead to one phase (hexane for instance) being separated out from the 1:1 methylene chloride-methanol and methanol extracts phase and slowly opening the tap of the funnel and collecting each immiscible fractions. The partitioned hexane, MTB, ethyl acetate and water fractions were labeled, sealed with aluminum foil and Parafilm® around the neck of the beaker, and placed in the laboratory refrigerator in Fogarty 41.

The MTB fraction was passed through Sephadex LH-20 loaded into a glass chromatography column using 1:1 methylene chloride-methanol as the mobile phase, yielding 8 fractions. The third and fourth fractions were collected are noted as (Sephadex fraction 3/4). An illustration of this protocol is further depicted in Figure 9,

all of which were analyzed by analytical techniques in section 3.3.

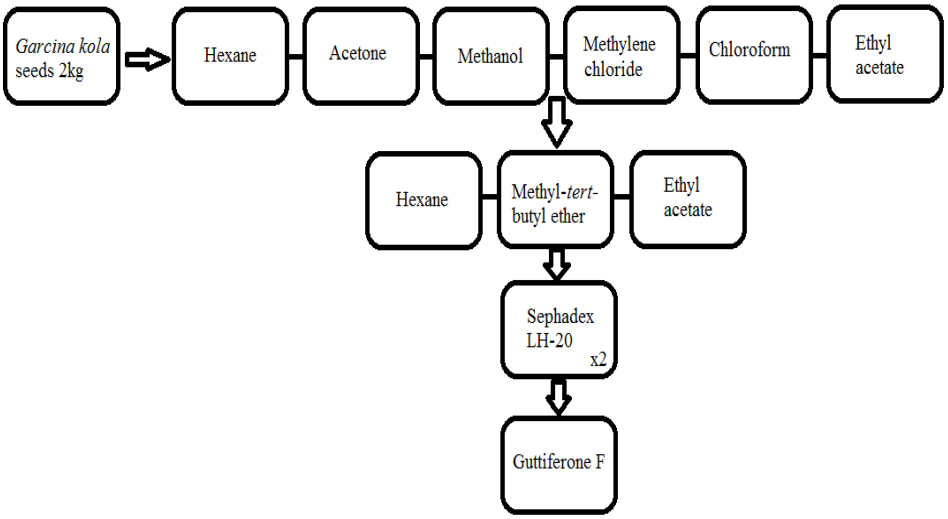


Figure 9: Flow chart of Guttiferone F isolation (Fuller et al 1999)

3.2.2 Supercritical fluid extraction (SFE)

Sample preparation for the SFE experiment was performed by weighing out 1 gram of *Garcinia kola* nuts which were placed in empty white tea bags, the bags were folded and stapled shut. The tea bags were placed in a metal vessel and sealed tightly. The vessels were then loaded into the oven portion of the SFE. Valves were connected, the instrument set with 100% carbon dioxide (CO_2), and organic solvent modifiers, including methanol (MeOH), ethanol (EtOH) and isopropyl alcohol (IPA) 5%, 10% and 30% each by w/w. Each sample collected was subjected to various analytical methods also mentioned in section 3.3.

3.3 Analytical analysis

The following sections include the analytical analysis of samples extracted and collected with both extraction techniques mentioned previously.

3.3.1 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

High Performance Liquid Chromatography (HPLC) is an analytical technique used to separate, identify and quantity components in a mixture. HPLC has been used in various industries including medical, legal and manufacturing. With a slightly higher operating pressure (50 bar-350 bar), it contains a mobile phase which is typically an organic solvent, and a hydrophobic stationary phase (hence, reverse phase) which is typically a solid absorbent material, 2-50µm in size. There are two types of phases in chromatography: normal phase and reverse phase. The term "reverse phase" refers to any chromatography methods that uses hydrophobic stationary phase. HPLC separation is due to the different degrees of interaction with the solid adsorbent material which is why it's so widely used. Components of a typically HPLC system includes: a liquid analyte (sample), pumps used to push the analyte through the column, an injector, a non-destructive detector, such as phased diode array or ultraviolet where the analytes can be recovered and a computer analyzing system.

In brief, the analytes are prepared, injected, carried to the column by a pump where it's separated and lastly detected then recorded yielding a spectra which shows the analyte's characteristic fingerprint. The HPLC method used was an isocratic acetonitrile-water (0.1% Glacial acetic acid) detection range from 190 to 400 nm (baseline check at 220 nm, 230 nm, 256 nm, and 282 nm). The column used was an Agilent Zorbax SB-C_8, 250mmx4.6mm, 5µ, at a flow rate of 1 milliliter per minute and the run time was 20 minutes. Detection wavelengths were set to collect within a range between 200 nm to 400 nm. The *Garcinia kola* extracts were subject to this isocratic method on each of the separation process found in Figure 9.

3.3.2 Ultra Performance Convergence Chromatography (UPC^2) coupled to quadrupole time-of-flight Mass Spectroscopy (Q-Tof-MS)

Mass spectrometry (MS) is an analytical technique used to measure molecular weights, observe elements present in a compound and provide more information about its molecular structure. To do so, the element must be ionized (remove one or more electrons creating a charged particle) by a magnetic field. Because of the magnetic field, the ions in the element are accelerated so that they all have the same kinetic energy and are measured according to their masses. There are various ways the elements can be ionized electrospray ionizer (ESI) being the most popular. ESI works by applying high voltage to a particle producing an aerosol of ions. ESI is great for producing ions from macromolecules because it takes into account fragmentation of ions. ESI is also used for producing multiple charged ions and widening the mass range of bigger compounds like proteins and polypeptides. Most mass spectrometer works in negative ion, ESI(-), and positive ion, ESI(+), electrospray ionization mode depending on whether the observed ions are negatively or positively charged.

After the ionization step, the data system then reads the digital information (mass to charge ratio, m/z) displaying it as a mass spectrum. There are also different ways that the mass to charge ratio can be analyzed: Quadrupole mass analyzer and Time of Flight (ToF) mass analyzer being two popular examples. As the name implies, quadrupole has four cylindrical rods which are set in parallel. The purpose for the quadrupole is to filter the mass based of the mass to charge ratio as electric field is being applied to the rods. Time of Flight on the other hand, determines the mass to charge ratio based on a time measurement, the time it takes the particle to get

to the detector at a known distance. The time depends on the mass to charge ratio: heavier particles takes longer and vice versa. When both the quadrupole and time-of-flight mass analyzer instruments are combined and used this way, a more accurate mass measurements and fragmentation experiments can be carried out.

Another type of chromatography used is Ultra Performance Convergence Chromatography (UPC^2). UPC^2 is also an analytical technique that incorporates the re-design of a chromatographic system using liquid carbon dioxide (CO_2) as the primary mobile phase. UPC^2 uses both gas and liquid as mobile phases; hence, the term convergence. A co-solvent or modifier, typically methanol, is used as an elution solvent. The technique follows the principles and selectivity of normal phase chromatography (hydrophilic stationary phase). UPC^2 also uses stationary phases consisting of smaller particle size (< 2 μm) therefore providing greater efficiencies that results in a greater resolving power capable of separating more analytes per unit time. UPC^2 works great for improving the productivity, efficiency, and throughput of samples. It reduces retention times, and thus processing cost, and drastically reduces solvent usage. Alternating high and low collision energy was performed during a single injection, a technique known as MS^E, which uses two different steps of mass spectroscopy, allowing for precursor and product ion determination. This method is used for finding out related substances of the compound of interest. The alternating scanning provides accurate mass measurement for all detectable ions for the system and provides faster speed and resolution. By applying a collision energy field, MS^E can be applied in an aim to measures the molecule's distinct fragmentation pattern which provides structural information. Note that Base Peak Intensity (BPI) was used

as a trace that subtracts the background noise in the spectra. UPC^2 may also be coupled to quadrupole and time-of-flight mass analyzers (Q-Tof MS) in order to target a broad range of compounds in complex samples. And this technology is used in order to comprehensively acquire data, locate and identify known compounds, identify unknown peaks of interest, and elucidate structures of unknown.

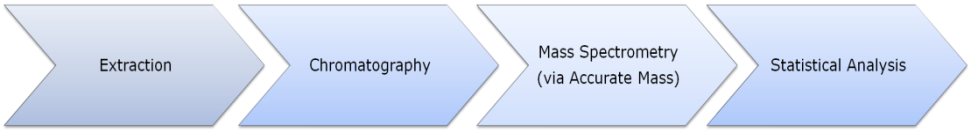


Figure 10: Workflow overview analysis (courtesy of Waters Corporation)

The workflow employed for the chemical profiling of *Garcinia kola* is described in Figure 10. Extracts from the samples listed in Figure 9, were analyzed using Waters ACQUITY UPC^2 instrumentation. An ACQUITY UPC^2 BEH column with dimensions 3.0 x 100mm; 1.7um was observed as providing the best separation with the greatest number of peaks. Ethylene Bridged Hybrid (BEH) columns are used in small molecule to large biopharmaceutical analysis. MS data acquisition was performed using Waters Xevo G2 Q-ToF. Alternating high and low collision energies, 5V and 30V, respectfully, allowed for precursor and product ion determination as stated above.

3.3.3 Nuclear Magnetic Resonance (^{H}NMR and ^{31}P-NMR)

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique used to study nuclei with non-zero nuclear spins. All nuclei contain protons hence they have charge and spin. Many nuclear spin values are possible. The most important are those with spin ½ such as ^{1}H and ^{31}P that are relevant here. When these non-zero nuclear

spin nuclei are placed in a magnetic field, two spin states form; one of higher energy and a more abundant lower energy state. NMR is detected by observing the spins moving between these energy states after an electromagnetic pulse. NMR can be used to investigate molecular structure using proton ^{1H}NMR as well as investigate lipid bilayer structure packing with phosphorus ^{31}P NMR.

The result of an NMR experiments is reported as a chemical shift (δ, delta) from a reference compound and is measured in parts per million (ppm). The ^{1}H chemical shift range is approximately 0 to 10 ppm (on a scale from right to left). The ^{31}P chemical shift range is approximately 250 to -250 ppm. ^{1}H nuclei resonate at different chemical shifts as a function of the electron density around them which is dependent on the molecular structure. As the electron density surrounding the nucleus increases, the chemical shift decreases.

^{1H}NMR is used to study bond connectivity which leads to identification and information about the molecular structure. Protons in different chemical environment have characteristic chemical shifts and these known chemical shifts can be used to determine molecular structures. In addition to the chemical shift data, protons near each other display the fine structure because they sense the presence of the neighboring protons through bonding electron pathways for protons less than three bonds away. These close connections can be determined with a Correlated Spectroscopy (COSY) experiment. COSY data allows groups of coupled protons to be identified and then these groups are used to build up network of protons to determine the molecular structure.

^{1H}NMR spectra observed the stability of the protons found in the garcinol molecule were acquired on a 300 MHz 1H NMR spectrometer. Testing was performed on garcinol and the hexane extract. The sample preparation comprised of dissolving 36

mg of garcinol and 36 mg of the hexane extract residue in 1 mL of deuterated methanol and each placed in a 5-millimeter NMR tube. The samples were then placed in the NMR system and results were generated and displayed as an intensity plot.

Phosphorus NMR can be used to assay purity and to assign structures of phosphorus containing compounds. In biological applications, this approach is used to study the lipid bilayer and membranes in various conditions. It can provide information about lipid bilayer packing, phase transitions, and lipid head orientation.

^{31}P-NMR spectra of DPPC liposomes were acquired on an Agilent NMRS 500 NMR spectrometer using 5mm NMRone probe operating at 202.3 MHz. The probe temperature was set at 37 °C for all experiments. Liposome formulations analyzed by NMR were prepared as described in section 3.5 with the exception that 10% deuterated oxide (heavy water, D_2O) in water was used as a solvent in order to provide a deuterium lock signal. NMR data were collected for 60,000 scans with a 35.7 kHz sweep width using 131 K data points. Acquisition time was 1.3 sec with a relaxation delay of 0.5 sec. The data were processed with Mnova program V8.1 Mesterlab research SL. A line broadening of 50 Hz was applied to all spectra. All spectra were indirectly referenced to phosphoric acid (H_3PO_4) set to 0 ppm. Data were acquired without spinning.

3.4 Biological Assay

Antiviral and cytotoxicity evaluation of three selected extracts of *Garcinia kola* (Table 1 and Figure 9) were conducted in the laboratory of Rega Institute for Medical Research at the University of Leuven in Belgium. The tetrazolium-based colorimetric (MTT) assay using MT-4 cells (C8166 cells) were used for the detection

of anti-HIV compounds. The MTT assay measures cell viability (mitochondrial activity), if cells are viable, tetrazolium dye is reduced and undergoes a color change, which can be detected and quantified. These tests were performed against HIV_{-1} (strain III_B) and HIV_{-2} (strain ROD) on MT-4 cells which are Human T cells isolated from a patient with adult T-cell leukemia which supports HIV growth. HIV_{-1} (strain III_B) was provided by the National Institute of Health and HIV_{-2} (strain ROD) was provided by Pasteur Institute, Paris France. The process for the replication of the HIV virus occurs within 5 days. In short, infected cells were cultured, treated, incubated, and evaluated to determine the IC_{50}s and CC_{50}s of the extracted compounds. These tests were carried out in quadruplicate. The 50% inhibitory concentration (IC_{50}) was defined as the inhibitor concentration that reduced the level of the HIV viral expression by 50%. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that inhibited MT-4 cell proliferation by 50%. Cell viability was taken into account relative to controls which were: nevirapine, lamivudine, azidothymidine, and dideoxyinosine, all of which are reverse transcriptase inhibitors (RTi) drugs effective against HIV and are available commercially.

Code	Sample
WOR/001A	1:1 (DMC-MeOH)-Hexane partitioned in Hexane
WOR/003/4	1:1 (DMC-MeOH)-MeOH combined
WOR/003/4 b	MTB extract

Table 1: Antiviral assay samples

3.5 Preparation of liposome formulations

Liposomes are hollow bi-lamellar or multi-lamellar spheres (vesicles) typically comprised of physiologically compatible lipids. These membrane-like structures have

two different phases: at low temperature (before transition temperature) rigidity occurs as a gel-like phase and at higher temperatures (above transition temperature) a liquid crystal phase is formed. These phases are dependent upon how the lipids in the bilayers of the liposomes are arranged.

