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Title:

α -Lipoic Acid Analogs: Preparation, characterization and anti-cancer activity

العنوان:

معالجة امراض السرطان باستخدام مشتقات حامض الليبوات

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α -Lipoic Acid Analogs: Preparation, characterization and anti-cancer activity

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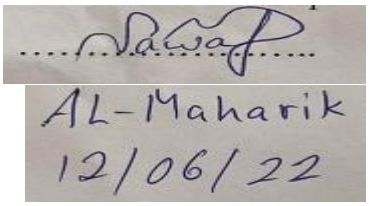

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Finally, I would like to dedicate this work to my triplet (Julia, Selia, and Rakan) and my beloved family

Contents

List of Figure

List of Tables

Schemes

Abbreviations

α-LA	Alpha Lipoic Acid
NO's	Nitric oxide donors
PEG	Polyethylene glycol
FTIR	Fourier infrared spectra
1HNMR	Proton Nuclear Magnetic Resonance
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
TLC	Thin-layer chromatography
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
BHA	Butylated hydroxyl
BHT	Butylated hydroxyl toluene
PG	Propyl gallate
DHLA	Dihydro lipoic acid
GSH	Glutathione
GSSG	Glutathione disulfide
AOS	Active oxygen species
ONOO-	Peroxynitrite
NO	Nitric oxide
$RONO_2$	Organic nitrates
GTN	Glyceryl trinitrate
PETN	Pentaerythrityl tetranitrate
ISMO	Isosorbide 5-mononitrate
DMSO	Dimethyl sulfoxide

RSSD	Disulphides
EPRF	Endothelium-derived relaxing factor
GTN	Glyceryl trinitrate
TTS5	Nitroderm
DCC	N, N′-Dicyclohexylcarbodiimide
DCU	N, N′-Dicyclohexyl urea
DCM	Di chloromethane
$NaHCO_3$	Sodium bicarbonate
Et OH	Ethanol
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
NMR multiplicities	s = Singlet d = Doublet t = Triplet m = Multiplate dt= Doublet Triplet

1. Abstract

Because drug-resistant cancer cells are the leading cause of death from malignancies, effective new medicines to combat tumor cell resistance to current chemotherapies are urgently needed. α -Lipoic Acid is a redox-active chemical found in nature that functions as a cofactor in a number of mitochondrial enzymes involved in metabolism and energy production. According to preliminary findings, α -Lipoic Acid and several of its derivatives may act as an anti-cancer.

Since its identification as a critical signaling molecule, there has been a lot of excitement about the possibility of new Nitric Oxide (NO)-based treatments for a number of ailments. We investigate the anti-cancer properties of Nitro-lipoate by combining the actions of α -Lipoic Acid mimics and NO. The addition of polyethylene glycol (PEG) to some of our proposed compounds represents a significant improvement. PEG is one of the most biocompatible polymers, and conjugating it increases the biological features of the linked substance in terms of circulation half-life, degradation resistance, and cell absorption. In this thesis, a new and creative approach is to synthesize new α -Lipoic Acid (R-LA) derivatives (bifunctional NO-donor/anticancer drugs) and assess their efficacy against cancer cell lines in vitro. The effects of α -Lipoic Acid and NO in one compound, PEGylated lipoate-NO hybrids, and others are more effective than α -Lipoic Acid in treating and preventing cancer because they retain the pharmacological activity of the parent compound while also having the biological actions of NO, a strategy that has never been used in cancer treatment and prevention before. Compounds

(1-9) compounds were synthesized and analyzed using different spectroscopic methods such as FTIR, ^{1}HNMR, HPLC, LC-MS, TLC, and other physical properties, as well as other techniques.

The anticancer activity of the synthesized compounds was tested in vitro against a variety of cancer cells. Compounds A, B, and Z present a considerable influence on HT29 colon cancer cells, MCF7 cells, and MDA MB231 breast cancer cells with percent's ranging from 20%-88%.

In the light of the above results, the ensuing experiments suggest that the tested compounds may serve as advanced dual-action, serve as nitric oxide donors, and anti-oxidant agents

which lead to the development of a new product for acquiring anticancer activity and serve as prototype candidates for the treatment of some aggressive cancer cells.

الملخص بالعربية

هنالك العديد من الامراض التي تهدد حياة الانسان وليس لها علاج محدد الى وقتنا هذا، من بينها السرطان. يعرف مرض السرطان على انه نمو غير طبيعي للخلايا التي تنقسم بدون رقابة ولديها القدرة على اختراق الأنسجة وتدمير أنسجة سليمة في الجسم، وهو قادر على الانتشار في جميع أنحاء الجسم. ونظرا لأنه مرض خبيث ومقاوم للأدوية هناك حاجة ماسة الى أدوية وعلاجات جديدة لمكافحة هذا المرض. واحدة من أهم الطرق التي يجري عليها بحوثا عالمية هي تقنية موت الخلايا المبرمج للخلايا السرطانية .حمض ليبويك هو مادة كيميائية فعالة في الأكسدة والاختزال توجد في الطبيعة وتعمل كعامل مساعد في عدد من إنزيمات الميتوكوندريا المشاركة في التمثيل الغذائي وإنتاج الطاقة. وفقًا للنتائج الأولية، يمكن أن يتصرف حمض الليبويك والعديد من مشتقاته كمضاد للسرطان.

العديد من الأبحاث السابقة أشارت الى أن مركب أكسيد النيتريك ومشتقاته لديها القدرة على مقاومة وعلاج عدة أمراض من بينها السرطان، كما أنها اثبتت بأن إضافة مركب ايثيلين جلايكول الى المركبات تزيد من السمات البيولوجية للمادة المرتبطة من حيث عمر نصف الدورة، ومقاومتة للتحلل، وامتصاصه السريع الخلايا.

بناءا على ما سبق سيتم استخدام تقنية جديدة في هذا البحث ومبتكرا يقتضي بتصنيع عدة مركبات مضادات الأكسدة القائمة على حمض ليبويك، مرتبطة بروابط كيميائية مختلفة مثل الإسترات وغيرها، ومضادات الأكسدة مثل ثنائي ليبوات مرتبطة كيميائية مختلفة ونوع جديد مشتقات حمض الليبويك ثنائية الوظيفة، تعمل كمضادات الأكسدة ومزودة بأكسيد النيتريك (NO).

تم تشخيص المركبات المصنعة باستخدام عدة تقنيات وطرق كيميائية طبقية، وتم فحصها مخبريا على عدة انواع من الخلايا سرطانية.

النتائج الأولية لمشتقات حمض الليبويك تشير بأن هنالك فعالية عالية لبعض المركبات ضد لمنع او قتل انواع مختلفة من الخلايا السرطانية.

Introduction

Cancer is the most pressing global issue, claiming the lives of over 8 million people each year [1]. As a result, scientists are working extremely hard to discover a cure, and in order to understand the treatments, it is necessary to first understand what cancer is.

Cells grow and divide in order to produce new cells. After a limited number of divisions, normal cells die. Cancer, however, is characterized by uncontrolled cell growth, which is defined as the condition where normal cells instead of dying continue to grow and form new abnormal cells (cancer). Unlike normal cells, cancer cells can't repair DNA damage, don't die when damaged, and just continue dividing and making new cells [2].

Although the reasons for the development of cancer cells are still unknown, some factors are known to be linked to cancer development. Some of these factors are summarized in figure 1 [3].

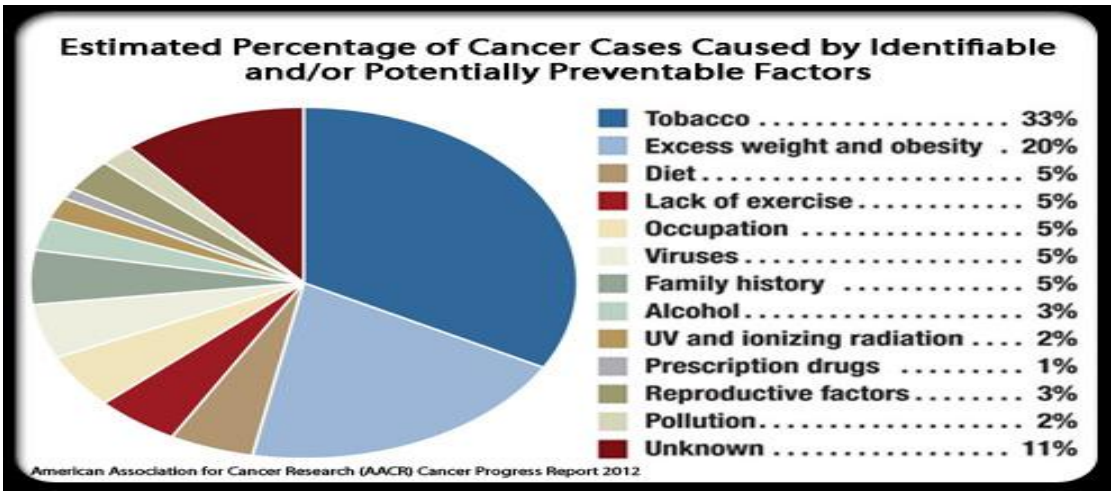


Figure1: Estimated percentage of cancer cases caused by identifiable and potentially preventable factors.

In the global arena, there are many methods for fighting cancer, but, currently, there is no cure. Examples of these methods include surgery, radiation and chemotherapy [4,5].

The most effective way to eliminate cancer cells is by forcing them to undergo apoptosis [2].

It is common for somatic cells to commit suicide by activating a program of intrinsic cell suicide [6], which is initiated by a cascade of biological events. These changes are typically associated with morphological and biochemical changes within the cells [7].

Cells in an apoptotic state lose their attachment to other cells and the plasma membrane becomes blebbed and disorganized. The chromatin of the cell condenses, and the nucleus fragments and phosphatidylserine flips into the outer leaflet of the bilayer [8,9]. As a result, nucleases are activated, resulting in the breakdown of chromosomal DNA into oligo nucleosome fragments (physiological death), of 50-300 kb [10].

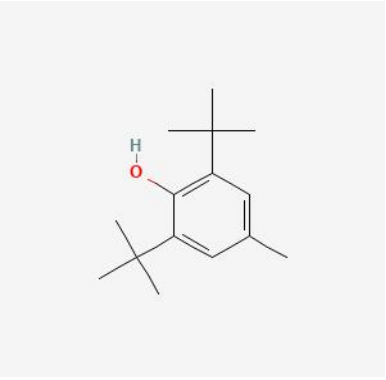
These events require metabolic energy to be synchronized and coordinated. The apoptotic cell is transformed into membrane-bound entities known as apoptotic bodies, which are identified and endocytosed by phagocytes, avoiding inflammation [11,12].

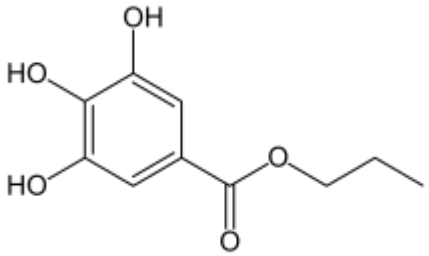
In the past few decades, the search for cures for many diseases has become increasingly competitive as research into chemicals or natural compounds has progressed. One such compound is antioxidants, which remove highly unstable species that have an unpaired valence electron, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) as superoxide. Free radicals are linked to sight loss, arthritis, Parkinson's, or Alzheimer’s disease, cellular damage in the brain, accelerated aging, coronary heart disease, among others, and cancers [13].

A free radical is formed as our body converts food into energy, also the environment can cause our bodies to produce free radicals. Such environmental factors include pollution, smoking, and sunlight. As a result, we need more antioxidants to prevent these diseases [14].

Food and medicinal plants are rich in antioxidants. Vegetables, nuts, whole grains, some meats, poultry, fish, and fruits are all high in antioxidants. The US government encourages people to eat these foods because they reduce the risk of several diseases. [15].

Vitamins such as polyphenols and carotenoids are antioxidants, which has a wide range of biological effects e.g., anticancer, anti-atherosclerosis, anti-aging, and inflammatory. As a result, scientists are focusing their efforts on extracting natural antioxidants and evaluating them for disease treatment. Synthetic antioxidants such as butylated hydroxy a (BHA), butylated hydroxyl toluene (BHT), and propyl gallate (PG) have been widely employed in place of natural antioxidants because they are more widely available, have superior stability, performance, and are less expensive. Natural acids are the most important antioxidants produced in human tissues and can be used to treat a variety of ailments including cardiovascular and bacterial diseases, and diabetes. One such acid is Lipoic acid which was studied in the current research [16].





Structure of BHT

Structure of PG

α-Lipoic acid (α-LA), also known as thioctic acid (1, 2-dithiolane 3-pentanoic acid) Figure 2, is a naturally occurring chemical found in a variety of mitochondrial enzymes. Reed et al discovered it in lactic acid bacteria in 1951[17]. The researchers identified it as an organosulfur compound derived from octanoic acid, α-LA is an eight-carbon molecule with two sulfur atoms (at C6 and C8) that are linked by a disulfide bond. As a result, despite the presence of

sulfur atoms in higher oxidation states, it is termed oxidized. Plants, animals, and humans can all synthesize it in small amounts. [18].

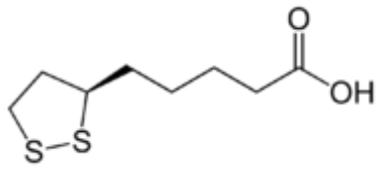


Figure 2: Structure of α-LA

α-LA is a golden needle-like crystal with a molar mass of 206.32 g.mol-1. It has a melting point of 46-48 degrees Celsius. It is soluble in ethanol and weakly soluble in water (0.24 g/L) [19]. α-LA LA is available in two enantiomers, R-LA and S-LA (Figure 3), as well as a racemic mixture (RS-LA), which contains 50 percent R-LA and 50 percent S-LA. In all in vitro models and clinical trials, R-LA is the natural form of α-LA, with more potent pharmacological activity than S-LA. The biological efficiency of R-LA and S-LA is not the same [20].