Liposome preparation was performed using the film rehydration method using garcinol as an active pharmaceutical ingredient (API). The garcinol liposomes vesicles were prepared by dissolving 43.75 mg of DPPC in 3.5 ml of methanol (2 mM lipid concentration). Methanol was removed by rotary evaporation at 50 °C starting at 450 mbar for 30 minutes, then at 300 mbar for 30 minutes and lastly at 200 mbar for 30 minutes. As a side note, the lipids were first rehydrated in 137 mM PBS (pH 7.4) in which garcinol degraded. From this observation, several biological buffers were tested in the presence of garcinol in order to determine garcinol stability in the optimal buffer solution. Results of this stability test are shown in section 4.5.5. Potassium phosphate buffer (PPB) at pH 7.4 was selected as the most stable buffer. The lipid film was then rehydrated with 2 mM garcinol in 50 mM PPB for 30 minutes at 50 °C above the DPPC transition temperature. Dynamic light scattering (DLS) was performed after sonication of the hydrated lipid film to confirm the particle size and its distribution. Finally, the liposome formulation was subject to release studies in order to assess the rate and extent of garcinol release.

3.5.1 Sonication

After the liposomes were make, its particle size was very large. To improve and decrease the size of the liposomes, they were carefully sonicated for 2 minutes at 50% amplitude.

3.5.2 nano-Differential Scanning Calorimetry (nano-DSC)

Differential Scanning Calorimetry (DSC) is used to measure melting temperature, heat of fusion, latent heat of melting, reaction energy and temperature, glass transition temperature, and precipitation energy and temperature, denaturation temperatures, and specific heat or heat capacity. DSC measures the amount of energy absorbed or released by a sample when it is heated or cooled, providing quantitative and qualitative data on endothermic and exothermic processes. DSC also measures the degree of crystallinity as well as the drug release profile which can be used to optimize formulation of suspensions/emulsions and predict long term stability. This is done by the change in the heat that flows through the sample and a reference at a certain temperature is observed as the test temperature changes. An empty aluminum pan serves as the reference sample. Data is measured as a function of temperature, which is typically varied over a period of several minutes

The liposome 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in Figure 11 which is a neutral phospholipid containing two palmitic acid (16-carbon saturated chains) esterified to glycerol with a phosphate group and choline attached at the polar end was used. The phase transition temperature of DPPC from gel to liquid crystal is 42 °C, higher than human body. DPPC is surface active and fervently forms liposomes, with the molecule actively comprising a high compaction capacity as a result of the free rotation and attraction of the palmitoyl tails to each other.

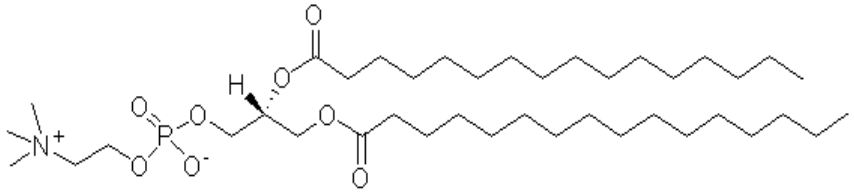


Figure 11: 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (courtesy of Google image)

Analysis of the DPPC liposome was performed using a DSC Q100 from TA Universal Instruments. Here, 300µL of the sample was weighed and placed in an aluminum pan with a cover. The heating rate was 10 °C/min from 0 °C to 180 °C under 40 cm^3/min nitrogen flow. The DSC apparatus was set equilibrated at 20 °C. Run time was roughly 2 hours. Upon the conclusion of the sample run, 1 L of deionized water was used to clean the DSC apparatus to ensure that there were no liposomes left stuck in the apparatus. Data was further assessed on the instruments software.

The same theory discussed in the previous paragraph is applicable to nano-DSC but on a nano scale (nanometers, nm, in size). Nano-DSC was performed using a TA Instruments Nano DSC (New Castle, DE, USA). Samples at a concentration of 0.1 mM lipid were degassed under vacuum for 30 min before loading into a 0.6 mL capillary cell. The cell was then pressurized with nitrogen to 1 atm and equilibrated at 25 °C. The sample was scanned at 1 °C min^{-1} over a range of 25 °C to 60 °C.

3.5.3 Zeta potential

The fundamental states of matter are solids, liquids and gases. A colloidal system occurs when one of the states of matter is finely dispersed in another. This effect is evident in measuring thermal analysis by observing a particles' zeta potential. Zeta potential, Greek letter (ζ), ζ-potential, measures a fluid containing particle (in this case the dispersion of the garcinol liposome) in a colloidal system. It measure the electrical potential difference between the double layer of a particle at the slipping plane with respect to the bulk liquid away from the interface as seen in Figure 12. This measurement provides detailed information about the stability of the colloidal system.

Particles within the dispersion with a zeta potential tends to move toward the electrode of opposite charge with a velocity relative to the amount of zeta potential. This velocity is observed when the particle mobility is captured by the laser which is then converted to zeta potential. A smaller molecule that has a high zeta potential (let's say ±60 mV) means that the molecules in solution resist aggregation (electrically stable) hence exposing charged phosphate groups. However, coagulation occurs at lower zeta potential (±5 mV).

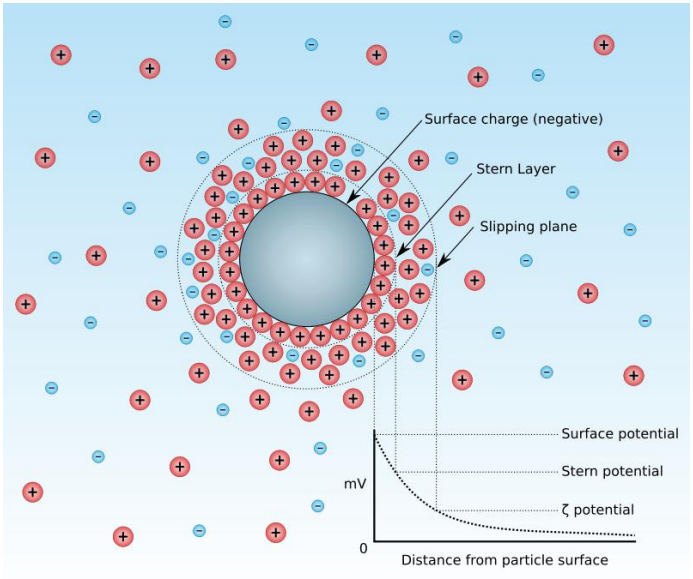


Figure 12: Zeta potential definition (courtesy of Google image)

There is no direct way to measure zeta potentials but there have been various theoretical models and experimental techniques used to calculate it. A known theoretical model used to describe the stability of a colloidal system is the DVLO theory; named after its discoverers Derjaguin, Landau, Verwey and Overbeek. DVLO theory states that the stability of a particle in solution depends on its total potential energy ($V_T = V_S + V_A + V_R$) which consists of the total energy (V_T) produced by the

solvent (V_S), total attractive forces (V_A) and the total repulsive force (V_R). In order to obtain stability in colloidal systems, there should be minimal attractive force and repulsion in order to resist the flocculation of particles. One experimental technique used to estimate zeta potential of a particle in other words measure colloidal stability is electrophoresis.

A small aliquot part of each formulation (17 mM DPPC) was diluted with 137 mM PBS to give a final lipid concentration of 1 mM. Zeta potential values were then determined using a laser doppler procedure with a Malvern Instruments Zetasizer Nano ZS at 25 °C. Air drop interference was eliminated before measuring the zeta potential.

3.5.4 Differential Light Scattering (DLS)

Differential Light Scattering (DLS) determines the size distribution profiles of particles in suspension. This occurs by a light (often a laser) hitting a particle (in this case the garcinol liposome), producing an electronic distortion that is emitted in all directions. An oscillating dipole in the electron cloud results. As the dipole changes, energy is scattered in all directions and a fluctuating (or dynamic) intensity which captures the size of the particle in solution is obtained. Smaller particles undergo Brownian Motion, random motion of particles in a liquid or gas resulting from collision molecules in the gas or liquid. And the distance between scattering changes consistently with time.

DLS was carried out after sonication of the hydrated lipid film to confirm the liposome particle size and its distribution. DLS measurements were performed using a Malvern Instruments Zetasizer Nano ZS with a backscattering detector angle of 173°

and a 4 mW, 633 nm He-Ne laser (Worcestershire, UK). For size distribution studies, 1 ml of the liposome formulations was analyzed in an optical grade polystyrene cuvette at 37 °C. Before analysis, the samples were stored at 37 °C and then evaluated after 24 hours.

3.5.5 Dialysis

During the film rehydration stage of liposome (vesicle) preparation, a certain amount of PPB that was not trapped in the vesicles had dissolved garcinol in it. Consider also that all of the garcinol might not have been entrapped and some may still be present in the untrapped PPB. To overcome these potential barriers, the garcinol liposomes were subjected to dialysis studies in order to remove any untrapped drugs so that only the release of drugs from the vesicles could be accounted for. The dialysis experiment was carried out until a constant drug concentration was obtained and that equilibrium had been achieved in order to ensure no unentrapped garcinol was present in the sample.

Dialysis experiments were conducted at room temperature (25 ± 0.5 °C) using MWCO 3500 kD cellulose membranes for 24 hrs in 50 mM potassium phosphate buffer solution with constant stirring so that any potential unencapsulated garcinol was removed.

3.5.6 Dissolution

Extensively used in the pharmaceutical industry, dissolution studies provide information about drug release for various dosage forms. Dissolution studies were conducted in order to determine the garcinol release profile from the liposome formulation. Dissolution studies are importanin in predicting the bioavailability of

various drug formulations. Dissolution was performed by transferring the dialysis tube into a smaller beaker of 50 mM potassium phosphate buffer (37 ± 0.5 °C) solution at pH of 7.4 with a magnetic stirrer at 75 rpm. As 1 ml buffer samples were collected for analysis of 24 hrs period, the buffer was replaced with new buffer in order to maintain a sink condition. Dissolution samples were collected at serial time points and were analyzed for garcinol content by HPLC in order to quantify release over time.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Liquid-liquid extraction technique results

After the extraction and fractionation of *Garcinia kola* as described in the previous chapter, the samples that, based upon Fuller *et al*., would most likely contain guttiferone F. were analyzed using HPLC using an acetonitrile-water mobile phase. The samples are: methanol extract, MTB extract, Sephadex fraction 3/4 and three other samples were also analyzed by HPLC: the hexane extract; the ethyl acetate extract; and chloroform extract. These samples were noted as solvent-solvent (S-S). The results obtained from the HPLC analyses are represented in the HPLC chromatogram displayed in Figure 13.

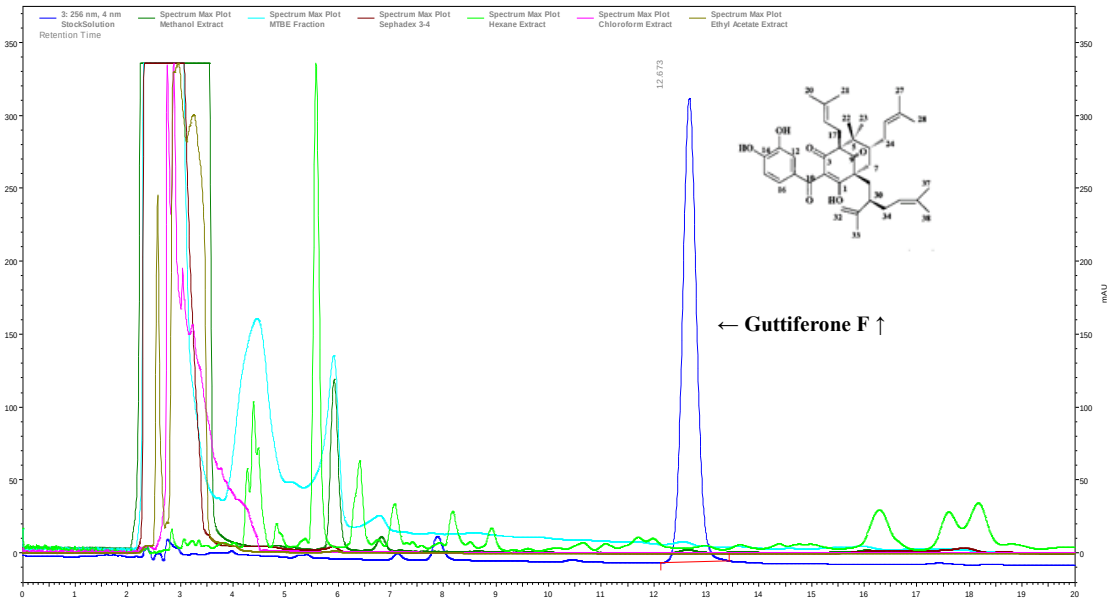


Figure 13: HPLC chromatogram overlaid of each solvent-solvent extraction sample (methanol, MTB, Sephadex 3/4, hexane, ethyl acetate, chloroform extracts) and the injection of the Garcinol standard using the 80:20 (w/w) acetonitrile-water mobile phase.

Results in Figure 13 shows the S-S samples were overlaid and compared to

that of the garcinol standard peak. The results indicate that of the majority of the analytes in the S-S extracted samples had a retention time before 9 minutes. The results also indicate that none of the other extracts contained detectable analytes with the same retention time as that of garcinol, which has a retention time roughly 12.5 minutes. This suggests that the antiviral compound of interest, garcinol was either not present in any of the S-S extracts or was present at a concentration below the limits of detection of the HPLC assay.

4.2 Supercritical fluid extraction (SFE) results

The goal of the SFE technique was to search for guttiferone F. via 100% CO_2, 5%, 10%, 30% modifier extractions which included methanol, ethanol, and isopropanol, and analyze via UPC^2 coupled with Q-Tof-MS. The second goal was to perform principle component analysis (PCA) of the SFE and S-S extracts in order to find correlations to streamline sample prep approach, exploit TransOmics workflows to aid visualization of data, targeted processing for garcinol, and comprehensive processing for feature differences found in the next section.

Results shown in Figure 14 are of the HPLC overlaid chromatograms for each of the SFE extractions and are compared to an injection of garcinol standard. Synonymous to the previous section, results indicate none of the extracts contained detectable analytes with the same retention time as that of garcinol, which has a retention time of roughly 12.5 minutes. This also suggest that the antiviral compound of interest, garcinol was either not present in any of the SFE extracts or was present at a concentration below the limits of detection of the HPLC assay.