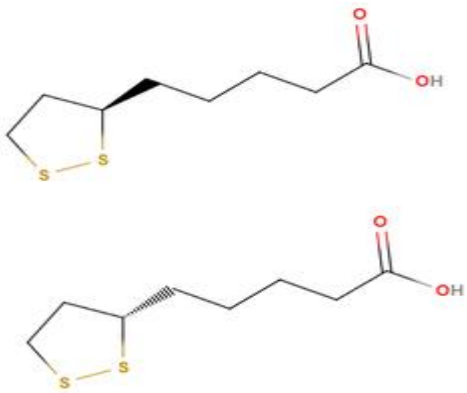


Figure3: Structure of R-LA & S-LA

In vitro, α-LA and its reduced form, dihydrolipoic acid (DHLA), have been found to have antioxidant action by interacting with various anti-species and scavenging hydroxyl radicals, hypochlorous acid, singlet oxygen, and transition metal [21], shown in figure 4.

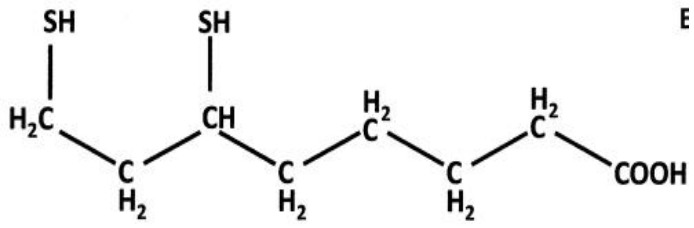


Figure 4: structure of DHLA

Glutathione/ glutathione disulfide (GSH/GSSG) has a redox potential of -0.24 V, while DHL α-LA has a redox potential of -0.32 V. As a result, ALA/DHLA is regarded as a universal antioxidant that can replenish a variety of different antioxidants. When ALA is given to cells under physiological conditions (PH=7.46), it is converted to DHLA. Figure 5 shows the equilibrium between the protonated and deprotonated species [22].

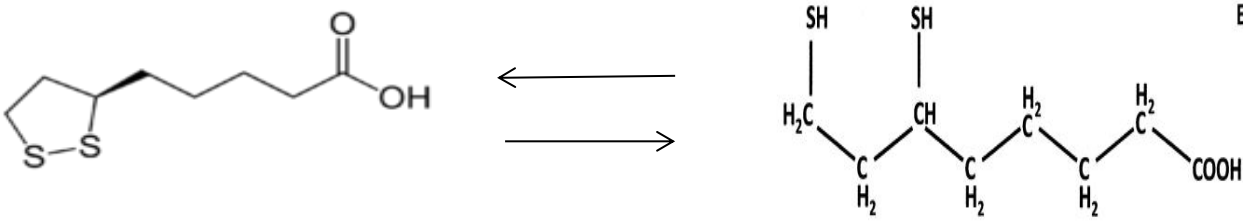


Figure 5: the equilibrium between the protonated and deprotonated species

Although oxygen is essential for aerobic species survival, it becomes harmful at larger concentrations. Dioxygen is unreactive in its natural state, but partial reduction results in active oxygen species (AOS), such as singlet oxygen, superoxide radical anion, hydrogen peroxide, and so on. Many diseases, such as arthritis and cancer, are caused by free oxygen radicals. To protect the body from these free radicals, antioxidant chemicals are used. Many features, such as specificity of free radical scavenging, metal chelating activity, and

interaction with other antioxidants, are required for a molecule to be a powerful antioxidant. Many studies have been done on antioxidants to see how they affect cancer cells, but none of them have been successful in preventing cancer. Following its release into the culture medium, where it converts cystine to cysteine, α-LA 's metabolic antioxidant activity was dependent on its conversion to DHLA. The neutral amino acid transport system rapidly absorbs the cysteine, which is then used to synthesize glutathione (GSH) [23,24]. Figure 6.

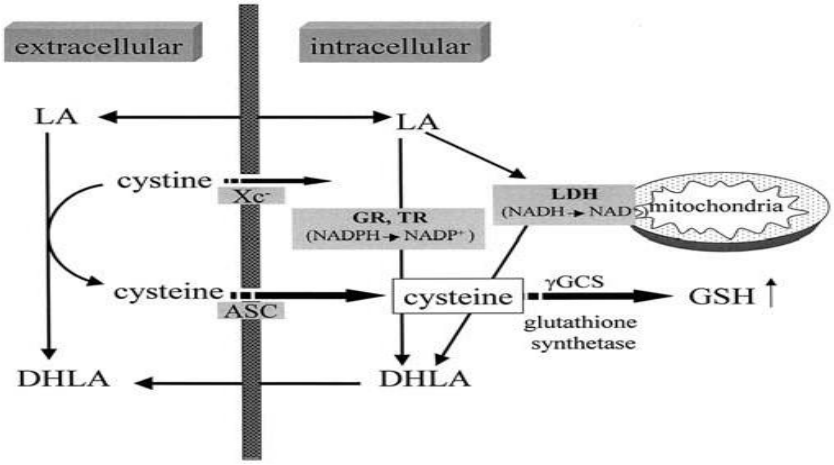


Figure 6: Cellular pathways for the bio reduction of *α*-lipoic acid (α-LA) to dihydrolipoic acid (DHLA).

DHLA is a key component for mitochondrial bio-energetic enzymes and can be regenerated from α-LA rather than being destroyed by quenching free radicals. As a superoxide dismutase, DHLA can inhibit super oxide-driven oxidation of spin probes. According to current research, α-LA and its reduced form can react with peroxynitrite (ONOO-), a product of the rapid reaction of nitric oxide (NO) with superoxide anion (O_2 -), and α-LA can also boost vitamin C cycle efficiency and activate the vitamin E cycle [20]. Figure 7.

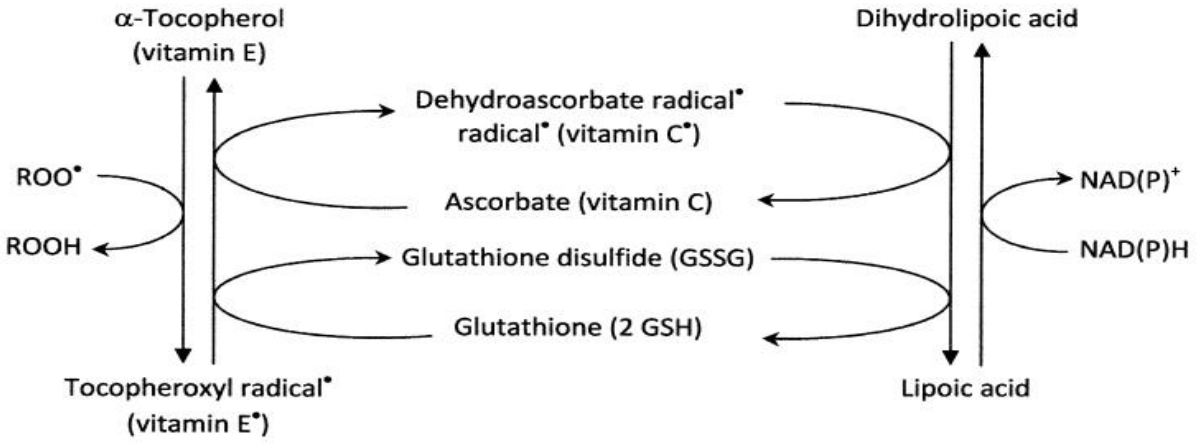


Figure 7: cycles of vitamins C and E activated by α-LA

Furthermore, α-LA reduced urinary F2-isoprostane levels, prolonged the lag time of LDL oxidation, and reduced cerebral lipid peroxidation, as well as prevented age-related decreases in endogenous antioxidant levels in elderly brain tissue [20].

By reacting with transition metal chelation and generating stable complexes with them (Mn^{+2}, Cu^{+2}, Fe^{+2}, $Zn^{+}2$), ALA and its reduced forms can also hinder antioxidant actions [20].

The reduced versions of the mitochondrial nicotinamide adenine dinucleotide and dihydrolipoamide dehydrogenase favours R (+)-α-LA as a chelator of divalent metal ions, correcting age-related build-up of iron and depletion of antioxidants in the rat cerebral cortex. The cytosolic reduced form of nicotinamide adenine dinucleotide and glutathione reductase, on the other hand, prefer to react with S (-)-α-LA [20].

In vegetables and animal tissues, a minor quantity of α-LA, which is lipoic acid connected to lysine residues, is present. The most R-LA-rich vegetables are spinach, broccoli, and tomatoes, which contain 3.2, 0.9, and 0.6 * 10-3 lipoyllysine per gram dry weight, respectively [20].

The largest concentrations of lipoyllisine in animals are found in the kidney, heart, and liver, which contain 2.6, 1.5, and 0.9 * 10-3 g lipoyllysine per gram dry weight, respectively [20]. Table 1.

Table 1: The amount of lipoyllysine in the tissue of experimental animals

Food	Lipoyllysine (mg/g Dry Weight)
Beef kidney	2.6
Beef heart	1.5
Beef liver	0.9
Spinach	3.2
Broccoli	0.9
Tomato	0.6
Peas	0.4
Brussels' sprouts	0.4
Rice	0.2
Egg yolk	0.05

Diabetes is a metabolic condition in which a person's blood glucose levels are high due to a lack of insulin synthesis, a failure of the body's cells to respond correctly to insulin, or both. In Germany, α-LA is used as a diabetes treatment. In type 2 diabetic patients, intravenous infusion of α-LA enhances the insulin-stimulated metabolic clearance rate of glucose as well as insulin sensitivity. Amide (10 mg kg day for 2 weeks) reduced blood glucose levels by 39%, while a larger dose of ALA (50 mg kg day for 2 weeks) reduced blood glucose levels by 30% [25].

It was recently discovered that α-LA promotes glucose oxidation in isolated working rat hearts while having no effect on glycolysis, lactate oxidation, or palmitate oxidation.

Because glutathionylation and activation of p21ras can cause insulin resistance, and GSH can initiate s-glut thiolation of p21ras in endothelial cells, most studies concluded that mitochondrial aldehyde dehydrogenase is an important enzyme for the detoxification of aldehyde and that DHLA could restore aldehyde dehydrogenase activity via reduction of

disulfide at the active site [20].

Nitric oxide donors are pharmacologically active substances that have been shown to release NO in both in *Vivo* and in *vitro* studies. All molecules with nitrogen and oxygen atoms bound together can be reduced, oxidized, and decomposed, releasing reactive nitrogen species. Table 3. Many nitro compounds, such as organic nitrate, furoxans, and nitrates, use thiols as a cofactor to release NO. NO donors are known to be heat, light, and PH sensitive compounds [25].

As a result, they can decay in a natural way. Organic nitrates (RONO2), such as glyceryl trinitrate (GTN), pentaerythrityl tetranitrate (PETN), isosorbide 5-mononitrate (ISMO), and nicorandil, are generated from mono and polyhydric alcohol esters that have been therapeutically employed [26]. Figure 8.

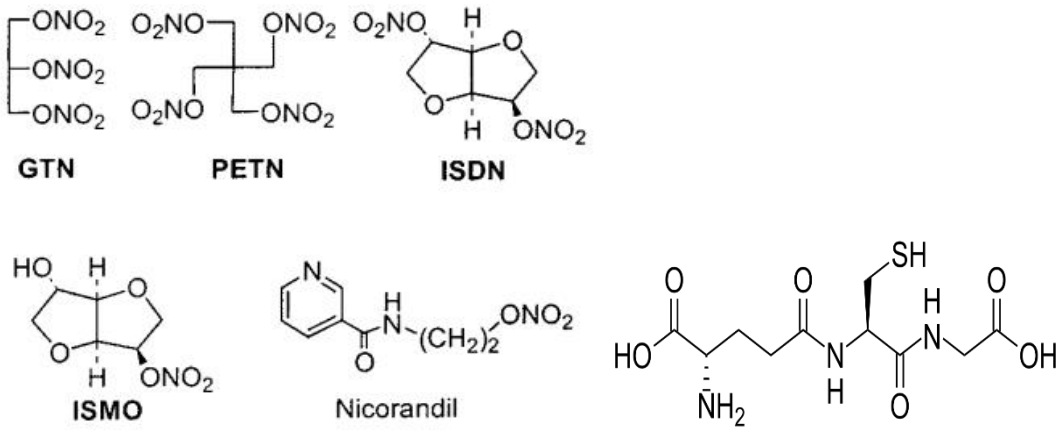
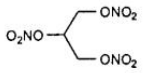
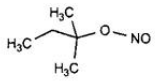
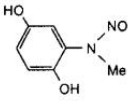
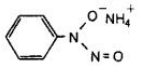
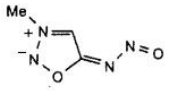
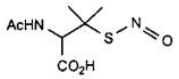
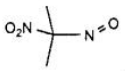
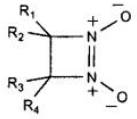
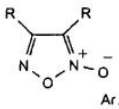
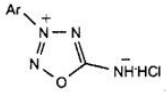
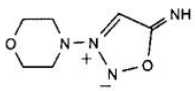
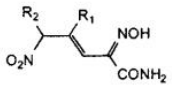
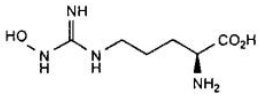
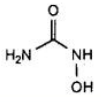


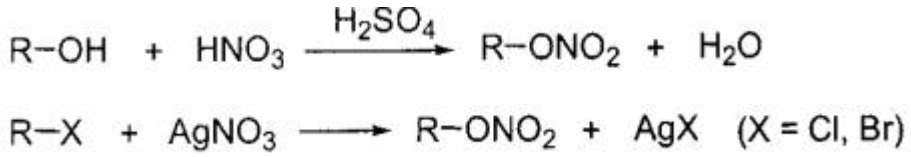
Figure 8: Nitric Oxide Donors

Table 2: The major classer of NO donors

Index, Name	Representative Compounds		
A. Organic nitrates			
B. Organic nitrites			
C. Metal-NO complexes	$Na_2[Fe(CN)_5(NO)] \cdot 2H_2O$		
D. *N*-Nitrosamines			
E. *N*-Hydroxyl nitrosamines			
		GSH	
F. Nitrosimines			
G. Nitrosothiols1			
H. *C*-nitroso compounds			
I. Diazetine dioxides			
J. Furoxans and benzofuroxans			
K. Oxatriazole-5-imines			
L. Sydnonimines		spontaneous, enhanced by light, oxidants and pH>5	prodrugs require enzymatic hydrolysis
M. Oximes		spontaneous; O_2/Fe^{III}-porphyrin	?
N. Hydroxylamines		autoxidation enhanced by metal ions	catalase/H_2O_2
O. *N*-Hydroxyguanidines		oxidants	NOSs, Cyt-P450
P. Hydroxyureas		H_2O_2/CuZn-SOD or ceruloplasimin; H_2O_2/Cu^{2+}; heme-containing proteins	peroxidase

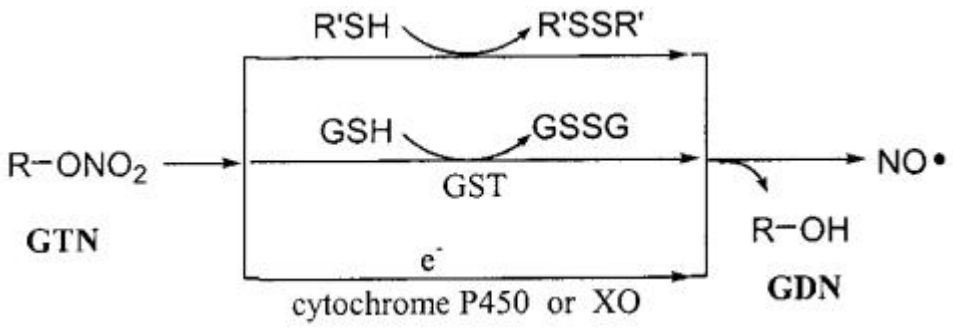
Esterification of alcohols or substitution of reactive alkyl halides with $AgNO_3$ can be used to make organic nitrates [26]. Scheme 1

Scheme 1: esterification of alcohols between reactive alkyl halides and $AgNO_3$



Organic nitrates can be converted to NO through enzymatic or non-enzymatic bioactivation that involves the reduction of three electrons, such as GTN, where NO is produced from the C-3 carbon [26].

Scheme 2: the intracellular and extracellular pathways of releasing NO from GTN.



Thiols can boost GTN's response to release NO by donating reducing equivalents from sulfhydryl groups to create disulphides (RSSD) and NO_2-, but only cysteine, N-Ac-cysteine, and this salicylic acid can stop NO from being released [26].

The NO thiol mediated reaction of nitrate esters will be generated by a two-electron reduction followed by a nucleophilic attack of thiolate on the nitrate group.

Organic nitrates have a wide range of biological applications, including relief of angina pectoris, coronary arteries, acute myocardial infarction, congestive heart failure, blood pressure regulation, relaxing of vascular smooth channels, orthent for children with anal fissures, and as the active substance of Viagra [26].

NO is a key cellular signalling molecule in mammals, including humans, that has a wide range of activities in the cardiovascular, neurological, and immunological systems, as well as smooth muscle relaxation, platelet inhibition, and neurotransmission.

NO is thought to be the most biologically significant kind of endothelium-derived relaxing factor (EDRF). Glyceryl trinitrate (GTN), the most well-known NO donor (used to alleviate acute angina pectoris episodes), is virtually entirely used to create cellular NO. Nitroglycerin, nitroderm TTS5, nitroderm TTS 10, isosorbide mononitrate (40 mg), and isosorbide-5-mononitrate (20 mg) are all utilized to accomplish the same goal [26].

Resistance of cancer cells to diverse cytotoxic stimuli continues to be a key challenge in oncology, necessitating the development of innovative therapeutic techniques. Nitric oxide is gaining attraction for overcoming cancer cells' resistance to conventional treatments [26].

Polyethylene glycol (PEG) is one of the most biocompatible polymers, and conjugating it improves the biological properties of the coupled substance in terms of an increased circulating lifetime [27], increased resistance to degradation [28-30], and increased cell uptake [31,32], and d. lack of immunogenicity and toxicity in the case of proteins [33-35].

PEGylation technology's therapeutic utility has been demonstrated in a range of settings. Over the last two decades, an increasing number of reviews on various aspects of PEGylation

and PEG modification have been published [36,39]. PEG appears to be a viable choice for a medication carrier in this aspect [40].

In the domains of pharmaceutical and biomedical engineering [41-45], PEGylation has grown increasingly relevant, and various PEGylated protein medication systems have been effectively used in clinical applications [46-49]. Monofunctional PEG containing only one reactive terminal group, such as hydroxyl, amine, thiol, aldehyde, carboxylic acid, or activated versions, are used in the bulk of these systems. Targeted drug delivery methods based on PEGylated therapeutics necessitate two reactive termini on the PEG, i.e., - heterobifunctional PEG; one bound to the therapeutic and the other available for conjugation to targeting ligands like peptides, antibodies, or proteins [50-51].

The ability of polymers to attach to nitric oxide and/or antioxidant chemicals has piqued interest in clinical research and medicinal applications. PEG appears to be a viable contender for drug carriers in this aspect [52-53].

2.1 Objectives

a) Preparation, purification, and characterization of different α-lipoic acid derivatives based on antioxidant and nitric oxide donors.

b) Study the anti-cancer activity of the synthesized compound on various cancer cells type.

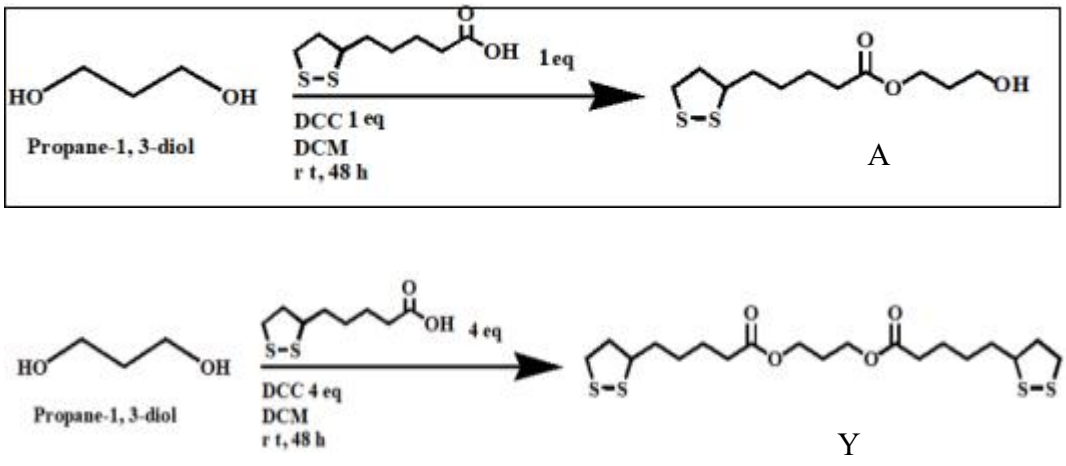
3. Methodology

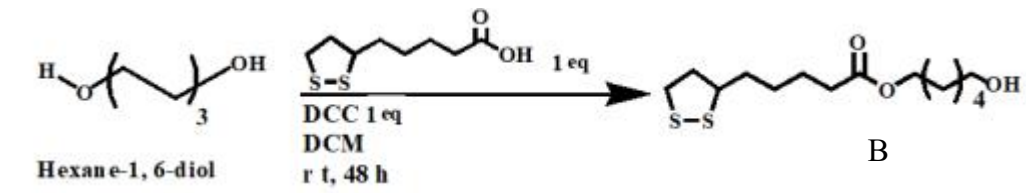
3.1 Chemical synthesis and characterization

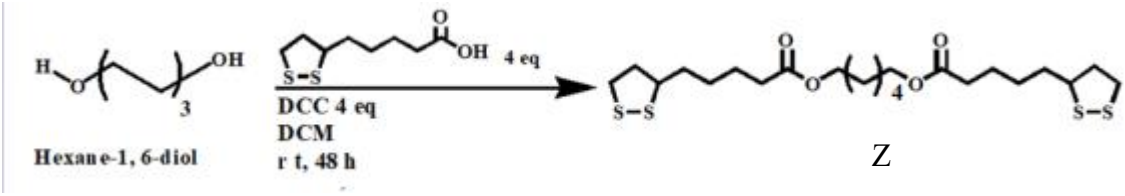
A new α-LA and PEG derivatives (NO-donor/anticancer bifunctional) compounds were synthesized, these compounds will comprise one or more α-LA residues and/or NO-donor coupled via a short or long flexible spacer.

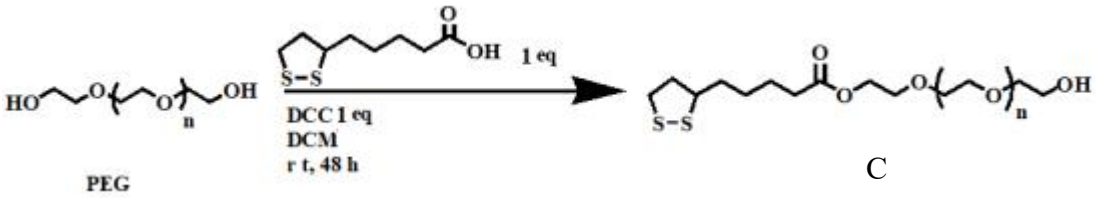
Our strategy for the synthesis of novel bifunctional compounds, anti-superoxide, and NO-donors is based on a unique, simple procedure and acceptable chemistry. The synthesized compounds are summarized in Schemes 3-4, purified by column chromatography (CC) and the compounds were characterized and verified by various analytical techniques like ^{1}H NMR spectroscopy, and liquid chromatography-mass spectrometry (LC-MS), and infrared spectroscopy (FTIR). The purity was determined by the reverse phase HPLC system. A mixture of 10% acetonitrile and 90% H_2O was used as the eluent at a flow rate of 1ml/min, coupled with UV/vis detectors.

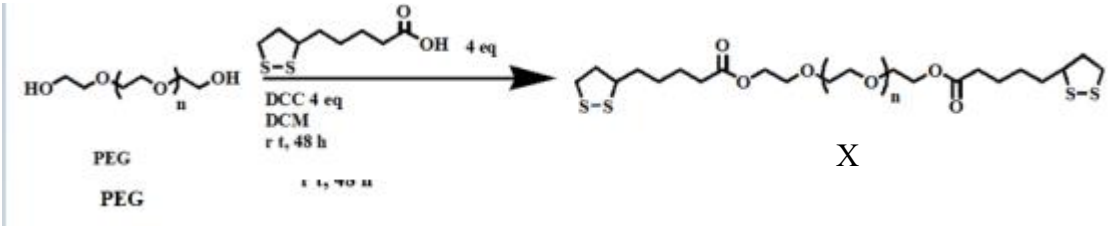
Scheme 3





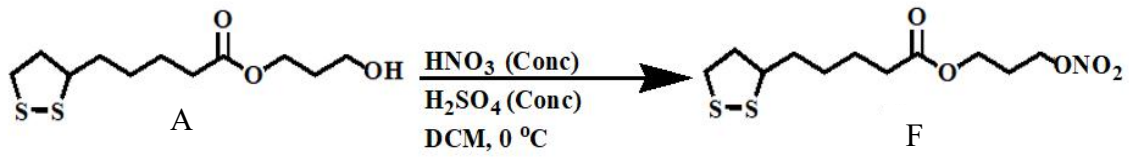


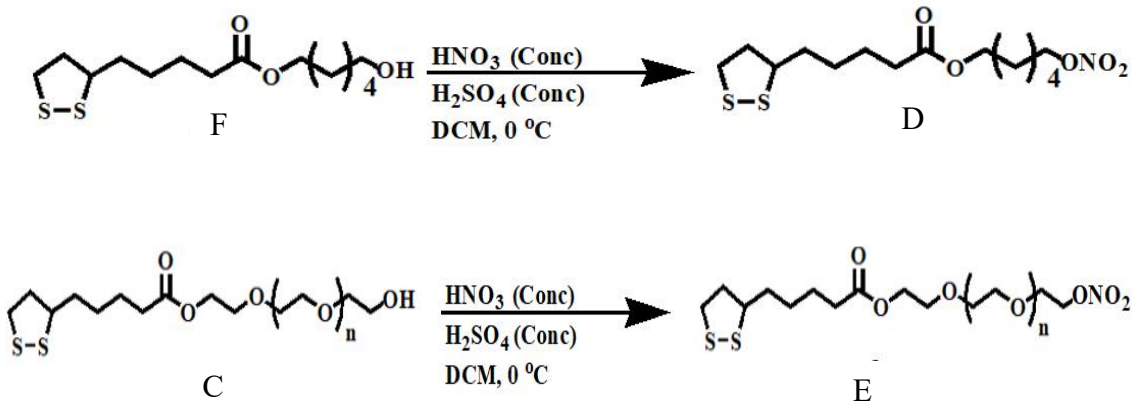




Compounds (A, B, C) shown in scheme 3 were employed as a starting material for the preparation of novel compounds, antioxidant/NO donors' compounds (F, D, E) shown in scheme 4

Scheme 4





Purification

Compounds A, Y, B, Z, C, and X were purified using flash column chromatography on Merck silica gel 60 (particle size 230-400 mesh), with the eluent being a combination of CH_2CL_2 and CH_3OH in various ratios. The eluents used in HPLC were acetonitrile and distilled water. To observe the chemicals, analytical TLC was done on silica gel 60F254-percolated plates supplied from Merck, utilizing UV light or I_2 vaper.

^{1}HNMR Measurements

The data was processed using the VNMR software and captured on a Varian VXR-300 (300MHZ) spectrometer with a 5-mm switchable probe. The internal standard was tetramethyl silane Me_4Si (0.00 ppm) and the solvents were chloroform-d and dimethyl sulfoxide-d6. The abbreviations for the splitting patterns are s, singlet; d, doublet; t, triplet; q, quartet; m, unresolved multiple due to instrument field strength; and dd, doublet of the doublet.