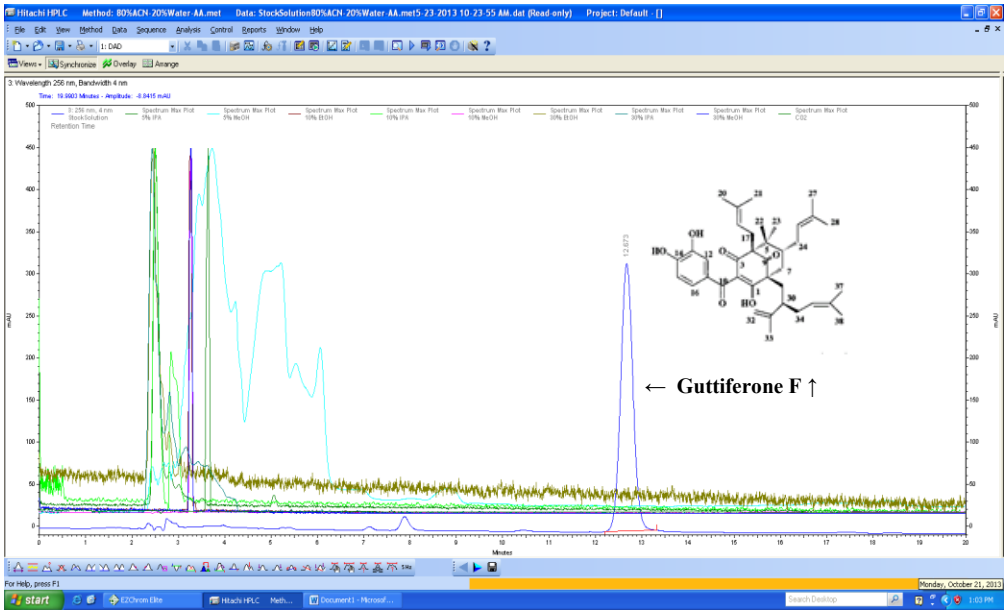


Figure 14: HPLC overlaid chromatograms of the SFE extractions and the injection of the Garcinol standard.

4.3 Comparison of both extraction techniques

After compiling the HPLC overlay as in Figure 13 and Figure 14, it was determined that the compound Garcinol was not detected in any of the extracts. In an effort to maximize our understanding of the various extraction methods employed, comparisons were performed such to determine the amount of coverage for each extraction technique by their mass. Questions regarding types of analytes and variations between the extractions became objectives in determining an appropriate guidance for future studies and protocols for researchers performing natural product profiling. Data was obtained on the UPC^2 coupled to Q-Tof MS by negative electrospray mode. Plots were constructed in order to observe trends related to components in the S-S extracts and the SFE extracts. Statistical analysis techniques were used to determine differentiation between techniques by utilizing principle component analysis (PCA). PCA measures statistical analysis of variability in the data

set in order to make understanding and find a correlation. PCA and quadrant assignment afford an opportunity to understand and appreciate the composition similarities between fractions and extractions techniques. The results, shown in Figure 15 are split up into four quadrants denoted as 1, 2, 3 and 4. Each quadrant indicates if the sample components are similar and associated near each other, or their extracted masses were found to be within close proximity of each other and or overlap. An analysis of the samples in both S-S and SFE techniques is summarized in Figure 15. The components derived from the Sephadex fraction 3/4, MTB, and chloroform are associated with quadrant 2, while the most of the SFE as well as hexane and ethyl acetate components are nearly overlapping in quadrant 3 of the trend plot. Implying that similar mass were extracted being that they are in proximity to each other. The one extraction profile that was observed significantly different the rest of the extraction groups was the 5% EtOH results, which is the outlier located on the bottom right.

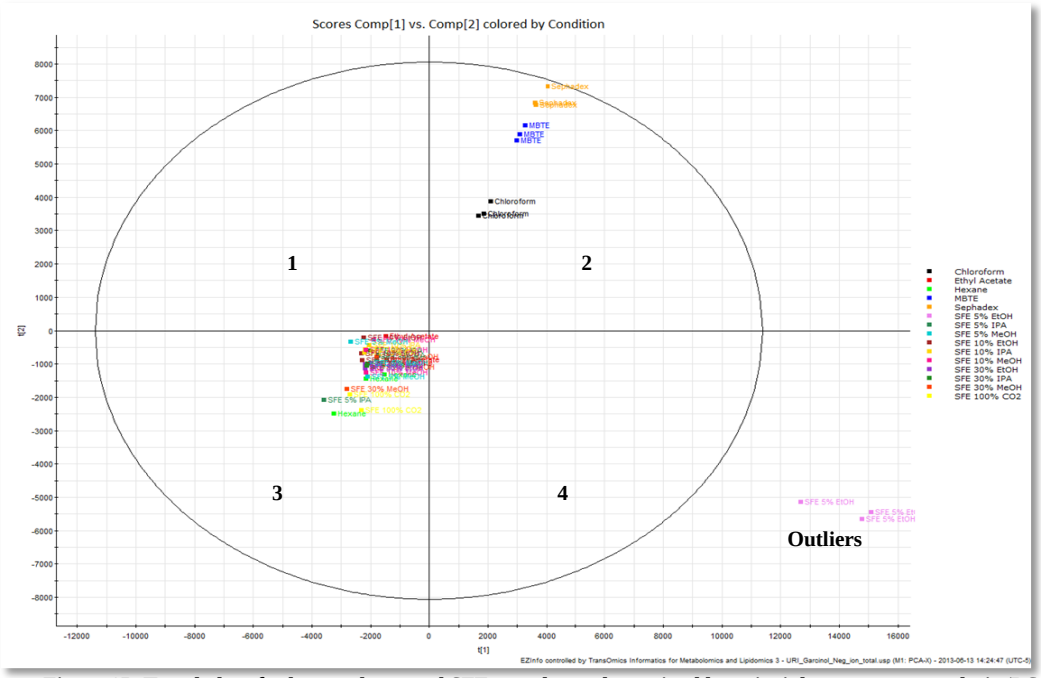


Figure 15: Trend plot of solvent-solvent and SFE samples as determined by principle component analysis (PCA)

In order to look more in detail at the SFE fraction, quantitative analysis was performed using mass spectroscopy. Figure 16 displays the results are of the TOIML dendrogram and abundance plot which shows an example of how a single feature of the quantitative data which was examined and aided justification for consideration to statistically group the S-S and or SFE. The top of the plot shows the different samples of both extraction techniques. As observed, there are three different replicates within the SFE 5% EtOH (left, indicated by □) and the Sephadex 3/4 (right, also indicated by □). Implying that for this single spectrum feature at retention time 1.05 minutes and m/z=499.2848, was grouped as being found in both the SFE (5% EtOH) and S-S (Sephadex fraction 3/4) techniques

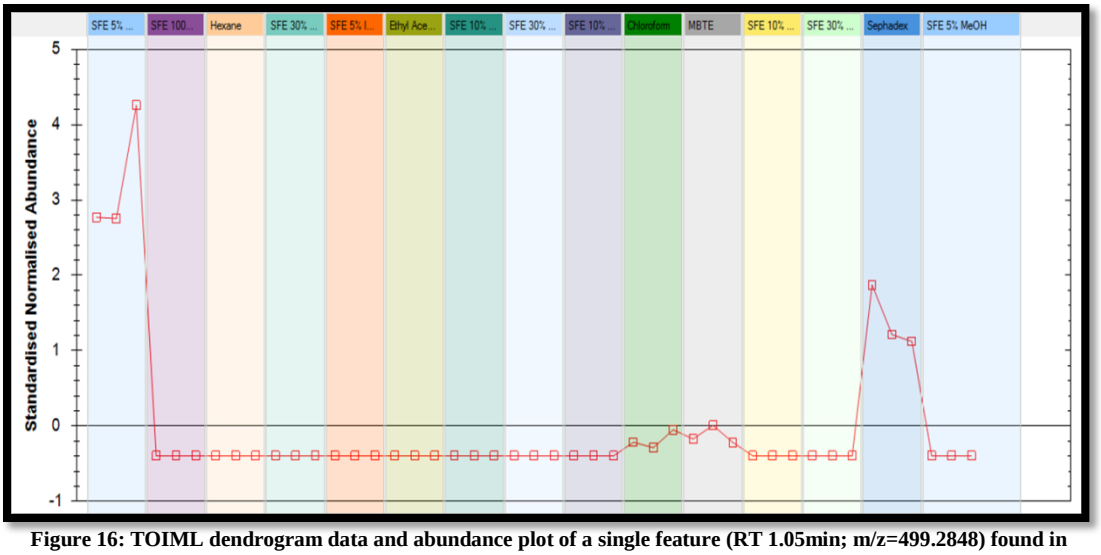
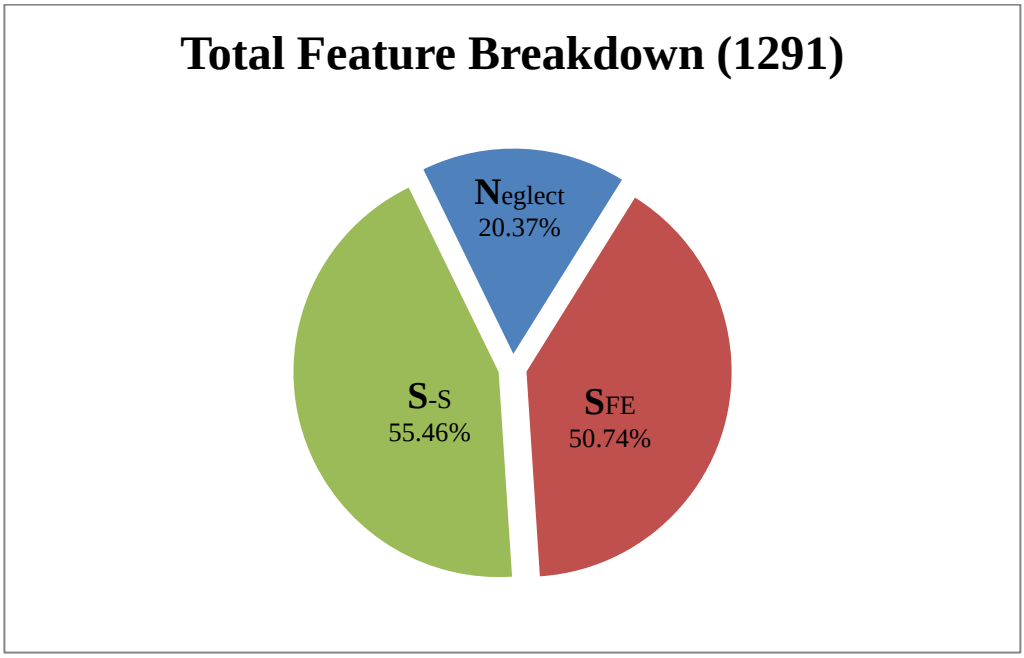


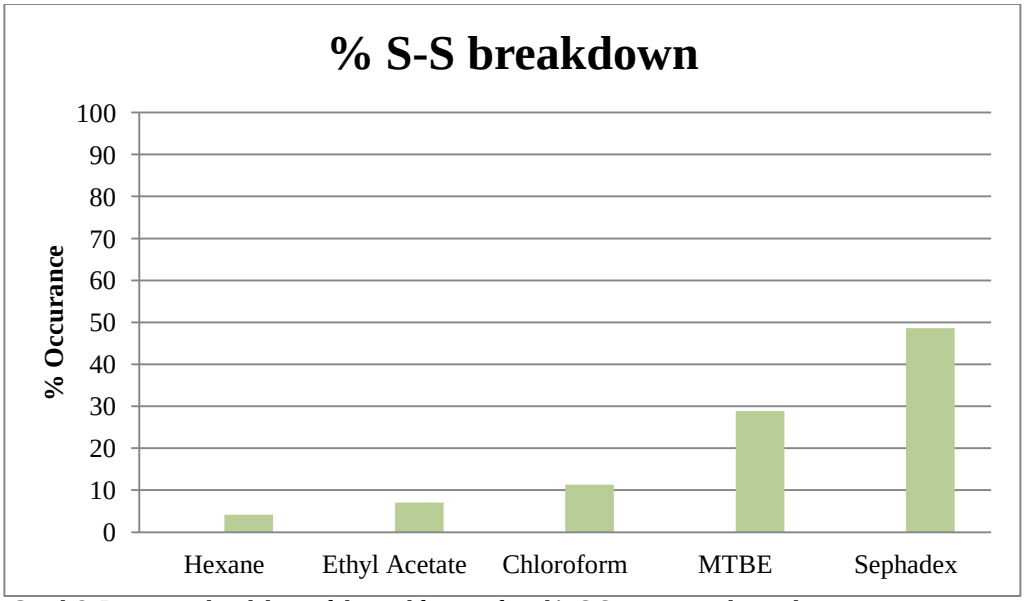
Figure 16: TOIML dendrogram data and abundance plot of a single feature (RT 1.05min; m/z=499.2848) found in both 5% EtOH (left) and Sephadex fraction 2 (right) extractions.

(see Graph 1, 2 and 3). This grouping is termed chemical features. A total of 1291 individual chemical features were observed in the entire S-S and SFE samples (Figure 17, in percent), analyzed and placed into the following three categories: SFE, S-S and neglect. Neglect represents neither of the S-S and or the SFE samples were observed with a mass standardized normalized abundance (items under consideration) of zero

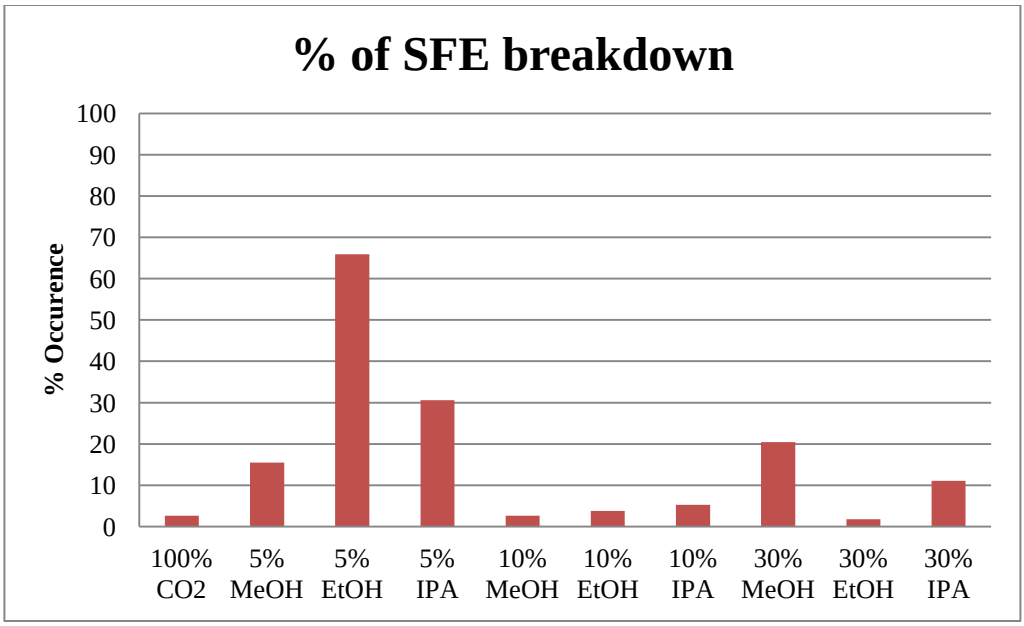
and or below zero.



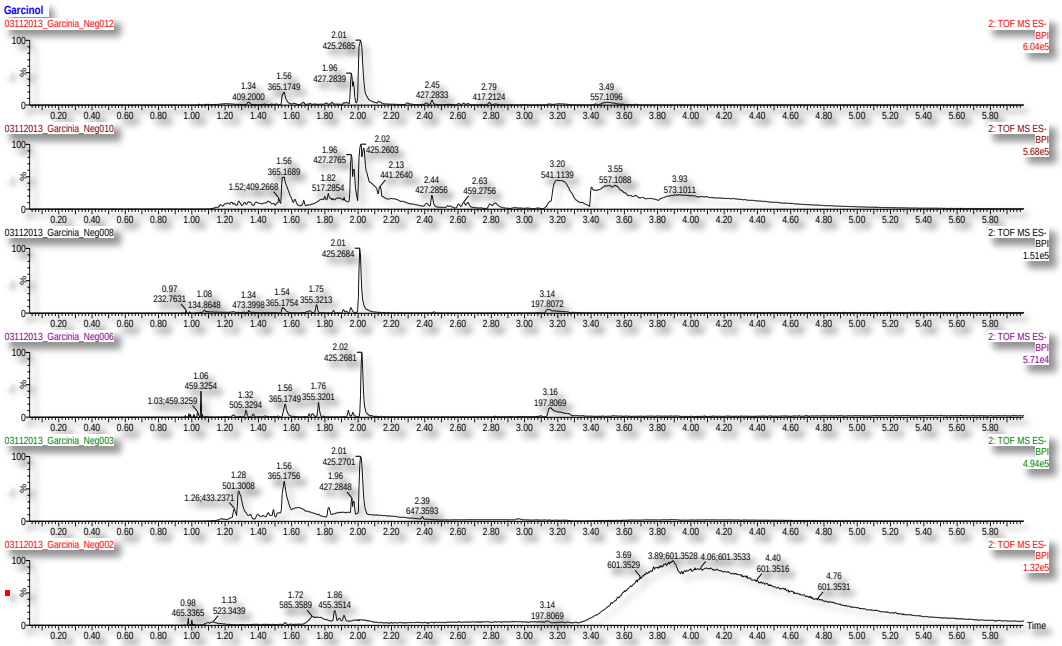
Graph 1: Breakdown of the total features found in solvent-solvent (S-S) and Supercritical fluid extraction (SFE) determined by a normalized abundance .1% based on the TOIML dendrogram data set



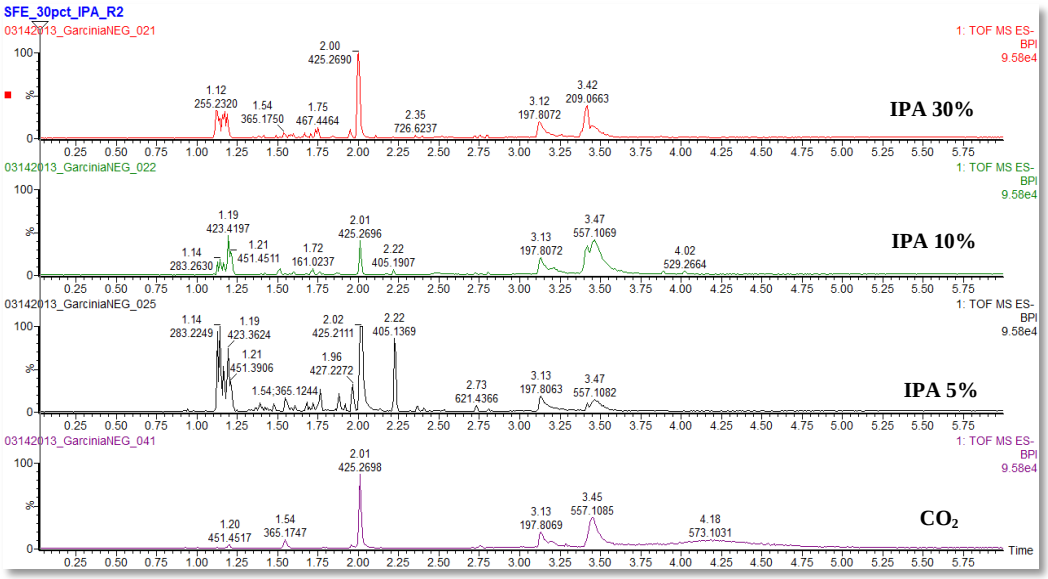
Graph 2: Percentage breakdown of the total features found in S-S extracts as observed.



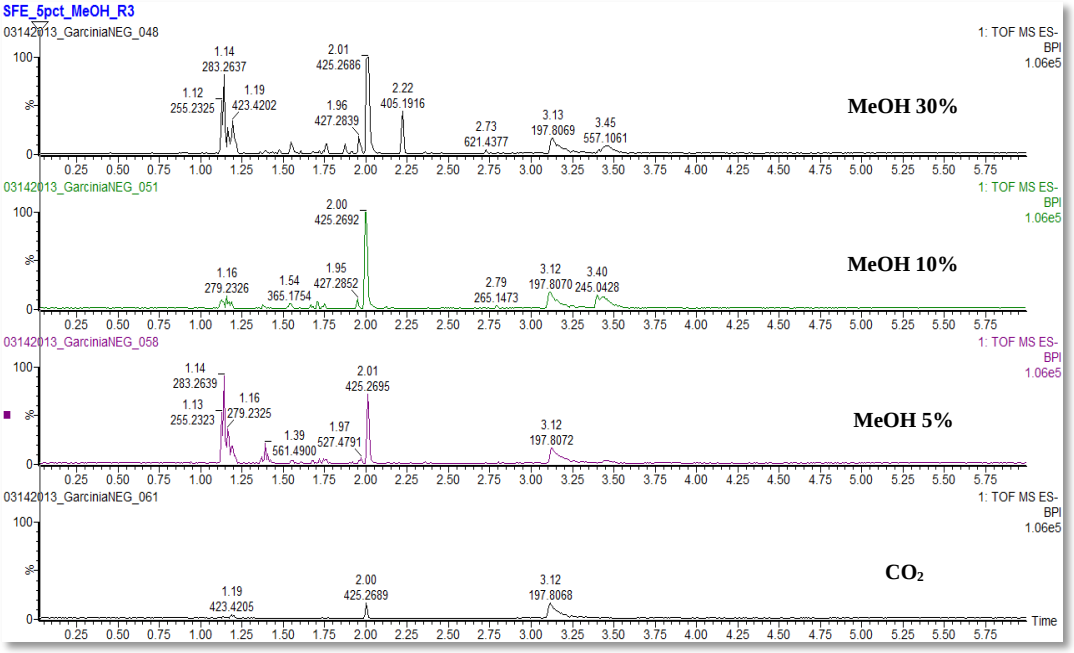
Graph 3: Percentage breakdown of the total features found in SFE extracts as observed.



Graph 4: Garcinol (bottom) in comparison to other solvent-solvent extracts (chloroform, Sephadex 3/4, hexane, MTB, and ethyl acetate, respectfully. Results indicate no presence of garcinol in any S-S fractions.



Graph 5: UPC^2/MS^E ES$^-$ BPI MS traces of the IPA (30%, 10%, and 5%) SFE and 100% CO_2 extractions from the top to the bottom. Garcinol was not observed to be present in these or any of the other SFE extractions with MeOH and EtOH



Graph 6: UPC^2/MS^E ES$^-$ BPI MS traces of the Methanol (30%, 10%, and 5%) SFE and 100% CO_2 extractions from the top to the bottom. Garcinol was not observed to be present in these or any of the other SFE extractions with IPA and EtOH

The results of the 1291 features in Graph 1, 20.67% are neglected. Of the remaining detectable compounds indicated, 50.74% of the extracted compounds can be extracted

by SFE technique and 55.46% can be extracted by S-S technique indicating both extraction techniques nearly extract the same amount of compounds/masses. Of the 55.46% extracted by S-S, majority was extracted using the Sephadex fraction, as shown in Graph 2. Likewise, of the 50.74% extracted by SFE, the majority was extracted by the use of 5% EtOH modifier, as shown in Graph 3. In addition, 26.6% of the abundant features are extracted by both extraction techniques (not shown). Results for garcinol standard indicate a broad UPC^2 chromatography peak eluting at 4.0 minutes. Mass accuracy of the garcinol standard by ESI(-) was measured to be 1.3 ppm. Results of the S-S and SFE techniques indicated no presence of garcinol in any extract when based on ESI(-) located in Graph 4-6. The SFE fraction of EtOH was not shown for simplicity purposes; it too, indicated no presence of Garcinol based on ESI(-). Garcinol was not found in *Garcinia kola* when following the extraction procedure described in the Fuller *et al.* publication. However, common ESI(-) m/z components found in all extracts in Graph 4 to 6 were m/z=425.26 and m/z=365.17. The observation of these common components could further provide a more insight into the extracts within *Garcinia kola*.

Next we examined garcinol at high and low collision energy as well as molecular fragmentation of garcinol. Figure 17 and Figure 18 represents that data.

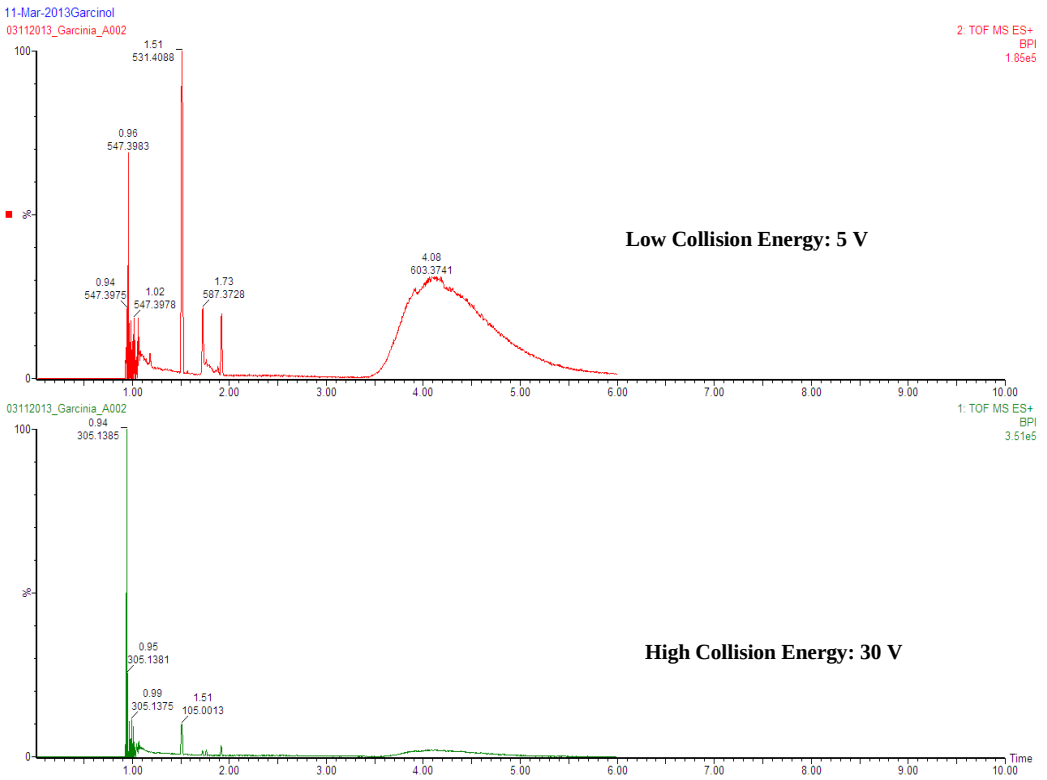


Figure 17: Garcinol MS^E data at high and low collision energy with BPI trace.

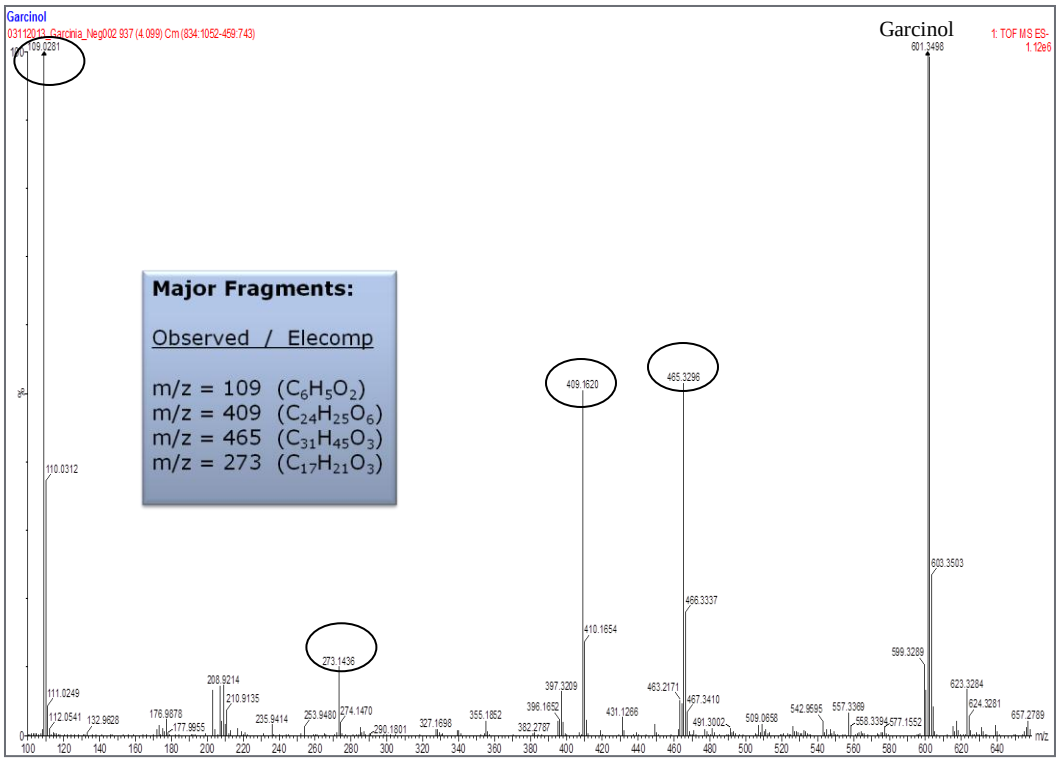


Figure 18: MS^E precursor and product ion data: major fragment used to confirm the presence of Garcinol.