Griess Method (assay):

This sample was made by combining 1 percent sulphanilamide and 0.1 percent naphthyl ethylenediamine in 2% phosphoric acid and mixing them to induce a colorimetric change when NO donors were added.

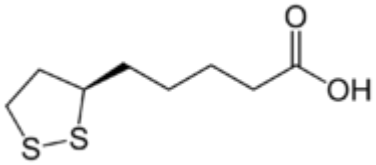
3.1.1 Synthesis of Lipoic acid and PEG derivatives

3.1.1.1 Materials and methods

All compounds were synthesized at Birzeit University laboratories, with NMR analysis performed at the Hebrew University of Jerusalem laboratories and LC-MS, HPLC, and FTIR performed at Birzeit University laboratories for characterization. Sigma Aldrich Chemical Co (St. Louis, MO, USA) provided all of the materials including α-lipoic acid, which was used exactly as they were.

3.1.1.2 Experimental

Characterization of α-Lipoic acid



HPLC chromatogram indicates one compound which means that the starting material α-LA was pure.

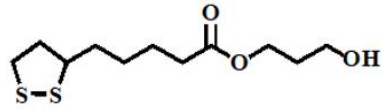
^{1}HNMR: 7.25 (1H, OH), 3.6 (t, 2H, S-S-CH_2), 3.2 (m, 1H, CH_2CH-S-S-), 2.45 (t, 2H, -CH_2-CO), 2.3 (dt, 2H, -S-S-CH_2CH_2-),1.9 (m, 2H, -OCO-CH_2CH_2), 1.7 (dt, 2H, -OCO-$CH_2CH_2CH_2CH_2$-), 1.65 (m, 2H, -OCO-$CH_2CH_2CH_2$-),

FTIR (cm-1, KBr): 2700, 1709.33, 2990,650,550.

LC-MS m/z: 206.3 (MH^+).

The general procedure used for the synthesis of Compounds A, B, and C

Synthesis of 1-hydroxy-3- lipo ester (LA-PRO-OH) A



DCC (2.058g, 10.0 mmol) and α-LA (2.058g, 10.0 mmol) dissolved in CH_2Cl_2 (50ml) were added to 1, 3-propanediol (1.086ml, 15 mmol) dissolved in CH_2Cl_2 (50ml) (50ml). At r t, the mixture was stirred for 48 hours. The solution was filtered to remove the precipitated N, N-Di cyclohexyl urea (DCU), and TLC with LA, DCU, and DCC was conducted. The remedy was to place it in the refrigerator in order to extract more DCU. The solution was then filtered once more before being evaporated under reduced pressure. The residue was dissolved in a hot 1:1 EtOH/CH_2Cl_2 solution (30 ml) and filtered once more.

After the evaporation of the solvent, the residue was dissolved in a small amount of CH_2Cl_2, and the solution was washed with 10 ml of 10% $NaHCO_3$ followed by10 ml of distilled water (twice). The solution was dried with $CaCl_2$, and TLC was performed. The solution was evaporated and the remaining solid was dissolved in a small amount of ethyl acetate, and crystallized by ether, put in the freezer until the crystal was formed, the crystal was filtered with suction filtration and was put in the suction oven.

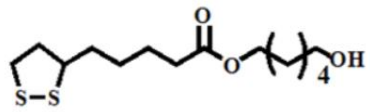
HPLC analysis shows that the compound was pure.

^{1}HNMR: 8.3 (1H, OH), 3.9 (t, 2H, -CH_2OH), 3.4 (t, 2H, S-S-CH_2), 3.5 (t, 2H, -CH_2-CO), 3.2 (m, 1H, CH_2CH-S-S-), 3.1 (t,2H, -CH_2-CO), 2.5 (dt, 2H, -S-S-CH_2CH_2-), 2.2 (m, 2H, -OCO-CH_2CH_2), , 1.8 (t, 2H, CH_2CH_2OH), 1.7 (dt, 2H, -OCO-$CH_2CH_2CH_2CH_2$-), 1.65 (m, 2H, -OCO-$CH_2CH_2CH_2$-).

FTIR (cm-1, KBr): 3296.71, 1697.01, 1229.60, 2929.74, 550, 440.49

LC-MS m/z: 264.4 (MH^+)

1-hydroxy-6- lipoester (LA-HEX-OH) B



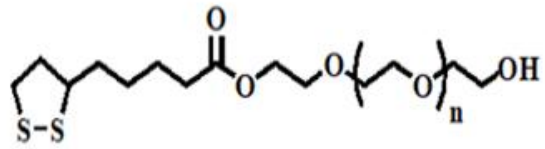
HPLC analysis indicates one compound.

^{1}HNMR: 8.3 (1H, OH),5.5 (t, 2H, CH_2OH), 4.0 (m, 2H, CH_2CH_2OH), 3.9 (t, 2H, -CH_2OH), 3.6 (m, 2H, $CH_2CH_2CH_2$OH), 3.5 (t, 2H, -CH_2-CO), 3.4 (t, 2H, S-S-CH_2), 2.2 (m, 2H, -OCO-CH_2CH_2), 3.2 (m, 1H, CH_2CH-S-S-), 3.1 (t,2H, -CH_2-CO), 2.5 (dt, 2H, -S-S-CH_2CH_2-), 1.8 (t, 2H, CH_2CH_2OH),1.7 (dt, 2H, -OCO-$CH_2CH_2CH_2CH_2$-), 1.65 (m, 2H, -OCO-$CH_2CH_2CH_2$-).

FTIR (cm-1, KBr):3295.85, 1710.27, 1540.85, 2851, 624, 443

LC-MS m/z: 306.4 (MH^+)

Synthesis of α-hydroxy-ω-lipoester PEG (LA-PEG-OH) C

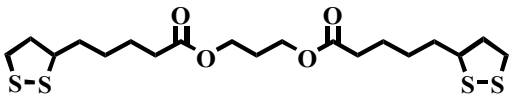


HPLC analysis indicates one compound.

^{1}HNMR: 4.6 (t, 1 H-CH_2-OH), 3.7 (t, 2H, CH_2-CH_2-O), 3.62 (m, 3H, -CH_2-CH_2-O, 1H, H-S-S), 3.4-3.7 (m, 168 H, (OCH_2CH_2)), 3.20 (m, 2H, -S-S-2H), 3.05 (m, 2H, -CH_2OH), 2.82 (t, 2H, -CH_2-CO-), 2.42 (m, 1H, CH_2-CH_2-S), 1.90-1.45 (m, 7H, -$(CH_2)_3$, CH_2-CH-S).

The general procedure used for the synthesis of compounds Y and Z

Synthesis of 1, 3-propane di-lipo ester (LA-PRO-LA) Y



DCC (5.145g, 25.0 mmol) and LA (5.145g, 25.0 mmol) were dissolved in CH_2Cl_2 (50ml) and added to 1, 3-propanediol (1.086ml, 10 mmol) dissolved in CH_2Cl_2 (50ml) (50ml). At r t, the mixture was stirred for 48 hours. The solution was filtered to remove the precipitated N, N-Di cyclohexyl urea (DCU), and TLC with LA, DCU, and DCC was conducted. The remedy was to place it in the refrigerator in order to extract more DCU. The solution was then filtered once more before being evaporated under reduced pressure. The residue was dissolved in a hot 1:1 EtOH/CH_2Cl_2 solution (30 ml) and filtered once more.

The residue was dissolved in a tiny amount of CH_2Cl_2, and the solution was extracted with 10 ml of 10% $NaHCO_3$ and washed with 10 ml of water after the solvent had evaporated (twice). TLC was done after the solution was dried with $CaCl_2$. The solution was evaporated, and the

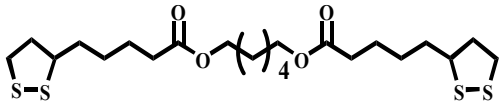
residual solid was diluted in a tiny amount of ethyl acetate and crystallized by ether. The crystal was then filtered with suction filtration and placed in the suction oven.

Two molecules of LA were linked to 1, 3-propanediol, the presence of the ester di-lipoate

HPLC analysis indicates one compound.

^{1}HNMR: 4.10 (t, 1 H, H-S-S-), 3.90 (t, 4 H, 2* -(OC-O-CH_2-CH_2)), 3.60 (m, 2H, -OC-O-CH_2-CH_2-), 3.16 (m, 2 H, -S-S-2 H), 2.40 (m, 1H, CH_2-CH-S-), 2.30 (t, 2H, -CH_2-CO-), 1.9-1.45 (m, 7H, -$(CH_2)_3$, CH_2-CH-S-) and the absence of the OH group at 4.60. FTIR (cm-1, KBr): 2930.15, 1709.33, 1234. The appearance of the OH group at 3296 indicates that two α-LA were coupled. LC-MS m/z: 452.4 (MH^+)

1, 6-Hexane di-lipo ester (LA-HEX-LA) Z



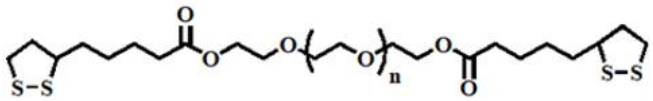
HPLC analysis indicates one compound

For ^{1}H NMR (DMSO-d6) δ (ppm) analysis, the appearance of the signals at 4.10 (t, 1 H, H-S-S-), 3.90 (t, 4 H, 2* -(OC-O-CH_2-CH_2)), 3.60 (m, 8H,-OC-O-CH_2-$(CH_2)_4$-), 3.16 (m, 2 H, -S-S-2 H), 2.40 (m, 1H, CH_2-CH-S-), 2.30 (t, 2H, -CH_2-CO-), 1.9-1.45 (m, 7H, -$(CH_2)_3$, CH_2-CH-S-) and the absence of the OH group at 4.60.

FTIR (cm-1, KBr): 2930.15, 1709.33, 1234, 664.06. the appearance of OH group at 3296 indicates that two α-LA were coupled.

LC-MS m/z: 495.3 (MH^+)

Synthesis of PEG di-lipo ester (LA-PEG-LA) X



Polyethylene glycol (HO-PEG-OH) 2KDa (5g, 2.5 mmol) was dissolved in toluene (100 ml), and traces of water were removed by the Dean-Stark apparatus. The solution was evaporated and reduced pressure and the residue was dissolved in CH_2Cl_2. DCC (2.06g, 10 mmol) and a solution of lipoic acid (2.06 g, 10 mmol) in CH_2Cl_2 were added, and the mixture was stirred for 48 h at rt. Continuation is like the previous compounds.

HPLC analysis indicates one compound.

For ^{1}H NMR (DMSO-d6) δ (ppm) analysis, the appearance of the signals at 4.10 (t, 4 H, 2* -(OC-O-CH_2-CH_2)), 3.60 (m, 8H,-OC-O-CH_2-$(CH_2)_4$-), 3.70 (t, 1 H, 1H, H-S-S-), 3.60 (m, 4H, -O-OC-CH_2-CH_2-O), 3.40-3.58 (m, 168 H, -(CH_2-CH_2-O)n), 3.16 (m, 2H, -S-S-2H), 2.40 (m, 1H, CH_2-CH_2-S-), 2.3(t, 2H, -CH_2-CO-), 1.9-1.45 (m, 7H, -$(CH_2)_3$, CH_2-CH-S-) and the absence of the OH group at 4.60.

FTIR (cm-1, KBr): 2885.62, 1734.47, 1241.94, 1111.80, 685.38. the appearance of the OH group at 3296 indicates that two α-LA was coupled.

3.1.2 Nitric oxide donors

General procedure for the synthesis of compounds (F, D, and ED)

The nitrate esterification of compounds (F, D, and E) followed the following procedure.

The purified and synthesized compounds (A, B, and C) were dissolved in CH_2Cl_2, to

which a 1:1 mixture of HNO_3 and H_2SO_4 was added. The final product was obtained after stirring the mixture for 24 hours.

1. 1-Nitrooxy–propane-3-lipoyl ester (LA-PRO-ONO_2) F. Yield: 92 %, modification percent: 95% (Griess Method).

2. 1-Nitrooxy–Hexane-6-lipoyl ester (LA-HEX-ONO_2) D. Yield: 89 %, modification percent: 97% (Griess Method).

3. α -Nitrooxy-PEG-ω-o-lipoyl ester (LA-PEG-ONO_2) E. Yield: 72 %, modification percent: 98% (Griess Method).

3.2 Biological Activities

Cell Culture: Different types of cancer cell lines were being grown in recommended cell culture media supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were being exposed to various LA analogs for different time intervals and apoptotic parameters were measured.

Determination of Cell Viability: Cells were exposed to the LA analogs for various time intervals. Cell membrane integrity was detected by trypan blue counting as a measurement of cell viability. For this assay, dead cells had leaky membranes through which the dye, trypan blue, could enter and stain DNA. Viable cells were not stained.

The synthesized compounds, were tested on a variety of cancer cells by using DMSO as solvent and LA as a reference.

4. Results and Discussion

4.1 Chemical synthesis

The synthesized compounds are divided into three different groups and classified according to their chemical structure and biological functions.

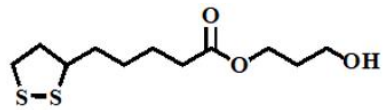
1- Antioxidants based on mono-lipoate, linked to different alkyl spacers.

2- Antioxidants based on di-lipoate, linked to different alkyl spacers.

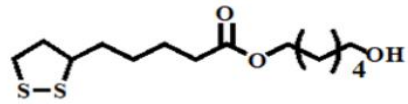
3- Bifunctional compounds, based on α-lipoic acid derivatives as antioxidants and nitric oxide (NO) donors.

Group 1: Antioxidants based on mono-lipoate, linked to different alkyl spacers.