The purposes of these experiments were to detriment the parent ion information at low CE and then get fragmentation data from the high CE. From the fragmentation data, we can use the spectra to look for common fragment ions in the spectra of other detected peaks. The four major fragments of garcinol are: m/z109, m/z 409, m/z 465, and m/z 273. Although not shown, UPC^2/MS^E ES-BPI MS trace of garcinol standard confirms a mass accuracy for the elemental composition of Garcinol ($C_{38}H_{49}O_6$). Retention time observed to be 4 minutes (Figure 17). The four major fragment ions (Figure 18) were used to confirm if garcinol presence were found in the extracted samples all of which came back negative.

4.4 Biological Study

The results for the cell-based, MTT anti-viral assay are listed in Table 2. The three *Garcinia kola* extracted samples tested were completely inactive against both HIV-1 (strain III_B) and HIV-2 (strain ROD). The extracted samples have a high cytotoxicity CC_{50} values >60 µg/ml as compared to the control lower CC_{50} values >20 µg/ml. These data are consistent with the HPLC and MS data which also suggest that garcinol is not present in any of the extracted compounds (S-S and or SFE); if indeed garcinol is an active in vitro antiviral agent.

Code	Strain	Exp_nr		IC_{50}(µg/ml)		CC_{50}(µg/ml)		SI	Max Prot(%)	Appr.	av.IC_{50}(µg/ml)	SD	av.CC_{50}(µg/ml)	SD	SI
WOR/001A	III_B	P3.5554	>	67	=	67	<	1	5	1					
		P3.5556	>	59.7	=	59.7	<	1	1	1	>60.55		60.55	5.01	<1
	ROD	P3.5555	>	54.8	=	54.8	<	1	1	1					
		P3.5557	>	60.7	=	60.7	<	1	1	1	>60.55		60.55	5.01	<1
WOR/003/4	III_B	P3.5554	>	125	>	125	X	1	1	1					
		P3.5556	>	164	=	**164**	<	1	0	1	>168.00		168.00	5.66	<1
	ROD	P3.5555	>	125	>	125	X	1	1	1					
		P3.5557	>	172	=	**172**	<	1	15	1	>168.00		168.00	5.66	<1
WOR/003/4B	III_B	P3.5554	>	125	>	125	X	1	6	1					
		P3.5556	>	138	=	**138**	<	1	6	1	>136.00		136.00	2.83	<1
	ROD	P3.5555	>	125	>	125	X	1	7	1					
		P3.5557	>	134	=	**134**	<	1	10	1	>136.00		136.00	2.83	<1
BOE/BIRG587	III_B	P3.5544	=	0.027	>	4	>	148	121	1					
= Nevirapine		P3.5546	=	0.0242	>	4	>	165	136	1					
		P3.5549	=	0.028	>	4	>	143	119	1					
		P3.5551	=	0.0297	>	4	>	135	112	1	0.027	0.002	>4.00		>147
	ROD		>	4	>	4	X	1	0	1	>4.00		>4.00		X1
CLO/3TC	III_B	P3.5546	=	0.423	>	20	>	47	93	1					
=Lamivudine		P3.5551	=	1.36	>	20	>	15	81	1	0.89	0.66	>20.00		>22
	ROD	P3.5547	=	1.75	>	20	>	11	96	1					
		P3.5552	=	5.37	>	20	>	4	97	1	3.56	2.56	>20.00		>6
DDN/AZT	III_B	P3.5544	=	0.00193	>	25	>	12948	90	1					
=azidothymidine,		P3.5546	=	0.00165	>	25	>	15110	88	1					
zidovudine,		P3.5549	=	0.00193	>	25	>	12979	80	1					
retrovir©		P3.5551	=	0.00189	>	25	>	13242	88	1	0.0019	0.0001	>25.00		>13514
	ROD	P3.5545	=	0.00187	>	25	>	13386	94	1					
		P3.5547	=	0.00162	>	25	>	15479	97	1					
		P3.5550	=	0.00132	>	25	>	18955	91	1					
		P3.5552	=	0.00168	>	25	>	14857	91	1	0.0016	0.0002	>25.00		>15408
DDN/DDI	III_B	P3.5544	=	2	>	50	>	25	104	1					
= dideoxyinosine,		P3.5549	=	3.12	>	50	>	16	115	1	2.56	0.79	>50.00		>20
didanosine	ROD	P3.5545	=	1.92	>	50	>	26	100	1					
		P3.5550	=	2.62	>	50	>	19	116	1	2.27	0.49	>50.00		>22

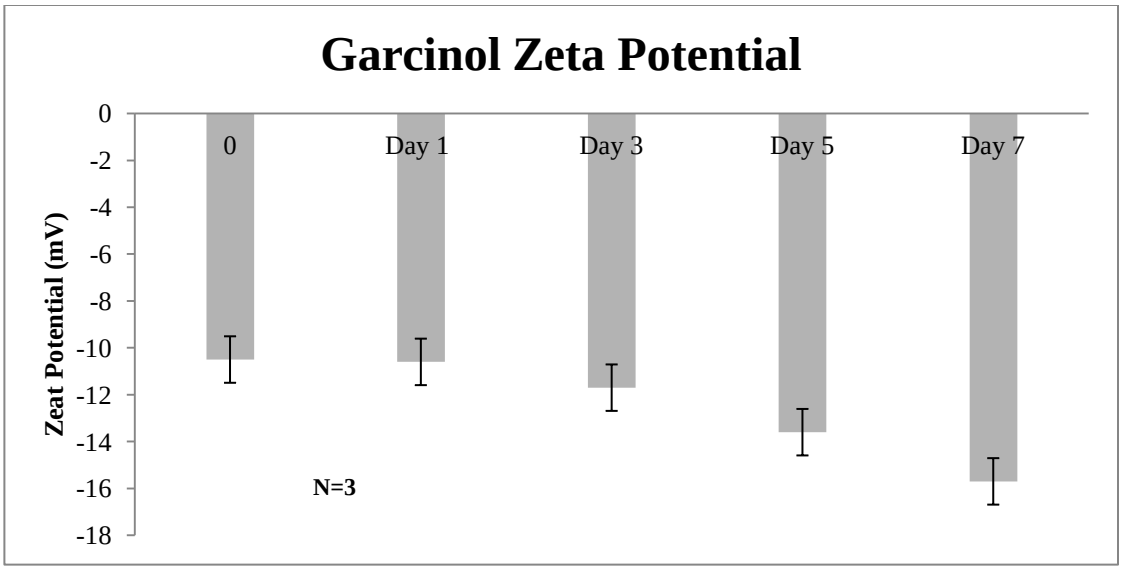
Table 2: Antiviral study of *Garcinia kola* extracts (1:1 methylene chloride/methanol-methanol, 1:1 methylene chloride/methanol-hexane, and MTB) versus controls (Nevirapine, Lamivudine, Azidothymidine, and Dideoxyinosine)

4.5 Garcinol formulaiton study

The following are the results represent the antiviral formulation studying where Garcinol acts as the active pharmaceutical ingredient.

4.5.1 Zeta potential

The surface charge was measured through Zeta potential measurements and the results predicting the stability of the colloidal system below in Graph 7.



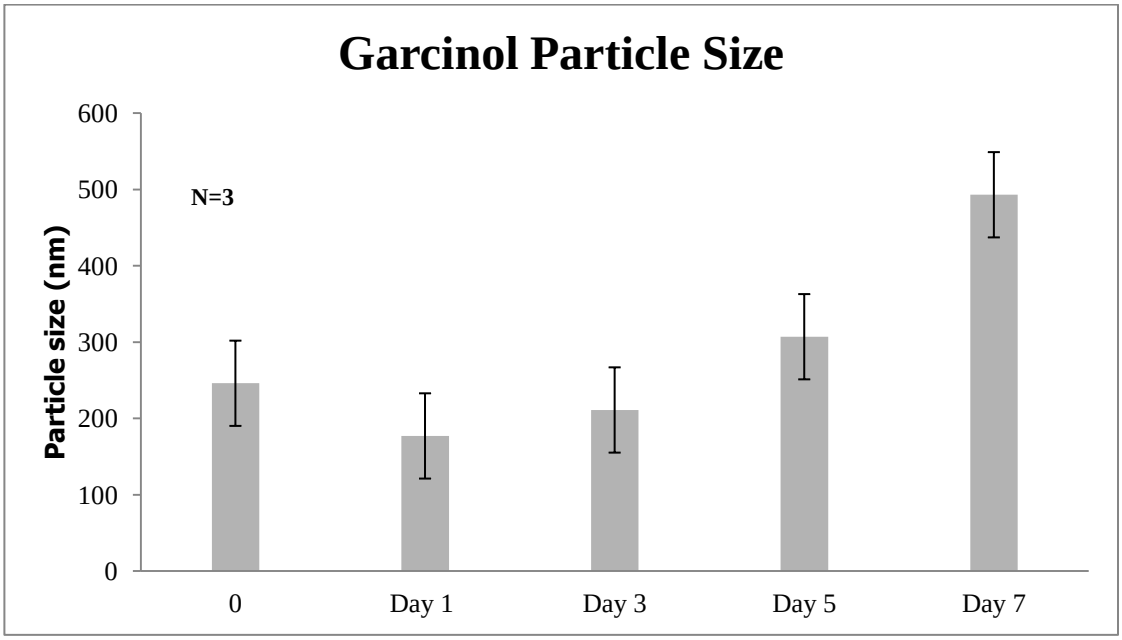
Graph 7: The results of surface charge through Zeta potential measurement

Zeta potential measurement provides detailed information about the cause of dispersion, aggregation or flocculation which can be used to improve the formulation of dispersions, emulsions and or suspensions. Results indicate a fairly consistent and, as compared to bare DPPC liposomes, negative surface charge for seven days in storage. The negative charge indicates that more phosphate head groups are exposed on the surface of the liposome as compared to that of the blank DPPC liposome (not shown), with a Zeta potential at -0.5mV. This reveals that garcinol may strongly interact with the phosphate head groups of DPPC, perhaps exposing the negatively charged phosphate groups to the surface and an average of -14 mV for day 3 to 7 indicating an incipient instability of the DPPC-containing liposomes.

4.5.2 Differential light scattering (DLS) results

DLS was used as a non-destructive method to characterize the size and size distribution of the garcinol liposomes in solution, in this case a biological buffer, (potassium phosphate buffer, pH 7.4). The results from DLS analysis of particle size are shown in Graph 8. The results suggest that Brownian motion (diffusion constant)

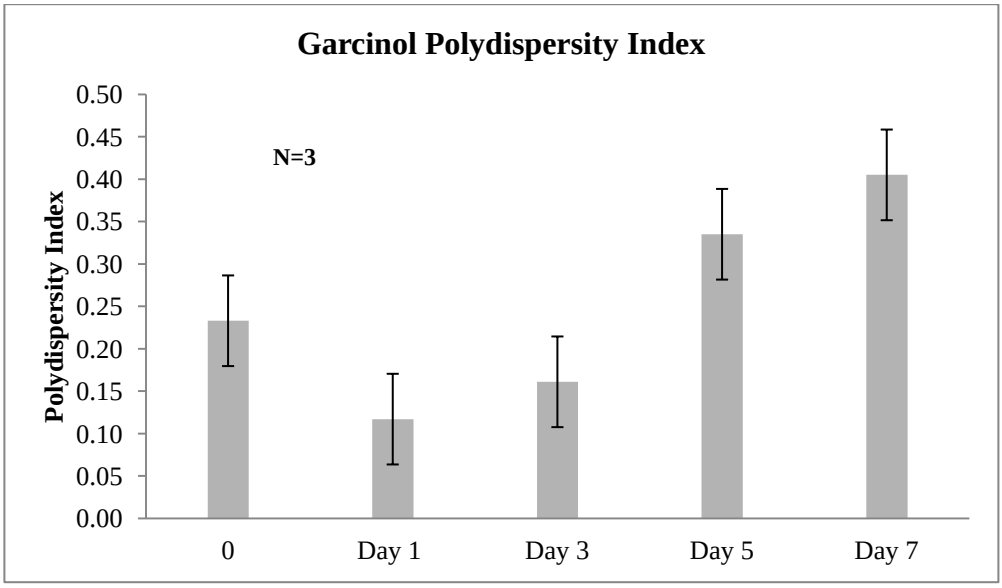
of the liposomes in solution causes the laser to scatter at different intensities. The intensity fluctuation produces the velocity of the Brownian motion resulting in the calculation of the particle size which is related to the hydrodynamic radius. Results indicate an increase in the liposome size over seven days. Results also indicate garcinol liposome is stable the first three days. At day five, aggregation starts to occur.



Graph 8: Garcinol particle size results

Next, the result in Graph 9 summarizes the Polydispersity index (PDI) of the garcinol liposomes. PDI takes into account the difference in size of the garcinol liposomes in solution, or distribution profile. The average PDI of the garcinol liposomes over a period of seven days was 0.024. There was also an increase in PDI over the seven days. The PDI results are consistent with the particle size data suggesting stability is enhanced the first three days at lower PDI. This lower PDI value means that there was a relatively small particle size distribution and

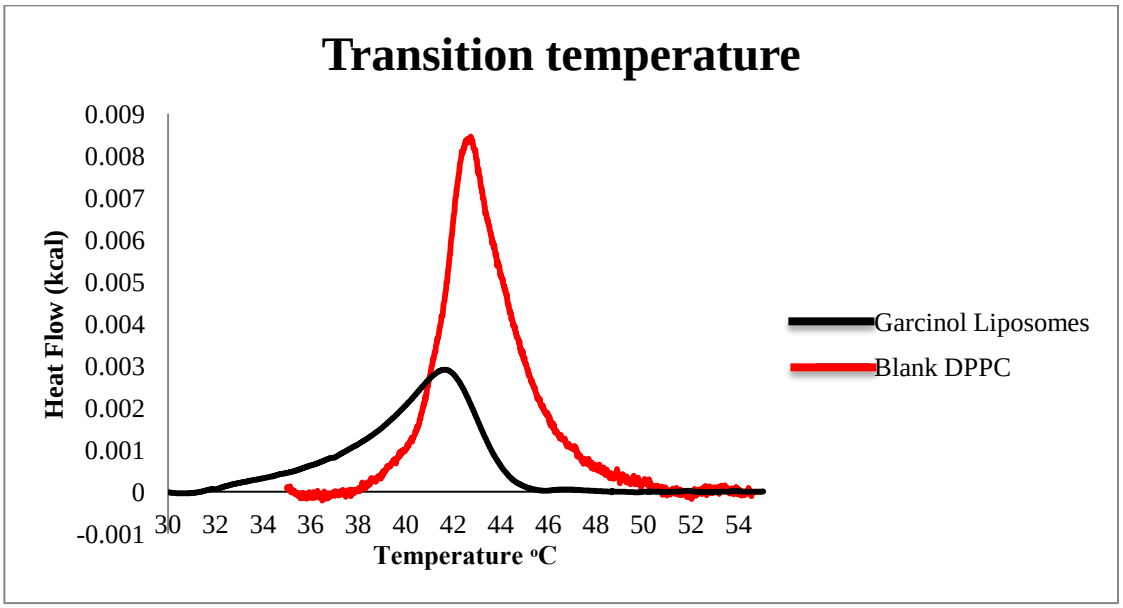
homogenous liposomal formulations and that the liposomes in solution were relatively stable over the course of the experiment.



Graph 9: Garcinol Polydispersity index results

4.5.3 Nano-Differential Scanning Calorimetry (nano-DSC) results

DSC measures the transition temperature of the polymer in comparison to the polymer with garcinol. Results from the nano-DSC study are in Graph 10.



Graph 10: Results of phase transitions through DSC measurements of blank DPPC liposomes and DPPC garcinol liposomes

Results from Graph 10 indicate an ideal exothermic reaction with the blank DPPC. An important characteristic of transition temperature depends on molecular weight. The transition temperature of DPPC is 43.3 $^{\circ}$C and molecular weight is 734.039 g/mol whereas the molecular weight of garcinol is 602.8 g/mol. Because of this, results show a slight polymer degradation with a lower transition temperature (41.6°C) when garcinol is added to the DPPC liposomes. A slightly higher polymer being combined with a smaller molecular weight API, leads to a lower transition temperature for the garcinol formulation.

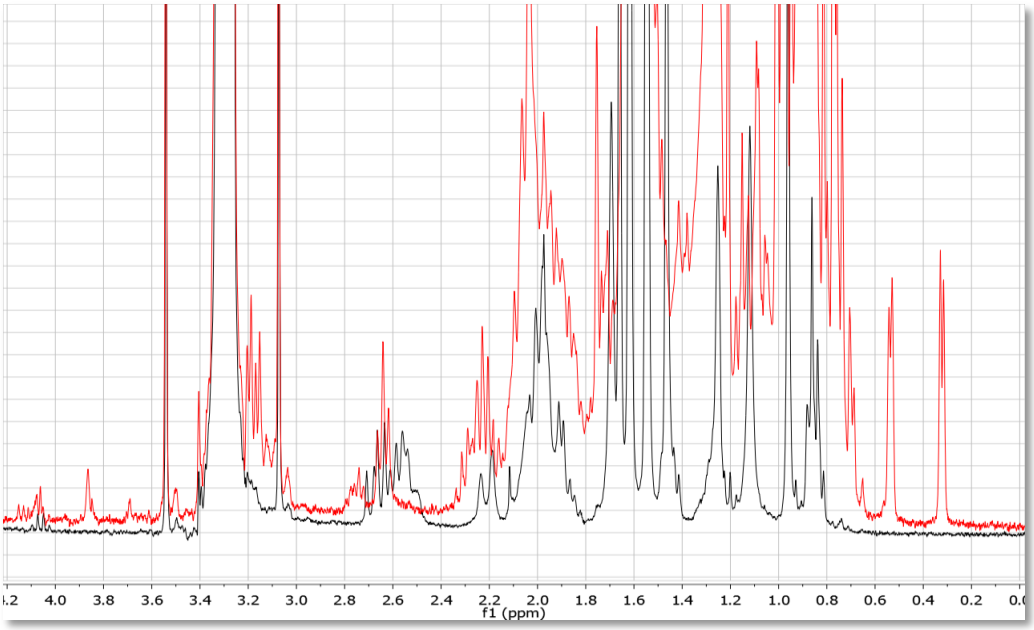
4.5.4 Nuclear magnetic resonance (^{1H}NMR and ^{31}P-NMR) results

The results in Graph 11 shows a proton NMR spectrum of Garcinol and Hexane, overlaid. This ^{1H}NMR spectrum was conducted in order to determine the structure of both Garcinol and hexane extract as well as to see if they overlap. Results indicate that although there are areas of the same hydrogen 1 nuclei within both extracts, the structures does not overlap so we can conclude that garcinol is not found within the hexane extract. A complete chemical shift analysis of garcinol as compared to that of guttiferone F as stated in Fuller *et al*, shows that both compounds are relatively the same compounds called by different names as in Table 3.

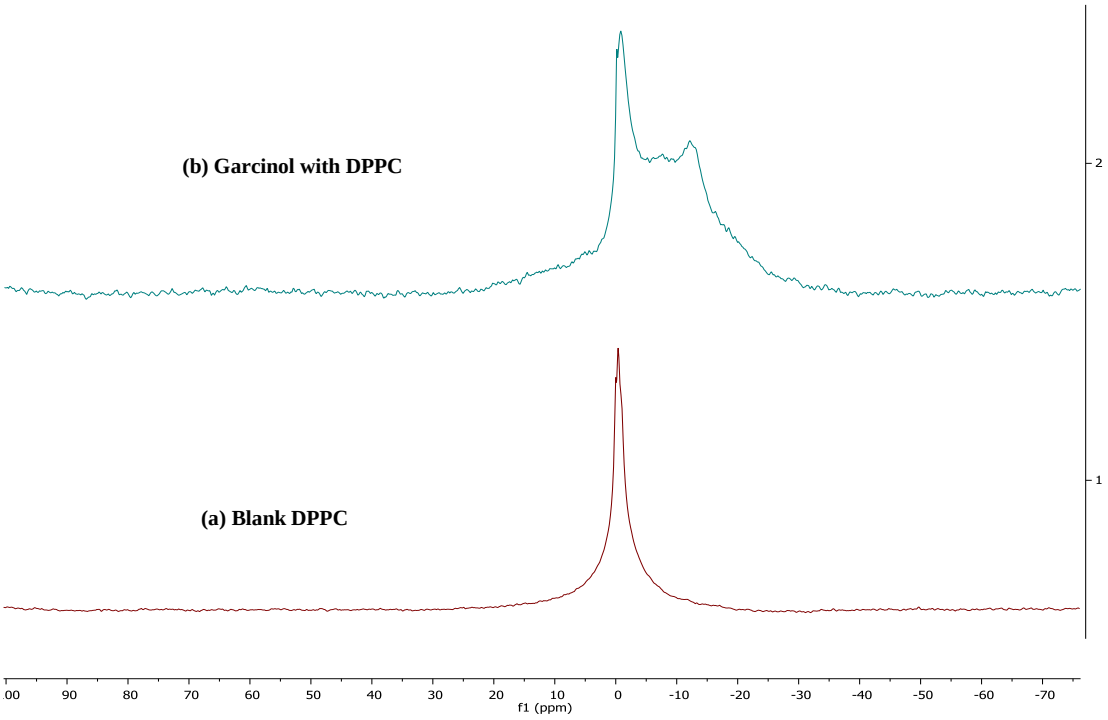
Atom	Chemical Shift (Δ)	Chemical Shift (Δ)	
Position	**Guttiferone F**	**Garcinol**	**Difference**
6	1.49	1.46	0.03
7	2.24	2.22	0.02
7	2.04	2.00	0.04
12	7.19	7.16	0.03
15	6.68	6.57	0.11
16	6.96	6.94	0.02
17	2.71	2.68	0.03
18	5.03	5.00	0.03
20	1.73	1.70	0.03
21	1.69	1.66	0.03
22	1.15	1.12	0.03
23	0.99	0.96	0.03
24	2.02	2.03	-0.01
25	4.87	4.83	0.04
27	1.65	1.62	0.03
28	1.49	1.47	0.02
32	4.45	4.43	0.02
33	1.58	1.55	0.03
34	2.01	2.00	0.01
35	5.03	5.00	0.03
37	1.65	1.62	0.03
38	1.57	1.55	0.02

Table 3: ^{1H}NMR chemical shift comparison of Garcinol and *Fuller et al*. 1999 Guttiferone F

The results in Graph 12 show a shielding of the phosphate nuclei of the garcinol liposome phospholipids, causing a slight change in transition temperature with a large tail. Based upon the NMR data, the orientation of the blank liposome phosphate head groups are saturated within the bilayer of the liposome, whereas the split NMR peak suggests orientation at the surface of the bilayer. With these interactions, results remain consistent with Zeta potential and DSC showing an issue with stability of the formulation.



Graph 11: ^{1H}NMR spectrum of garcinol in red and hexane extract in black.



Graph 12: Phosphorus NMR results of: (a) blank DPPC liposomes versus (b) garcinol DPPC liposome

4.5.5 Formulation study

In order to select the appropriate biological buffer for producing DPPC

liposomes wherein garcinol would be stable, an HPLC stability study was conducted using garcinol in the presence of various biological buffers. This was conducted in order to select the best, non degrading buffer solution that garcinol would be stable in. The biological buffers used were: 137 mM phosphate buffer saline (PBS); 0.9% sodium chloride (NaCl); 5 mM sodium phosphate; 5 mM sodium chloride; 5 mM Tris (hydroxymethyl aminomethane); 5 mM sodium citrate; and 50 mM potassium phosphate (PPB) all at pH=7.4 while using the previously developed acetonitrile:water isocratic HPLC method for analysis. The HPLC chromatograms for each buffer system were as shown in Figure 19 to Figure 30.

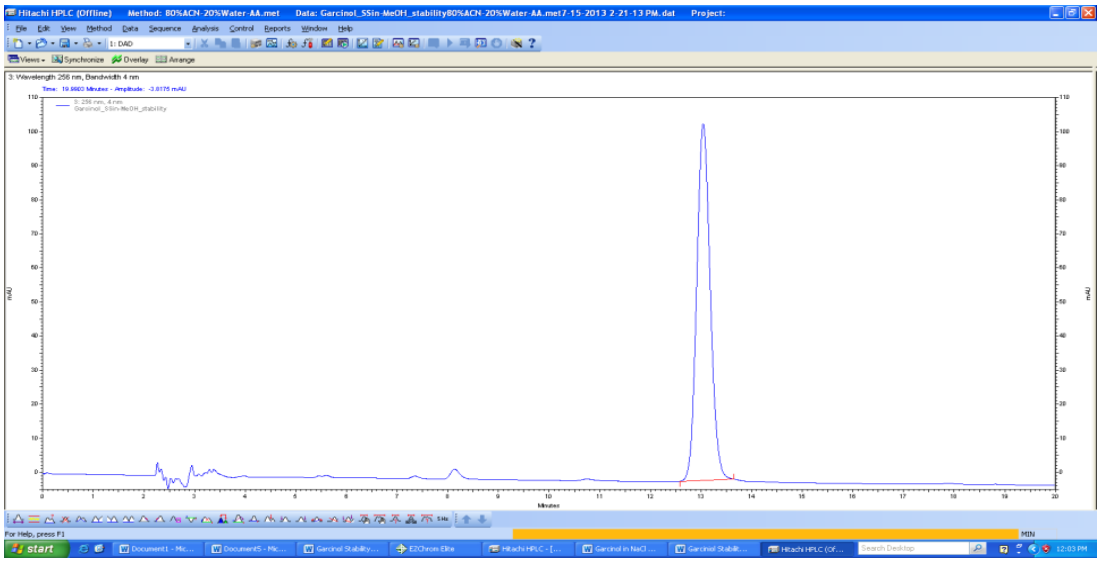


Figure 19: 137 mM Phosphate buffer solution initial.

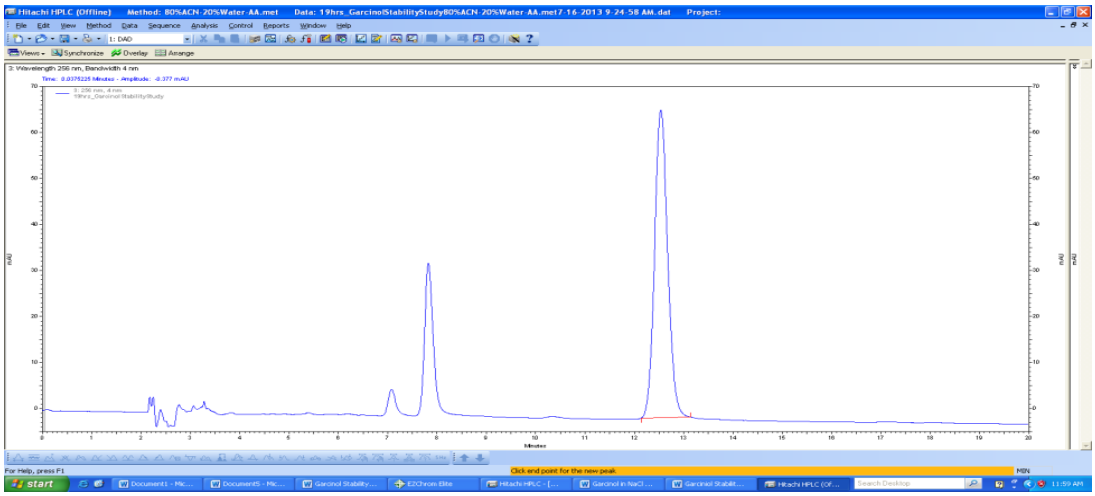


Figure 20: 137 mM Phosphate buffer solution 19 hours.

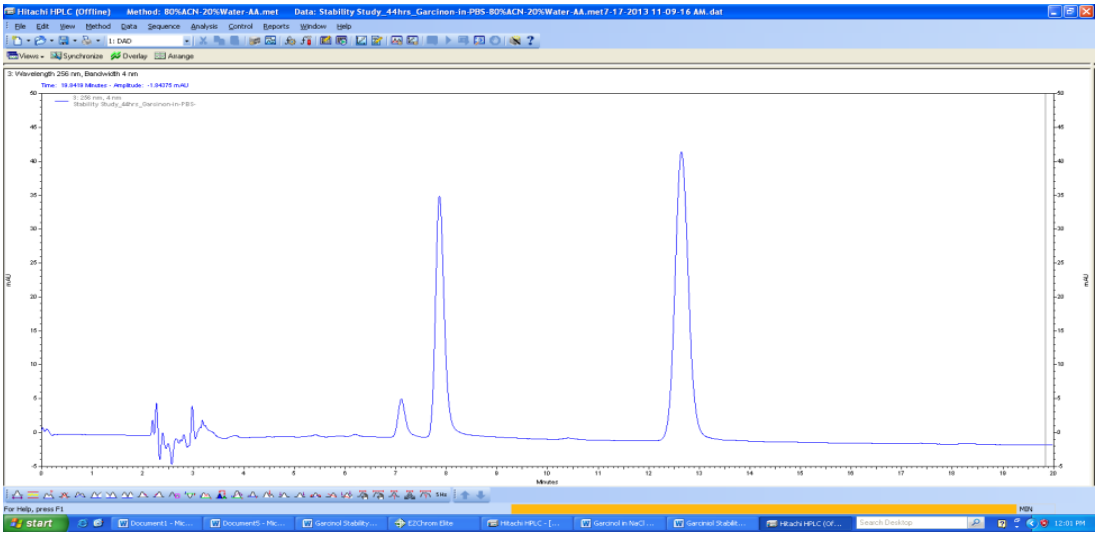


Figure 21: 137 mM Phosphate buffer solution 44 hours.