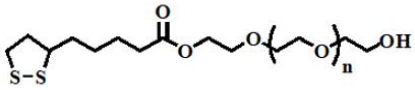
1. 1-hydroxy-3-lipoester (LA-PRO-OH) A



2. 1-hydroxy-6- lipoester (LA-HEX-OH) B

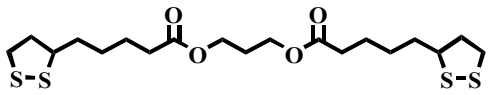


3. α-hydroxy-ω-lipoester PEG (LA-PEG-OH) C

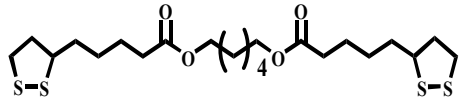


Group 2: Antioxidants based on di-lipoate, linked to different alkyl spacers.

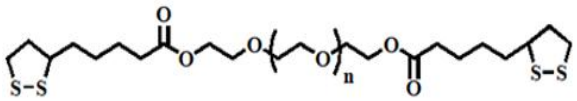
1- 1,3-propane di-lipo ester (LA-PRO-LA) Y



2- 1,6- Hexane di-lipo ester (LA-Hex-LA) Z

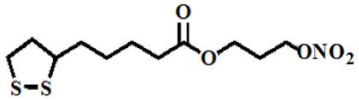


3- PEG di-lipo ester (LA-PEG-LA) X

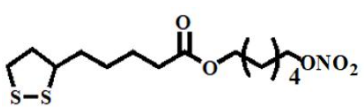


Group 3: Bifunctional compounds, based on α-lipoic acid derivatives as antioxidants and nitric oxide (NO) donors

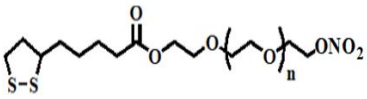
1- 1-Nitrooxy–propane-3-lipoyl ester (LA-PRO-ONO_2) F



2- Nitro -1,6- Hexane lipo ester (LA-HEX-ONO_2) D



3- Nitro- PEG lipo ester (LA-PEG-ONO_2) E



Antioxidants based on mono-lipoate, linked to different alkyl spacers

Various kinds of antioxidants are presented in this study. The major are α-lipoic acid and its derivatives that were designed as mono-lipoate derivatives and/or PEGylated [see section 4.1 chemical structures, group 1]. In this regard, α-LA is linked with only one terminal 1,3 propanediol, 1,6-hexanediol, and PEG, by ester pond using DCC as coupling reagent synthesis. Purification, characterization, and the biological activity of these compounds have been previously summarized.

Compounds A, B, and C were characterized by sing HPLC, ^{1}HNMR, FTIR, and LC-MS

For compound 1: The presence of one lipoate group was approved by the presence of the signals at 8.3 (1H, OH), corresponding to a hydroxyl group, and also the appearance of signals 3.9 (t, 2H, -CH_2OH), 3.4 (t, 2H, S-S-CH_2), 3.5 (t, 2H, -CH_2-CO), 3.2 (m, 1H, CH_2CH-S-S-), 3.1 (t,2H, -CH_2-CO), 2.5 (dt, 2H, -S-S-CH_2CH_2-), 2.2 (m, 2H, -OCO-CH_2CH_2), 1.8 (t, 2H, CH_2CH_2OH), 1.7 (dt, 2H, -OCO-$CH_2CH_2CH_2CH_2$-), 1.65 (m, 2H, -OCO-$CH_2CH_2CH_2$-).corresponding to the lipoate group. Compound 1 as an example.

The appearance of the hydroxyl group at 3296 in FTIR analysis indicates that the coupling is from one side.

LC-MS was used also and the experimental molecular weight was equal to theoretical values for these compounds. See section 3.1.1

Antioxidants based on di-lipoate, are linked to different alkyl spacers.

diesters of lipoate, compounds: Y, Z, and X were derived from propane-1,3-diol, compound Y, hexane-1,6-diol, compound Z, and PEG HO-PEG-OH, compound X [see Section 3.1.1.2 di-lipoate derivatives].

Two molecules of ALA were linked to OH producing ester pond by using DCC as a coupling reagent. The presence of the ester di-lipoate conjugate was verified by the appearance of signals at 4.10 (t, 1 H, H-S-S-), 3.90 (t, 4 H, 2* -(OC-O-CH_2-CH_2)), 3.60 (m, 2H,-OC-O-CH_2-CH_2-), 3.16 (m, 2 H, -S-S-2 H), 2.40 (m, 1H, CH_2-CH-S-), 2.30 (t, 2H, -CH_2-CO-), 1.9-1.45 (m, 7H, -(CH_2)3, CH_2-CH-S-) and the absence of the OH group at 4.60 for compound 2 as an example.

Bifunctional compounds, based on lipoic acid derivatives as antioxidants and nitric oxide (NO) donors as novel nitric oxide donors (i.e., $RONO_2$).

Hundreds of nitric oxide donors (NO) donors have been developed during the last two decades and have widely been used in biological research. This includes the disclosure of new NO donors, the development of NO-drugs and tissue specificity NO donors, and the preparation methods for various kinds of NO donors have been demonstrated [55,56,57,58,59,60,61,62].

Among these are Nitrosothiols (RSNOs) that were first synthesized in 1909.

Organic nitrates ($RONO_2$) as nitric acid esters of mono and polyhydric alcohols, representing the oldest class of NO donors that have been clinically applied. They are generally stable in neutral or weakly acidic aqueous solution and the homolytic bond dissociation energy of their O-NO_2 bond in aliphatic nitrates is around 41 kcal/ mol [63].

Organic nitrates ($RONO_2$) as drugs include glyceryl trinitrate (GTN), pentaerythrityl tetranitrate (PETN), isosorbide dinitrate (ISDN), isosorbide 5-mononitrate (ISMO), and nicorandil. Organic nitrates can also be prepared by nitration of the corresponding alcohols or reactive alkyl halides with $AgNO_3$[64].

During the present work, a variety of PEGylated and/or nonPEGylated, lipoate, and nitric oxide donors' derivatives were synthesized where part of the synthetic methods follows literature procedures [65], and others were prepared according to modified procedures. All procedures were optimized and simplified to obtain a high degree of modification [see Section 3.1.1.2 Experimental part]. Here we focus our attention on diverse combinations of antioxidants and nitric oxide donors linked to various carriers as PEG/alkyl chain through ester bond [see Scheme 4]. Several modifications were implemented by using N, N-dicyclohexylcarbodiimide (DCC) as coupling reagents.

Our new type of PEGylated and/or alkylated nitric oxide donors, organic nitrates ($RONO_2$) are distinct from other previously prepared PEGylated nitric oxide donors [65],

in the aspect that the NO molecule is conjugated directly to the hydroxyl group without any spacer or carrier. This feature confers simplicity in preparation, purification, and characterization.

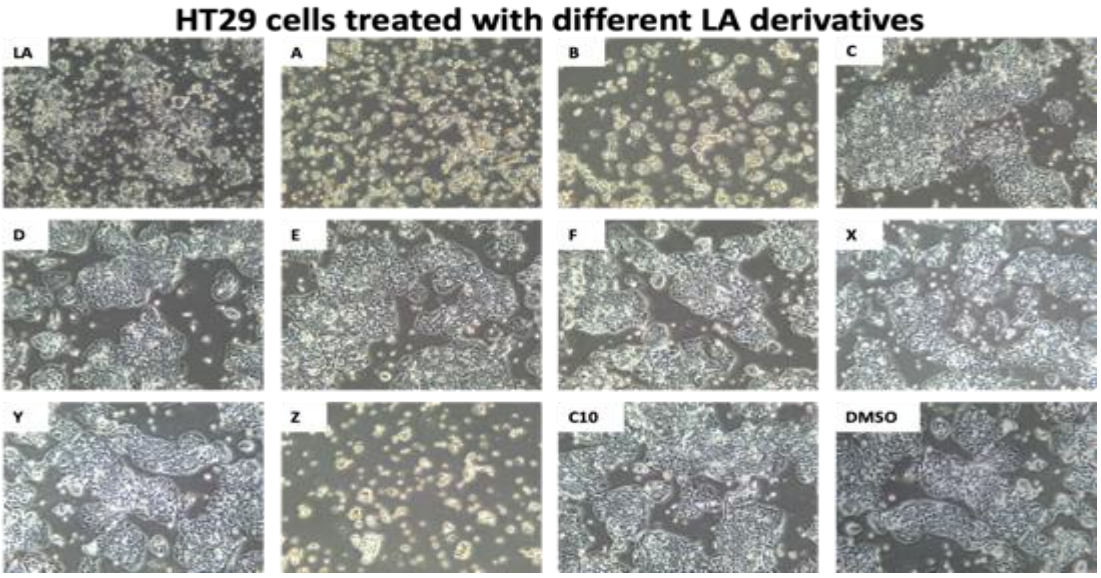
Since the nitric oxide structures are relatively unstable in aerobic aqueous media; the percentage of modification and the kinetic studies for the NO-releasing profile have not been studied by HPLC or NMR techniques. However, the percentage of modification of these compounds was determined by UV spectroscopy based on the Griess Method. The NO release profile compared to its release from standard Na_2NO_3 was analyzed by UV spectroscopy based on the Griess Method.

Compounds 1,3 and 5 were used as starting material for nitric oxide donors. Griess methods were used to ensure the presence of ONO-.

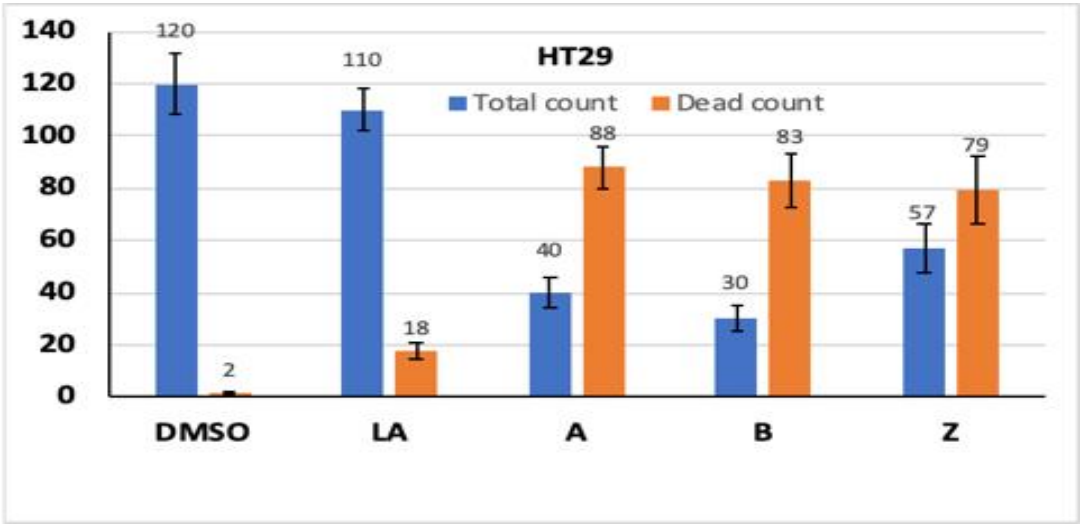
4.2 Biological Activity

The major aim of this study was to synthesize unique molecules that act as nitric oxide donors, antioxidant and bifunctional molecules acting as antioxidant and nitric oxide donors simultaneously. Many of these compounds were expected to have anti-cancer activity, in order to prove that, MDA MB231 and MCF7 breast cancer cells and HT29 colon cancer cells were cultured and treated with lipoic acid as a reference compound and the synthesized compounds.

The preliminary results show that compounds A and B which are possessing LA linked to alcohols as spacers and compound Z with two LA moieties linked with a short spacer are potent inducers of cell death in cancer cells. (Figures 9, 10, and 11). The other analogs didn’t have any anti-cancer effects

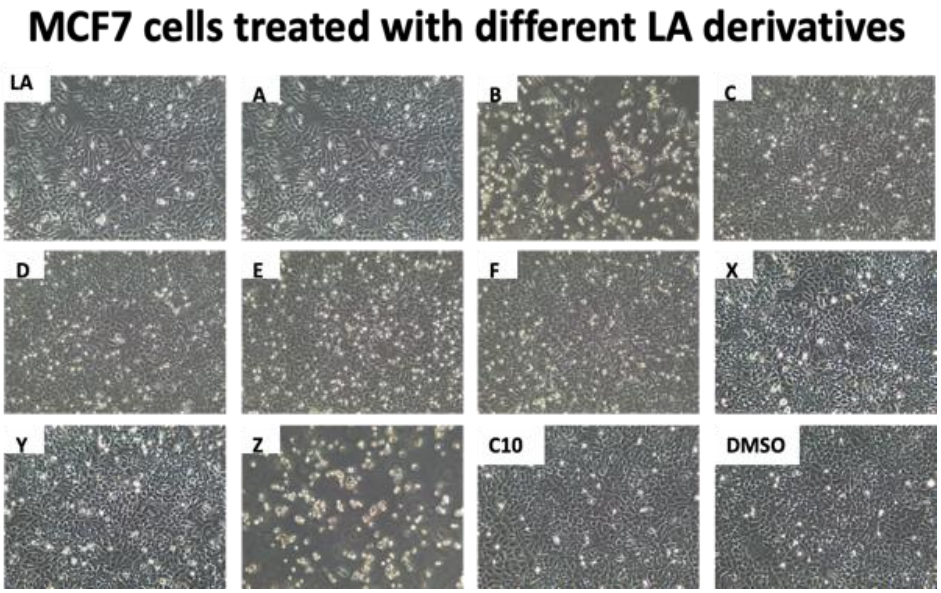


A

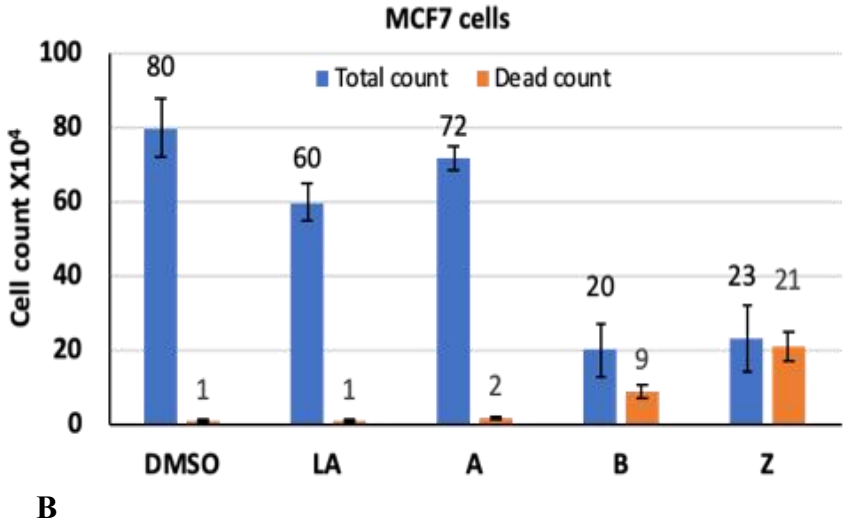


B

Figure 9: The effect of different Alpha-Lipoic Acid Analogs on HT29 colon cancer cells. A. representative micrograph showing the effect of the indicated compounds on the morphology of treated cells. B. Statistical analysis of the effect of different treatments on the cells. Cells were treated with 20nM of the indicated compounds for 48hrs. Bars represent the standard error of the mean of three replicates.