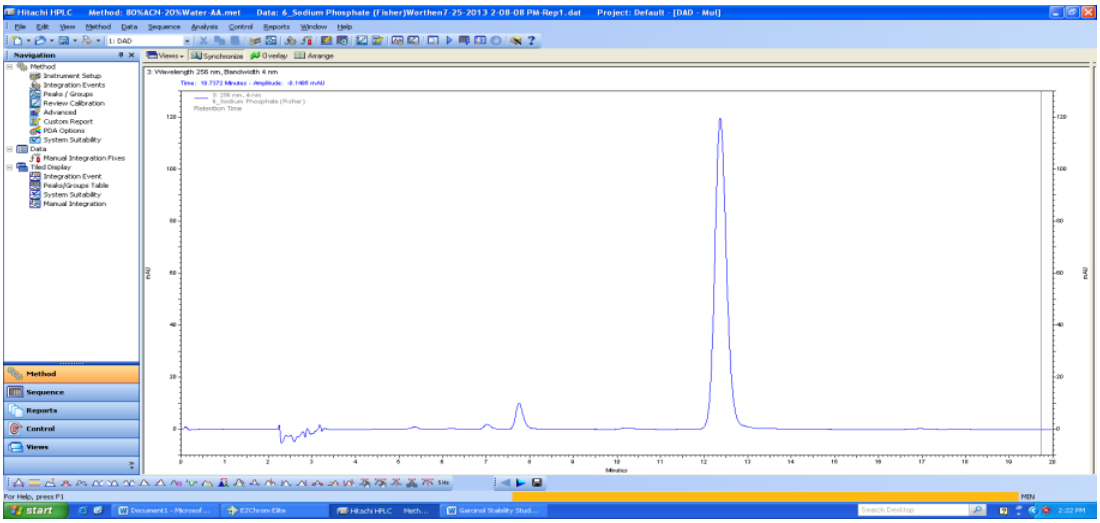


Figure 22: 5mM Sodium chloride initial.

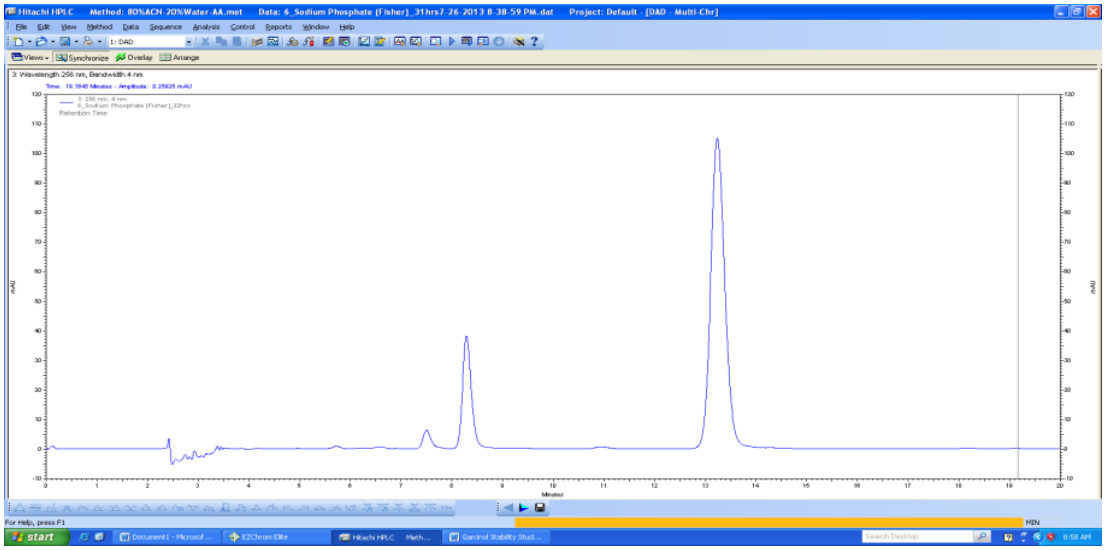


Figure 23: 5 mM Sodium chloride 31 hours.

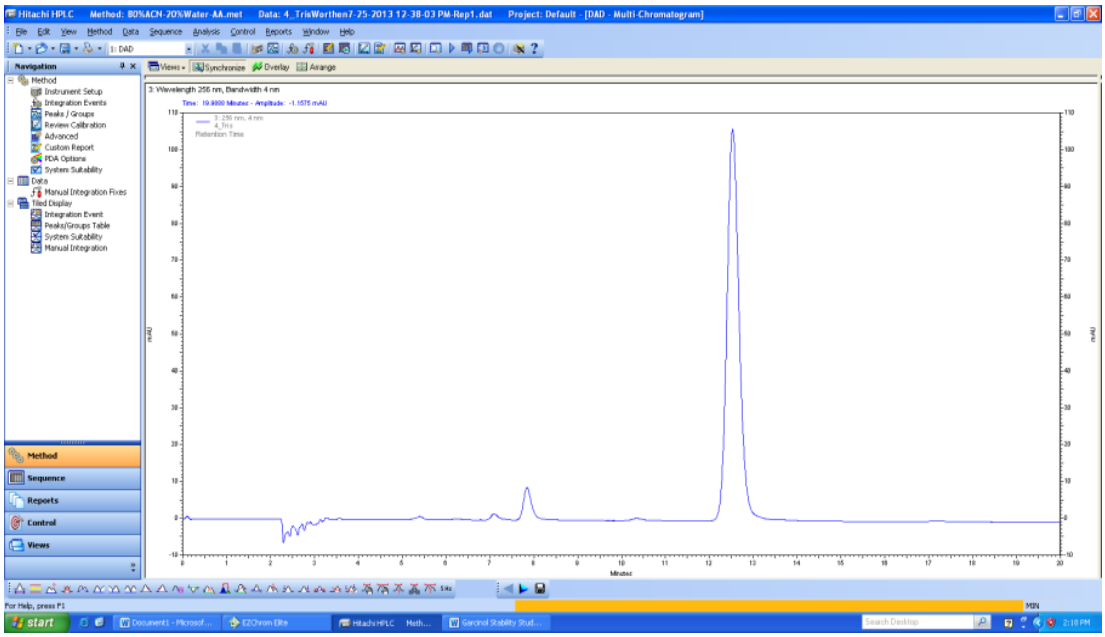


Figure 24: 5 mM Tris initial.

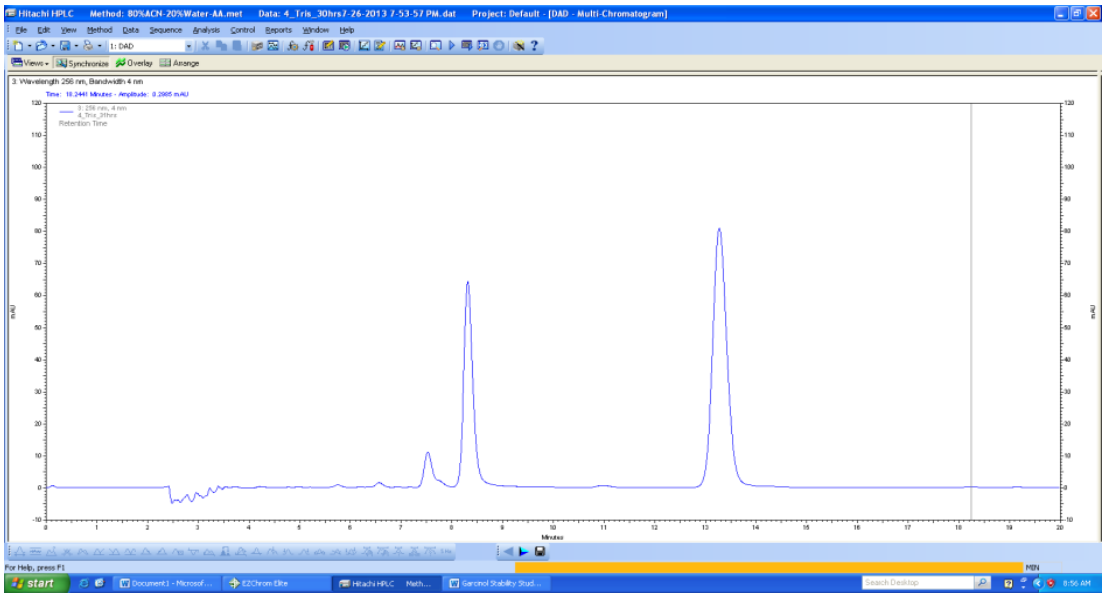


Figure 25: 5 mM Tris 31 hours.

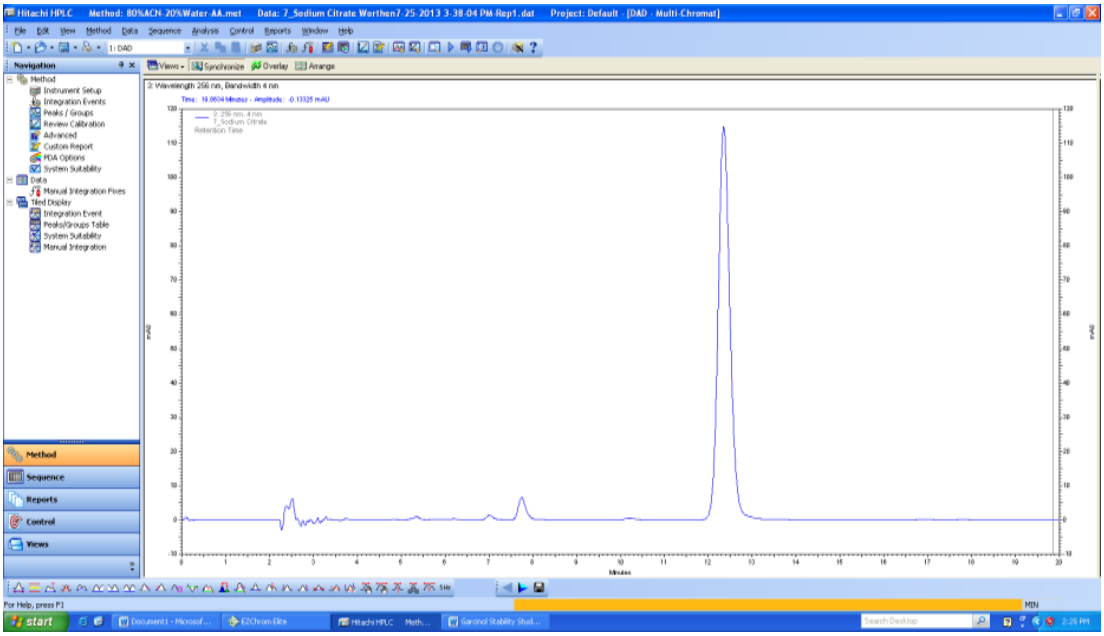


Figure 26: 5 mM Sodium citrate initial.

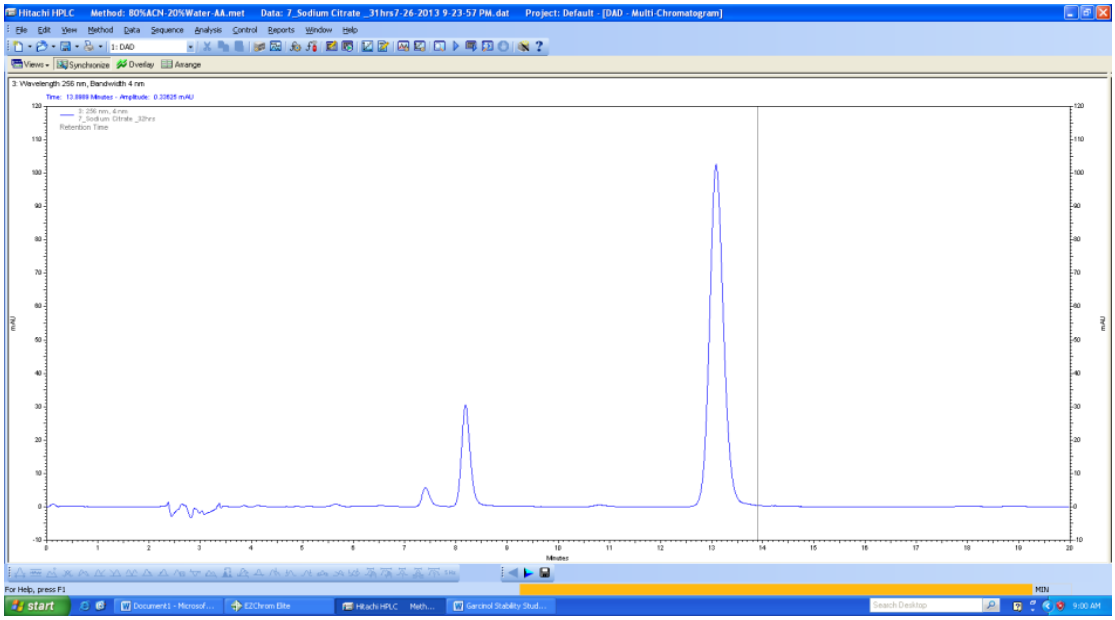


Figure 27: 5 mM Sodium citrate 32 hours.