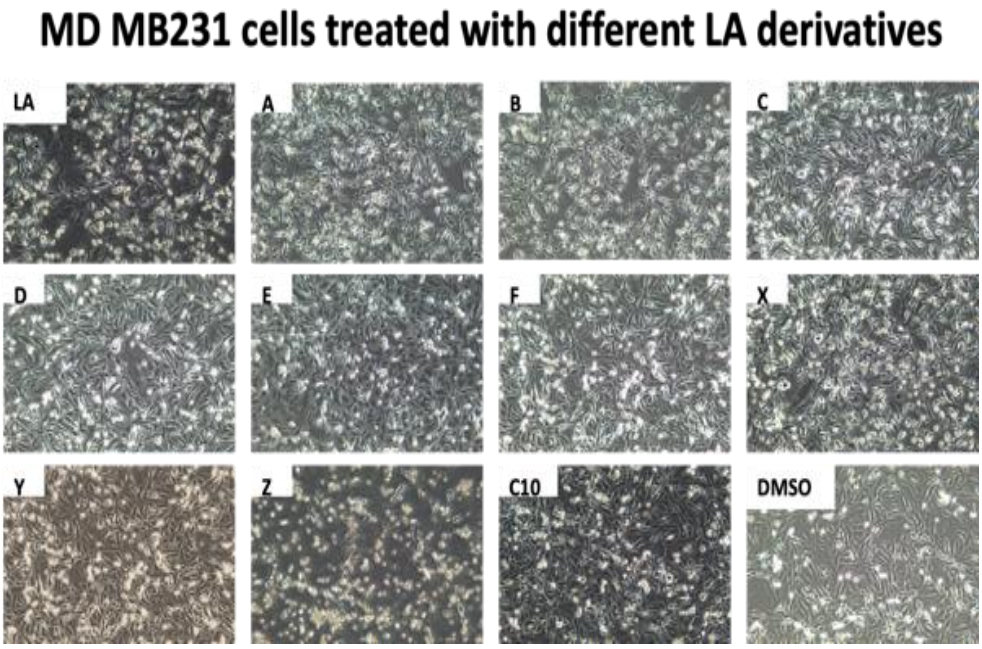


A

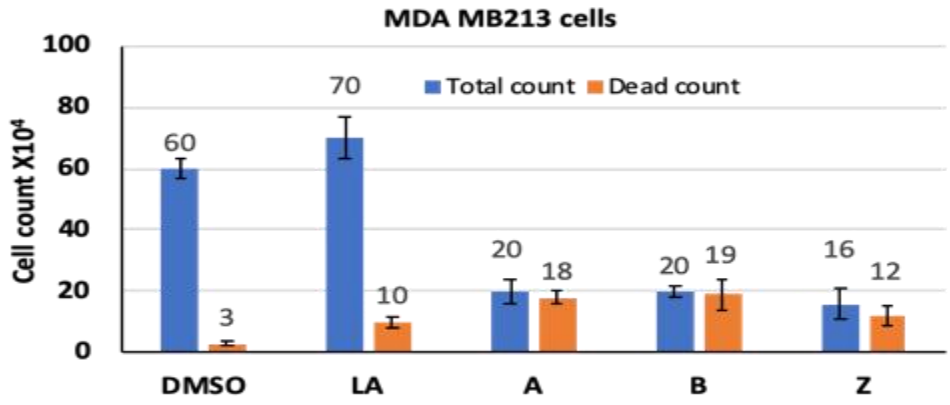


B

Figure 10: The effect of different Alpha-Lipoic Acid Analogs on MCF7 breast cancer cells. A. representative micrograph showing the effect of the indicated compounds on the morphology of treated cells. B. Statistical analysis of the effect of different treatments on the cells. Cells were treated with 20nM of the indicated compounds for 48hrs. Bars represent the standard error of the mean of three replicates.



A



B

Figure 11: The effect of different Alpha-Lipoic Acid Analogs on MDA MB213 breast cancer cells. A. representative micrograph showing the effect of the indicated compounds on the morphology of treated cells. B. Statistical analysis of the effect of different treatments on the cells. Cells were treated with 20nM of the indicated compounds for 48hrs. Bars represent the standard error of the mean of three replicates.

All of the synthesized compounds were tested on HT29 colon cancer cells, and when compared to the starting material LA, compounds A, B, and Z have a greater effect on this type of cancer cell than LA, with compound B having the greatest effect, most likely due to the presence of PEG spacers, which increase the solubility of this compound. 9th Figure

Furthermore, all samples were tested on MCF7 breast cancer cells in the same way; figure 10 shows that compounds A, B, and Z have an effect on this type of cancer cells that is superior to LA, with compound Z having the greatest effect.

Figure 11 shows that compounds A, B, and Z have the greatest effect on MDA MB213 breast cancer cells than LA, with compounds A and B nearly having the same effect.

Because none of the nitric oxide donors' compounds have any anti-cancer effect on the treated cells, the above results do not match our expectations. Alcohol esters, on the other hand, produced good results. This is not to say that the other compounds have no effect on different types of cancer cells.

These findings do not imply that our theory is incorrect, as other types of cancer cells should be tested and may yield different results.

5. Conclusion

Lipoic acid is a chemical compound that is used in many medical areas, especially in Europe. LA is used as an antioxidant, and stabilizer, for diabetes and for diet. New derivatives of LA which are considered both antioxidants and NO donors, were synthesized, characterized, purified, and tested on various cancer cells to show their anti-cancer activity.

The synthesized compounds are divided into two main categories according to their functional groups [see section 3.1 chemical synthesis]

Compounds were studied in vitro in order to evaluate their anti-cancer activity. LA and the synthesized compounds were tested on MDA MB231 cells, HT29 cells, and MCF7 KHOS cells. Compounds 1,3 and 4 have an effect only

In general, our preliminary results and evidence presented here suggested that several of the synthesized compounds are considerably anti-cancer active.