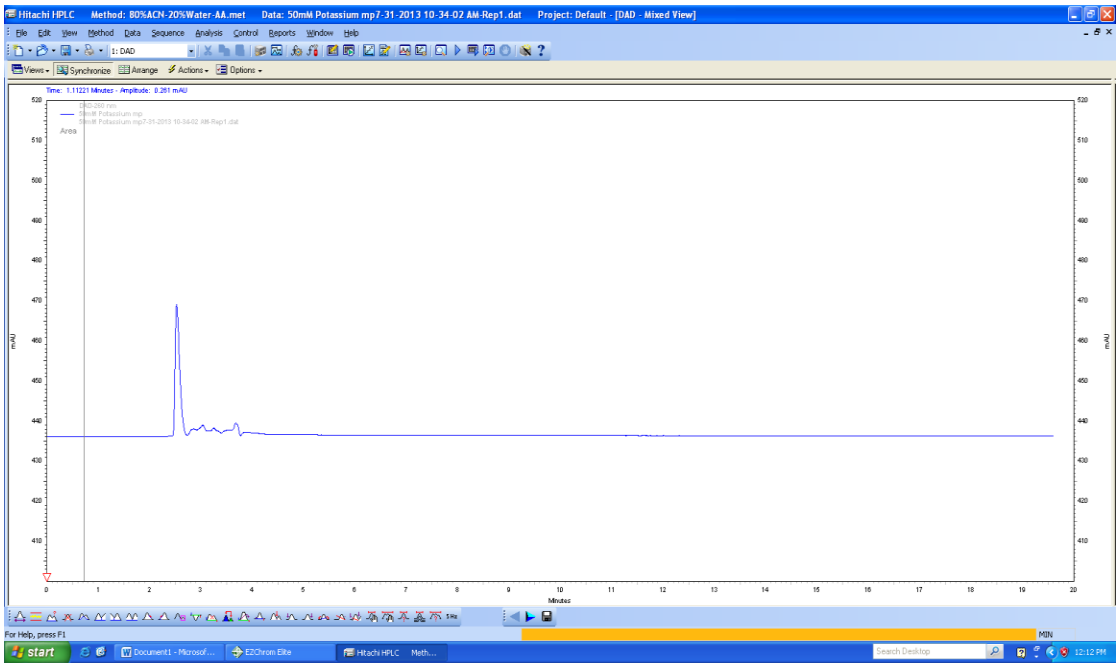


Figure 28: 50 mM Potassium phosphate.

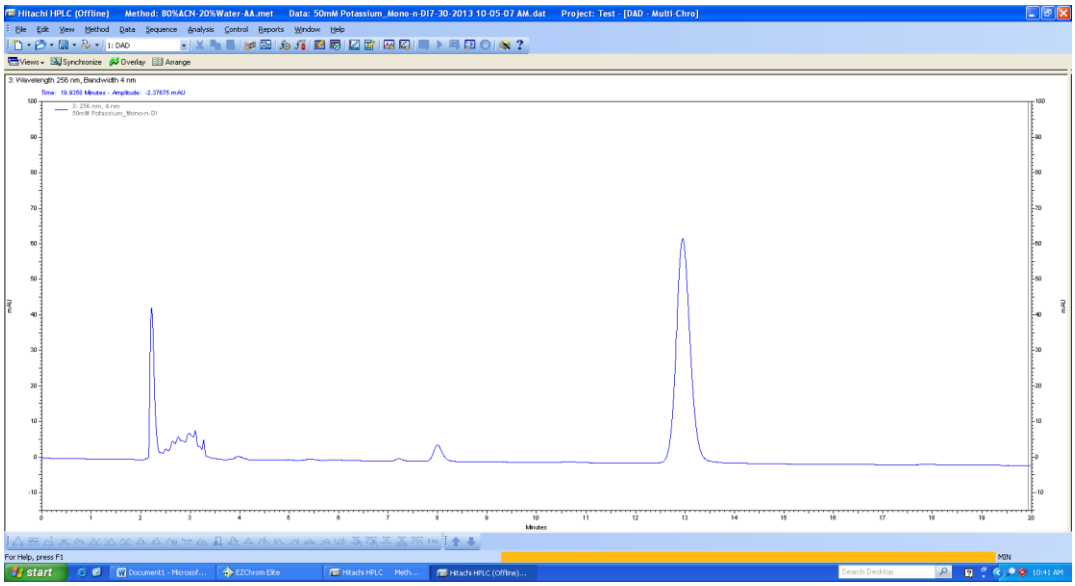
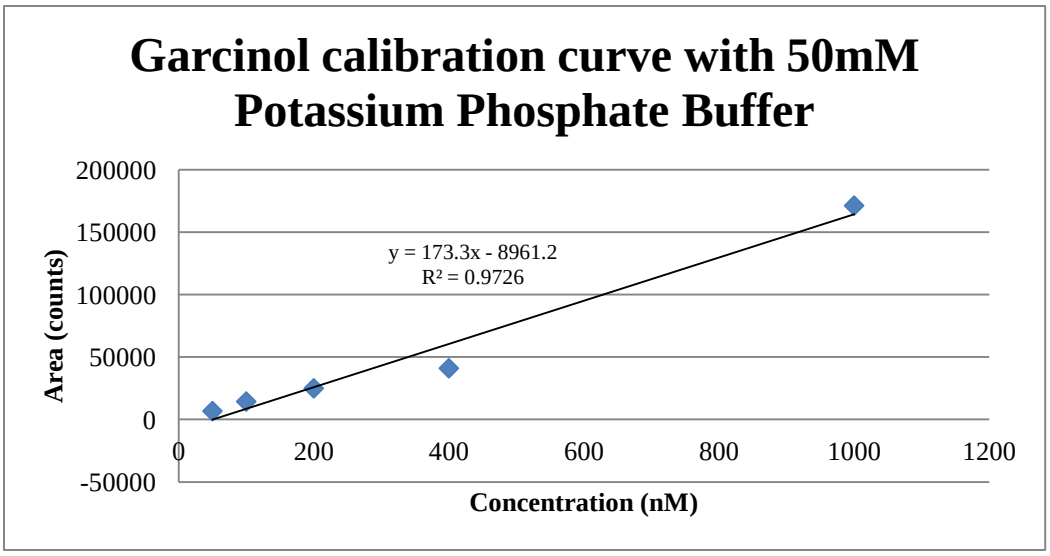


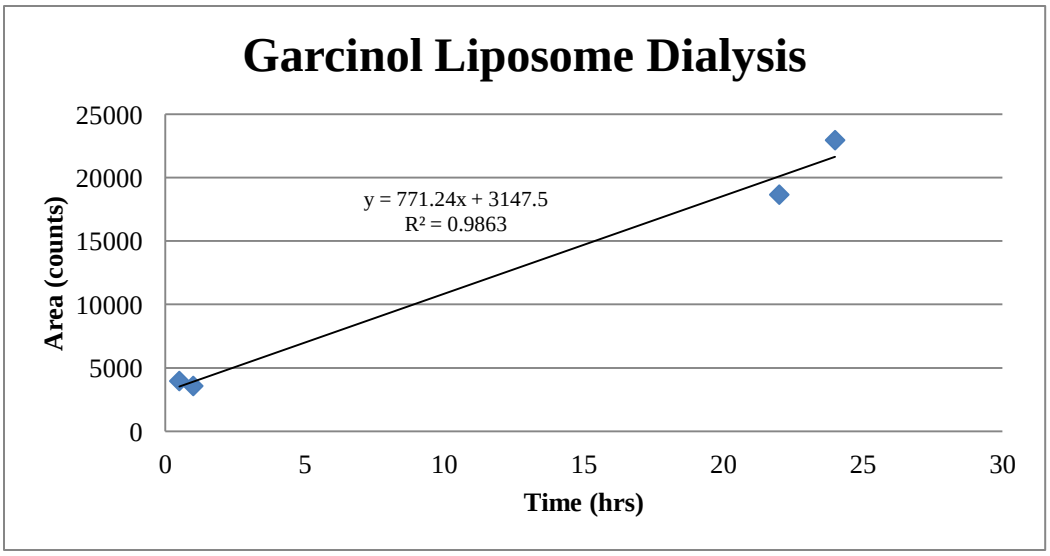
Figure 29: 50 mM Potassium phosphate 85 hours.

Results in Figure 19 to Figure 29 show the injection of garcinol has a retention time of roughly 12.5 minutes. However, a number of degradants increased in size and were observed in the various buffer systems at about 7-9 minutes. The concentrations also increased over time in several buffer systems. Accordingly, potassium phosphate buffer solution was selected as the suitable liposome hydration medium for formulating garcinol in DPPC liposomes.

The results of liposomal garcinol formulation are summarized in Graph 13 and 14 below.

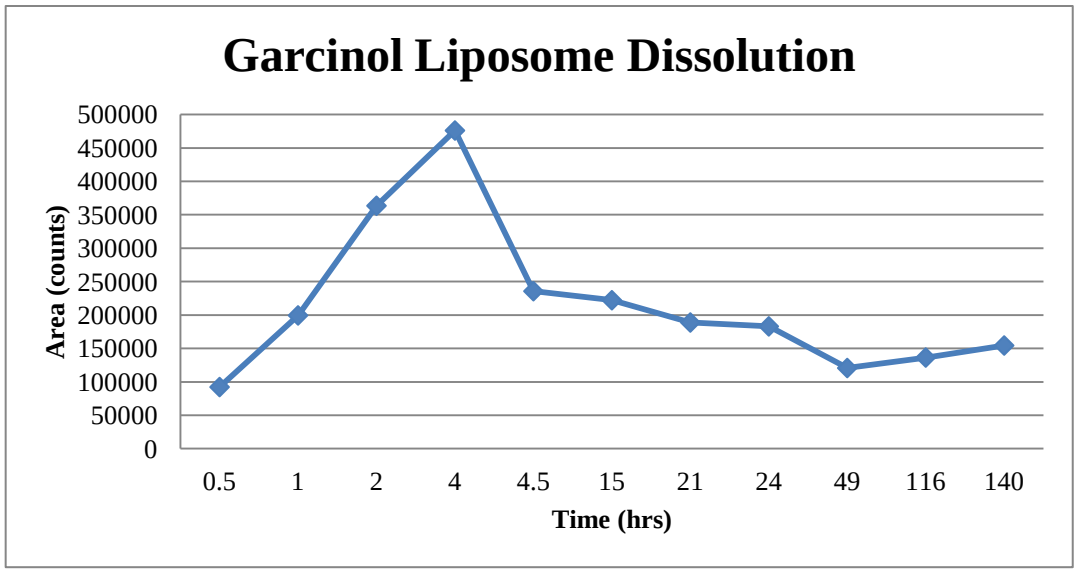


Graph 13: Garcinol calibration curve with 50 mM Potassium Phosphate Buffer solution.



Graph 14: Garcinol dialysis release study.

Graph 15 shows the garcinol HPLC calibration curve indicating a 97.26% linear trend line of concentration versus area under the curve. In order to study the release of the garcinol formulation, a dialysis study was conducted as seen in Graph 14. Over the 24



Graph 15: Garcinol dissolution release study.

hour period, there was an increasing trend in concentration of the dialysis study. Garcinol released over time during the 24 hours was proportional to time. In the dissolution study, results in graph 15 indicate a fast release follow by a sharp (65%) drop in concentration after 4 hrs, perhaps due to saturation and precipitation of garcinol in solution. Given the high affinity of garcinol for DPPC lipid, a significant amount of garcinol might still be present in the bilayers.

CHAPTER 5

CONCLUSION

In this research, *Garcinia kola* was extracted using two different extraction techniques: liquid-liquid (same as solvent-solvent, S-S) and supercritical fluid extraction (SFE). Both techniques have their advantages and disadvantages. The extracts from each technique were subjected to analysis in order to isolate, identify and formulate garcinol, a reported antiviral compound present in related plants. *Garcinia* extracts were also assayed for antiviral activity which was not observed in vitro. SFE extracts chemical compounds from *Garcinia kola* seeds using carbon dioxide instead of organic solvent as in HPLC. In the SFE technique, three different modifiers were used (IPA, EtOH, MeOH) and in the S-S techniques, methanol, MTB, Sephadex, hexane, ethyl acetate and chloroform were used. Both extraction techniques were compared and analyzed by HPLC, UPC^2 coupled to Q-Tof MS and compared using statistical analysis via PCA. PCA and dendrograms were used as visual aids to understand the different groupings, relevant use data, and understanding of the extraction coverage. Results indicated no presence of garcinol in any extract. The results indicated both techniques relatively extracted the same percent of extracts, 50.40% and 55.49% respectfully. In addition, 26.6% of the abundant mass features were extracted in both the SFE and S-S extracts meaning they overlapped in the compounds extracted. Both techniques serve as a means for complete chemical profiling of compounds. The compound of interest garcinol, was fragmented in a collision cell and that result yield structural information of four fragmented ions.

Although garcinol was not present in both the extraction techniques mentioned above, the aim of this research was to formulate an antiviral compound. garcinol, available commercially and found in other *Garcinia spp.*, was used as a reference standard for comparative means. Based upon the methods and techniques employed, it was determined that garcinol was not isolated from *Garcinia kola* in detectable amounts. Garcinol was used as a model compound for a representative liposomal formulation which was prepared and characterized. Preformulation experiments were conducted in order to identify an appropriate physiological buffer for preparing the liposomes, as it was found that garcinol was unstable in several representative buffer systems as well as release studies.

Garcinol liposome were made using DPPC which is widely used to study liposomes. ^{H}NMR and ^{31}P-NMR analysis were performed for particle size separation as well as to observe the amount of hydrogen and phosphorus, in the garcinol compound. The proton NMR confirmed that garcinol and guttiferone F is relatively the same compound and both names are used interchangeable. Phosphorous NMR observed any shielding effect of the garcinol liposomes. Nano-DSC studies were performed for the transition temperature of the liposome lipid component (DPPC) blank and to predict the transition temperature of the garcinol liposomes. Results indicated a lower transition temperature in the garcinol-DPPC formulation. DLS studies were performed in order to characterize the size and size distribution of the garcinol-DPPC liposome. The results indicate a uniform shape after sonication as well as an increase in size over time (7 days) which could be an indicator of stability issue. Zeta potential was conducted for the characterization of the surface charge of the

vesicles. The results showed that garcinol had a strong interaction with the phosphate head groups in the DPPC vesicles which may cause stability issues of the garcinol formulation. Both dialysis and dissolution study show garcinol was released in the DPPC liposome although in very small quantities.

In conclusion, most of the applied techniques mentioned above including SFE, nano-DSC, UPC^2 coupled to Q-Tof MS, ^{31}P-NMR and liposomal formulation have not previously been performed on *Garcinia kola* preparations or garcinol. This research is original, innovative and multidimensional. Although *Garcinia kola* fractions did not inhibit HIV virus as compared to the controls, the techniques used in this research is trendsetting in the investigation of antiviral compounds found in *Garcinia kola*. Further investigation (structural elucidation for example) is necessary in order to identify the exact compound that corresponds with the common mass mentioned above. In addition, analyzing the mass of the compound in positive mode would also be useful in a more complex analysis. Overall, both extraction techniques are complementary and may be necessary to achieve a comprehensive profile of the constituents found within *Garcinia kola* nuts and other natural products. Another future study suggestion includes further separating the extract in the S-S technique as well as the modified in the SFE technique which contained the largest number of detectable compounds and applying statistical analysis. Lastly, isolating other biological activities in those extracts including antibacterial and or antifungal would show the diversity of this plant natural product. With *Garcinia kola*, the possibilities are endless.

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