6. References

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7. Appendices

Appendix A: ^{1}H-NMR spectral for compounds 1, 2, 3, 4, 5, 6 and LA

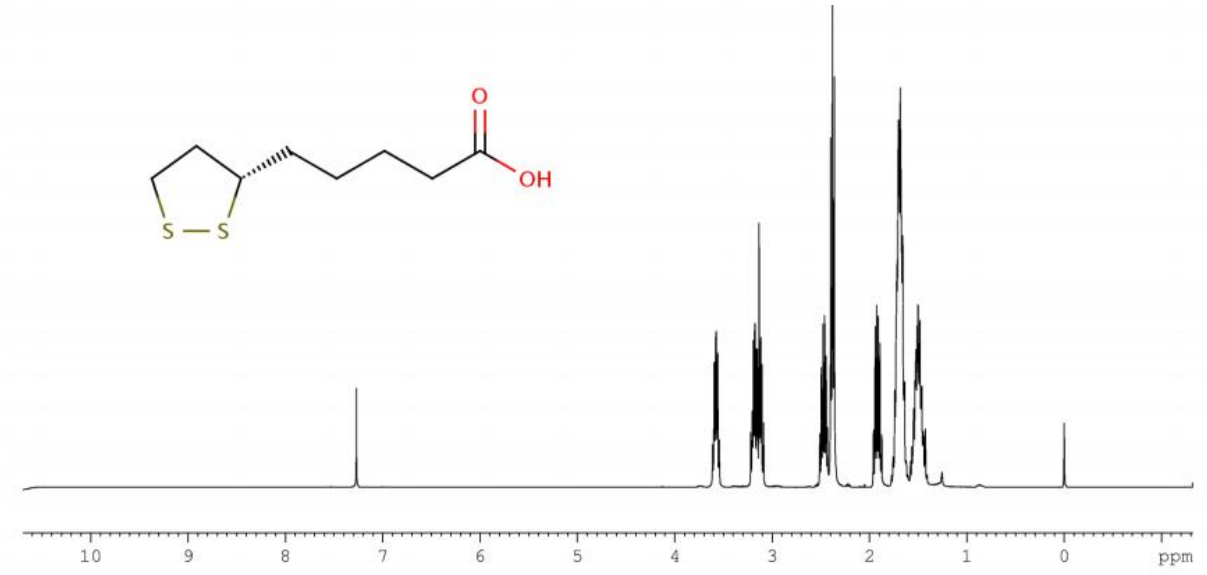

Figure 12: ^{1}H-NMR spectral for LA

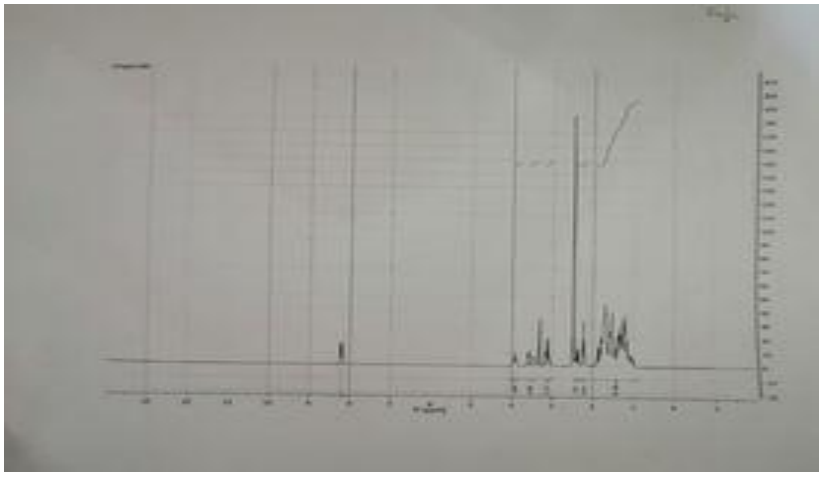

Figure 13: ^{1}H-NMR spectral for compound 1

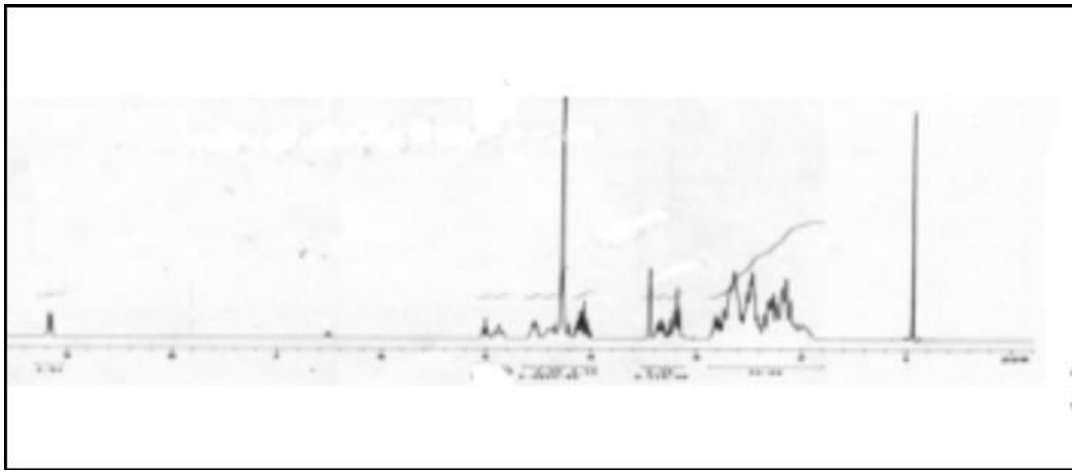

Figure 14: ^{1}H-NMR spectral for compound 2

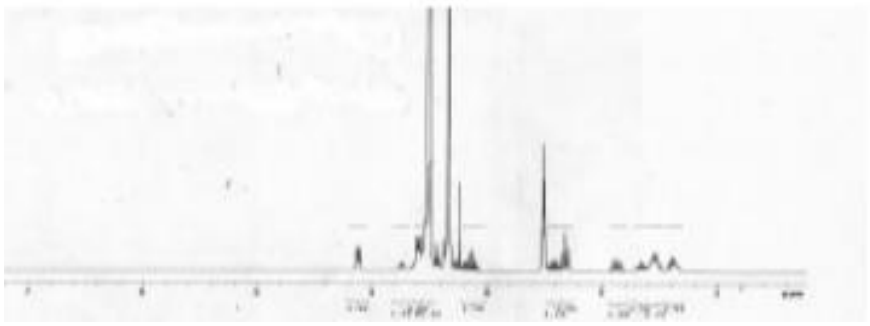

Figure 15: ^{1}H-NMR spectral for compound 6

Appendix B: HPLC spectrum for compounds 1, 2, 3, 4, 5, 6 and LA

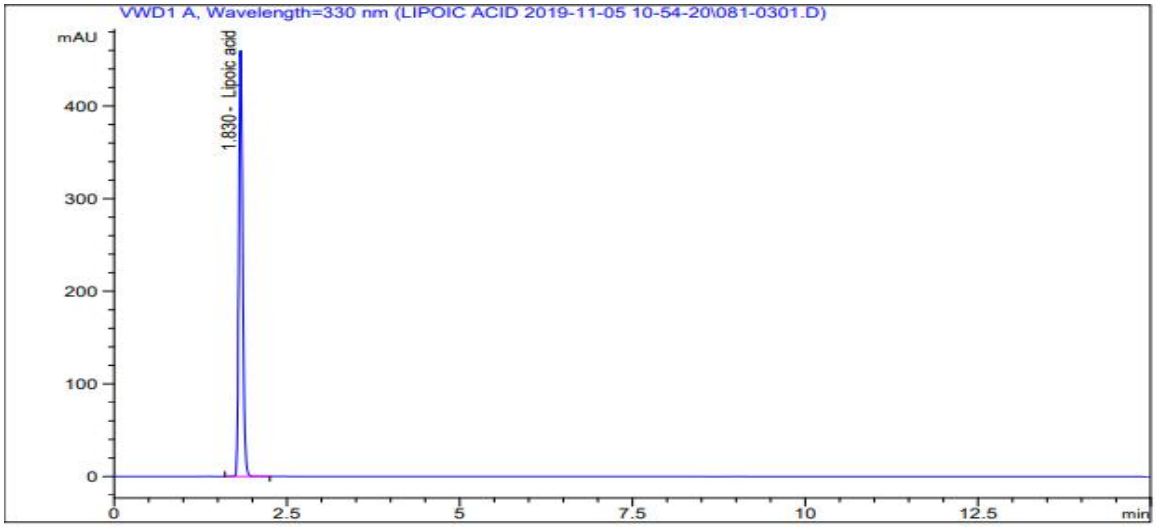

Figure16: HPLC spectrum of LA

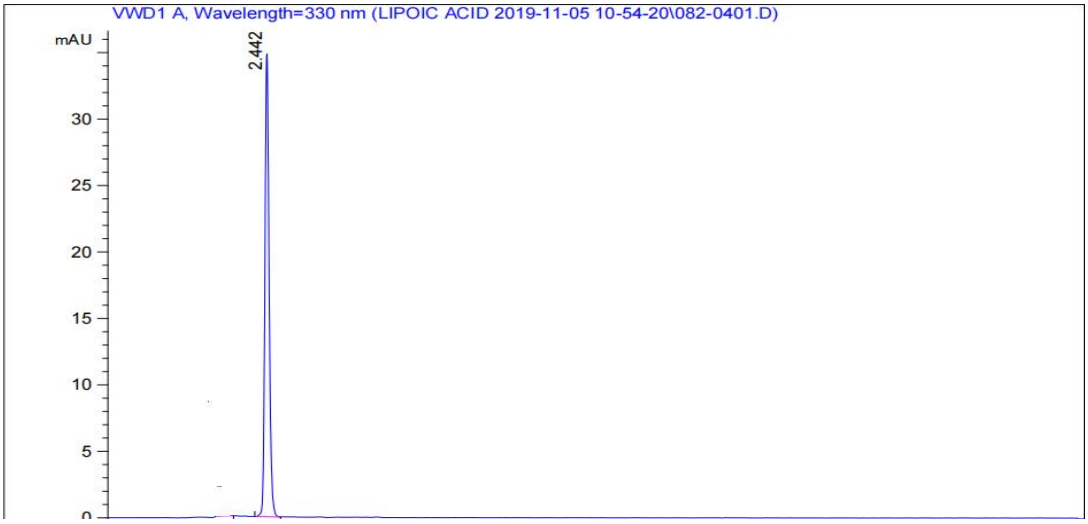

Figure 17: HPLC spectrum of compound 1

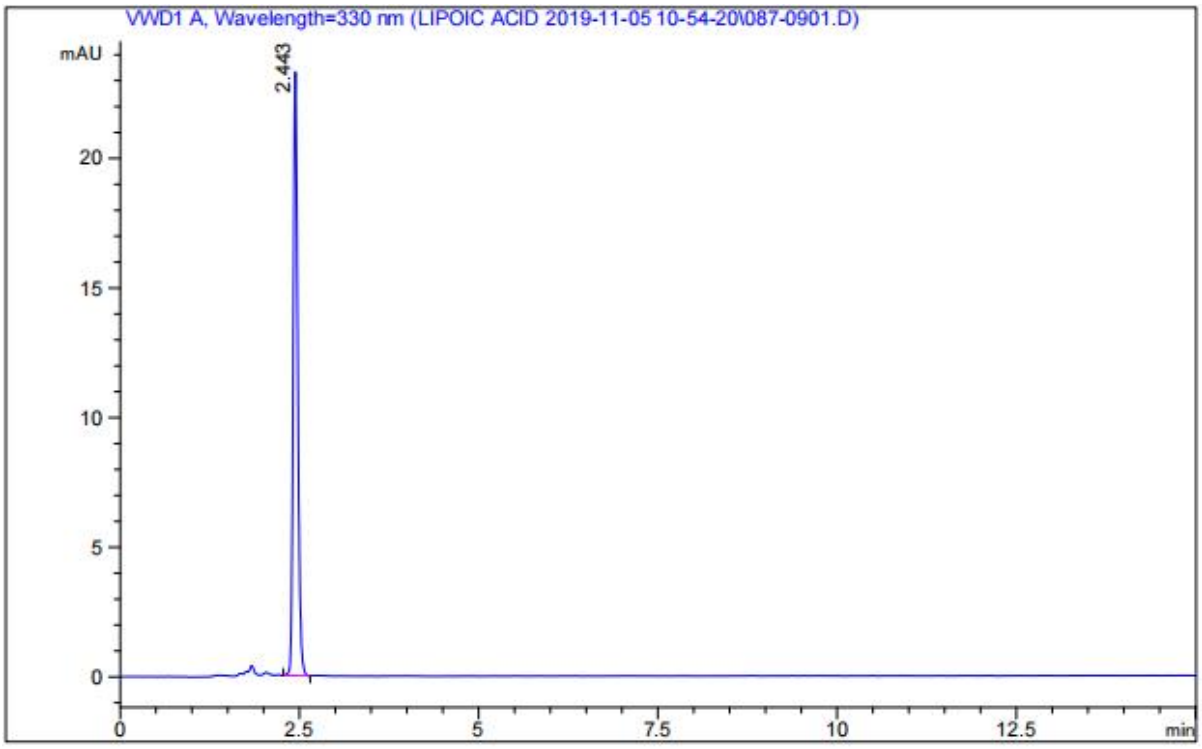

Figure 18: HPLC spectrum of compound 2

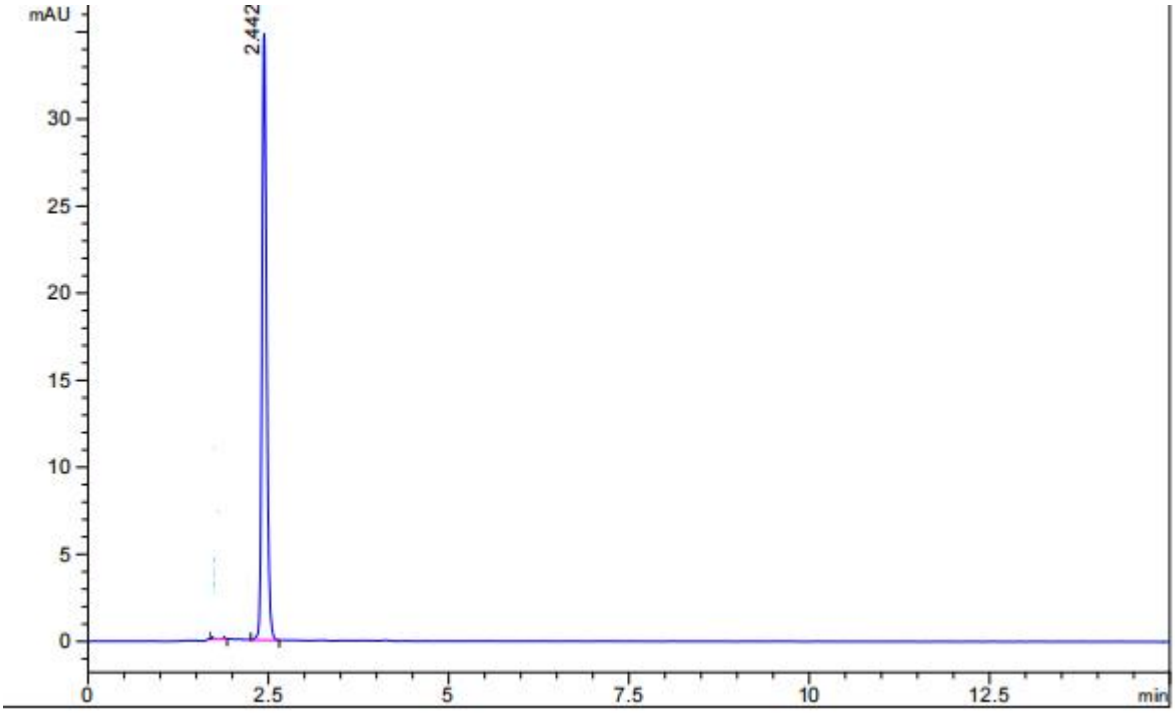

Figure 19: HPLC spectrum of compound 3

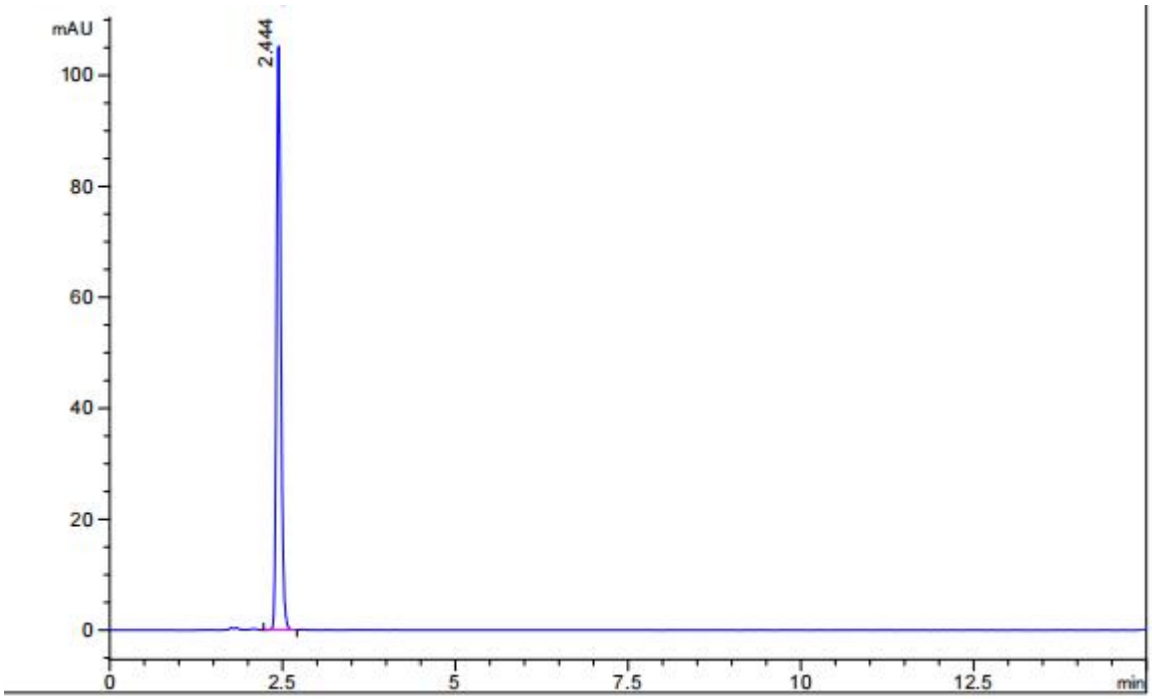

Figure 20: HPLC spectrum of compound4

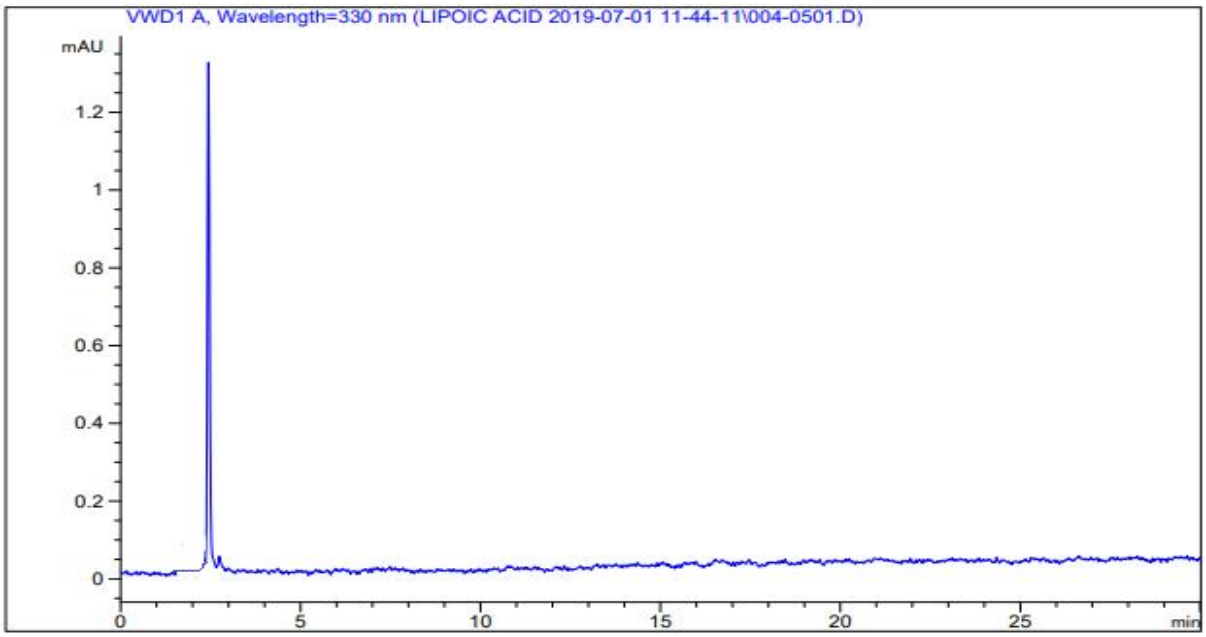

Figure 21 : HPLC spectrum of compound 5

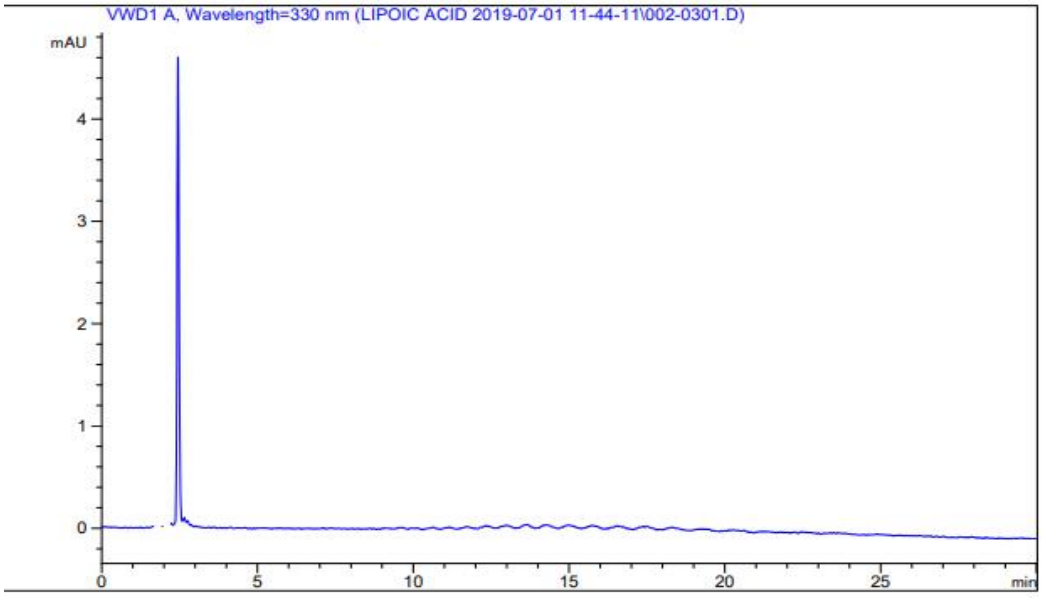

Figure 22: HPLC spectrum of compound 6

Appendix C: LC-MS spectra for compounds 1, 2, 3, 4, 5, 6 and LA

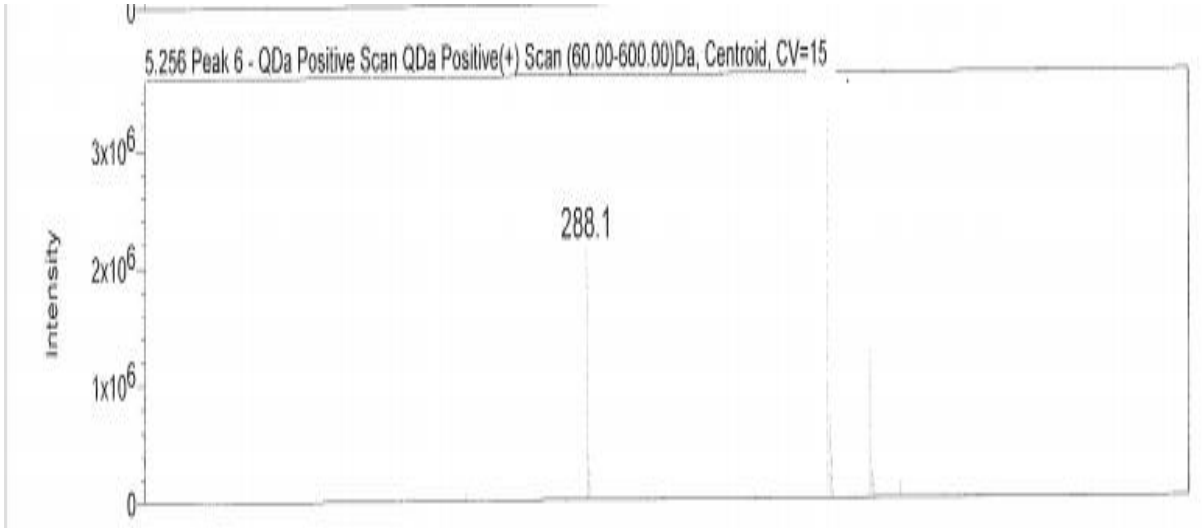

Figure 23: LC-MS for compound 1

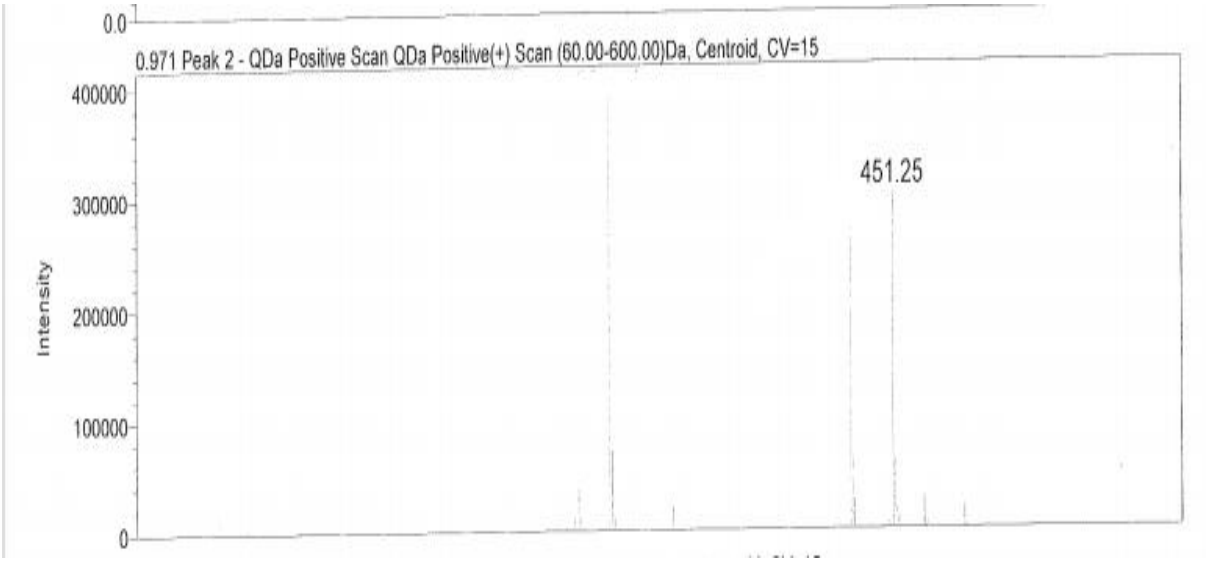

Figure 24: LC-MS for compound 2

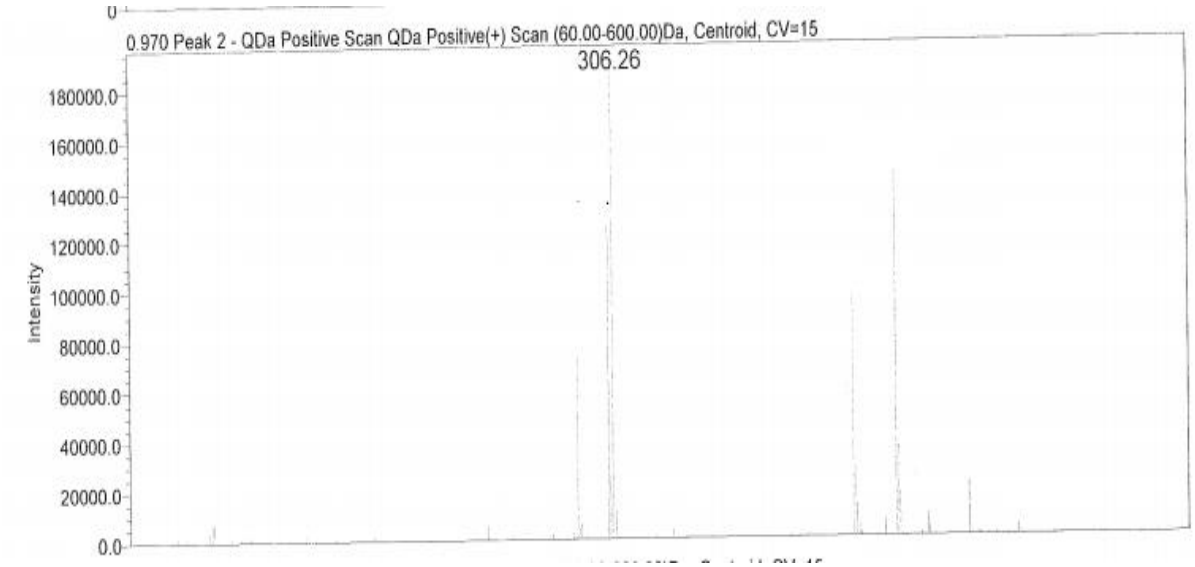

Figure 25: LC-MS for compound 3

Appendix E: FTIR analysis for 1, 2, 3, 4, 5, 6 and LA

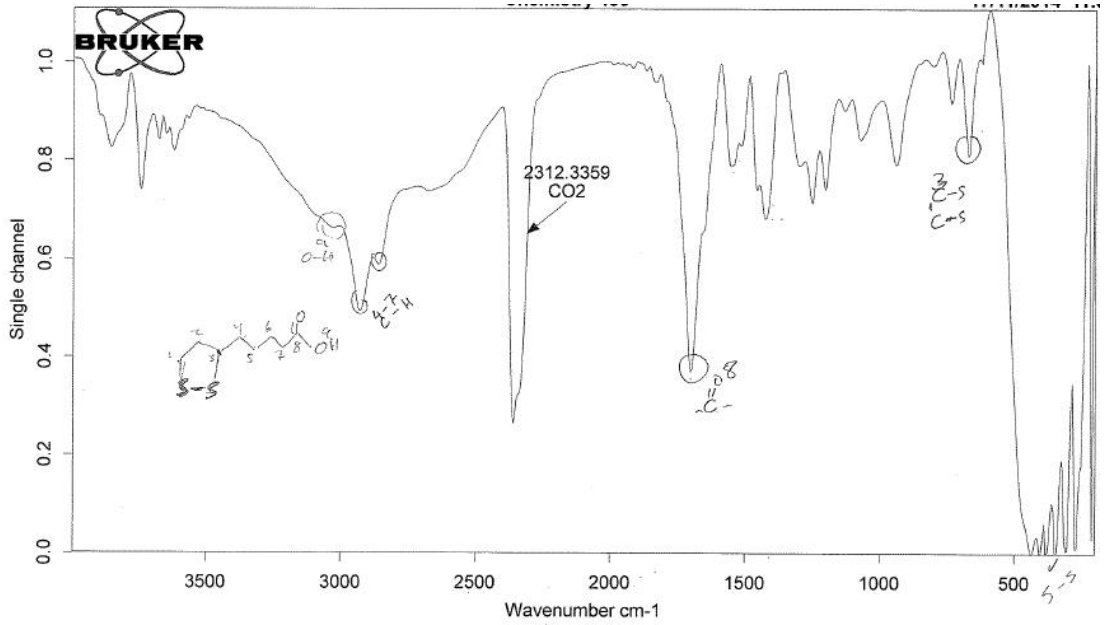

Figure 26: FTIR for LA

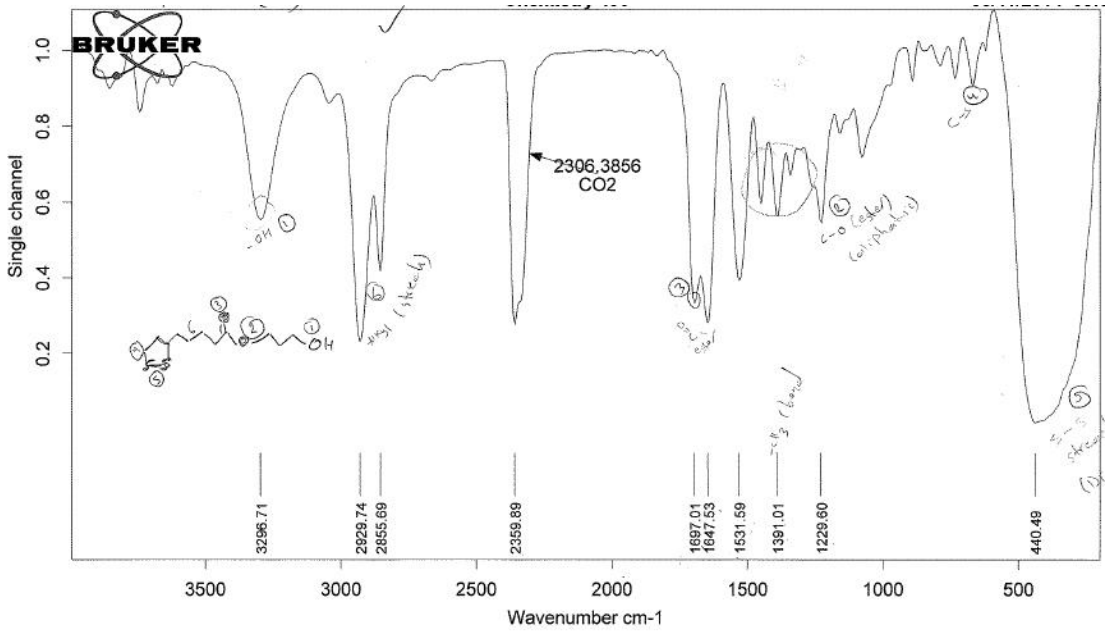

Figure 27: FTIR for compound 1