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Use of Natural Leaves Extract as Substituent of Synthetic Preservatives and Antioxidants in Pharmaceutical Preparations

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Use of natural leaves extract as substituent of synthetic preservatives and antioxidants in pharmaceutical preparations

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Dedication

This thesis is dedicated to the sake of Allah, my Creator. To my great teacher and messenger, Mohammad (May Allah bless and grant him), who thought us the purpose of life. To those who encouraged me and helped me fulfill my potential and made the cloud rain all the love and passion I have to the most precious Mom and Dad "True love and truth that does not change", to my brothers, sisters my precious family and my dearest friends, to my supervisors Dr. Fuad Al-Rimawi & Ms Reem Yaghmour, to my teachers in collage of Science & Technology, to my dearest colleagues, to the Palestinian martyrs and prisoners, to Al-Aqsa Mosque, to my beloved University; Al-Quds University, at last but not least to my beloved homeland; Palestine.

Declaration

I certify that this thesis submitted for the degree of master, is the result of my own research, except where otherwise acknowledged, and this thesis has not been submitted for the higher degree to any other university or institution.

Signed: .....

Mahmoud Neim Mahmoud Sbeih

Date: 2018/7/14

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I wish to express my deepest gratitude to my supervisor Dr. Fuad Al-Rimawi for his encouragement to start this work and for his great leadership and valuable comments. My warmest thanks belong to my parents for their confidence in me and for being always so supportive and interested in my work and well-being. I would like to thank them. Finally, my dearest thanks are addressed to my family.

Special thanks for the family Beit Jala Pharmaceutical Company (BJP) for their help during my experimental work.

Abstract

Although synthetic preservatives and antioxidant may provide high antimicrobial and antioxidant effectiveness, they are usually associated with adverse reactions Currently, there is an increasing desire for high quality, with fewer chemicals, and long-term pharmaceutical products. Thus, there is an urgent need to develop moderate pharmaceutical products use of protected natural materials from microbiological growth during storage and use. The aim of this study was to evaluate the antimicrobial activity and antioxidant activity of olive leaves extract and oleuropein .The main active compound of the olive leaf extract) as well as thyme oil against three bacteria (one gram-positive and 2 gram-negative) and two fungi (one yeast and one mold) in agar method. Those with such activity (working against two gram-negative bacteria, one gram-positive bacteria, one yeast and one mold) can be used as preservatives and will, therefore, be used as a preservative in a pharmaceutical syrup to replace chemical preservatives (methyl-propylparaben and benzalkonium chloride), and replacing Butylhydroxytoluene as chemical antioxidant.

In this work, oleuropein with different concentrations (0.2, 0.4 and 0.6 % w/v) and extracts of olive leaves with concentrations (0.2, 0.3 and 0.4% w/v) and thyme oil with concentration (0.1% v/v) and a mixture of oleuropein and thyme oil with concentration (0.4% w/v and 0.1% v/v) were examined as natural antimicrobial preservatives in pharmaceutical syrups and very promising results were obtained. Results showed that oleuropein can be used as preservatives with concentration (0.6 w/v), extract of olive leaf with concentration (0.4 w/v) and mixture between oleuropein and thyme oil with concentration (0.4 and 0.1 v/v) The results showed that thyme oil and oleuropein have synergistic activity of against microbes. Laboratory pharmaceuticals were analyzed in terms of the percentage of active substance using HPLC and antimicrobial activity in

microbiological laboratories and physical properties. The results showed that pharmaceuticals preparations were stable and effective for three months on accelerated Condition (40±2 °C /75%±5% RH).

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List of Abbreviations

TABC	Aerobic bacterial count
ATCC	American Type Culture Collection
SCDA	Casein Digest Agar
CDSLP	Casein Digest- soy leithin poly sorbate
CFU	CFU colony-forming unit
DPPH	2,2- diphenyl-1-picrylhydrazy
DD	disc-diffusion
FLM	Fluid Lactose Medium
FIC	FIC Ferrous ion chelating
HPLC	High performance liquid chromatography
OH	OH hydroxide
LF	Laminar Flow
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MIC	Minimum Inhibitory Concentration
NO	Nitric oxide
OLE	Olive leaves extract
O.D	Optical density
ROS	reactive oxygen species
SABDA	Sabouraud Dextrose Agar
SABD, SABDA	Sabouraud Dextrose Broth, Agar
NaCl	Sodium chloride
SCD, SCDA	Soybean-Casein Digest Broth, Agar
SCDM /SCDA	Soybean-Casein Digest Medium /Agar
SOP	standard operating procedure
TS	sterile saline

Eos	thyme oil
TYMC	Total Aerobic Yeasts and Molds
ZOI	zone of inhibition

Chapter One

: Introduction

1.1 Antimicrobial activity

Antimicrobial preservatives are materials added to pharmaceutical products to inhibit the growth of microorganisms introduced through the manufacturing process or during usage and storage (E.O. Akinkunmi et al., 2010). Adding antimicrobial preservatives to sterile products packaged in multiple-dose containers can inhibit the growth of microorganisms that could be accidentally introduced during repeated withdrawal of individual doses. These preservatives in finished dosage forms meet the requirements for added substances under limitations (V.W.C. Sutton et al., 2002). Antimicrobial agents may contain toxic materials. Have been reported (J. breilkreutz et al., 2007), (N. Shehab et al., 2009) e.g. for benzyl alcohol, coloring agents, propylene glycol, ethanol and propylparaben Antimicrobial agents may contain toxic materials. Thus, it is essential to add the effective concentration of the preservatives to packaged products taking in consideration that it is under a level that may be toxic to humans (J. breilkreutz et al.,2007) and An antioxidant is a molecule that inhibits the oxidation of different molecules. oxidation is a chemical process that transfers electrons or hydrogen from a substance to an oxidizing agent. oxidation reactions will produce free radicals. In turn, these radicals will begin chain reactions, once the chain reaction happens in a cell, it will cause damage or death to the cell. Antioxidants stop these chain reactions by removing free radical intermediates, and inhibit different oxidation reactions (Marino et al., 1999).

Recently, natural antimicrobial agents have been extensively tested and used instead of chemical agents in the pharmaceutical industry. Chemical preservatives are notorious for their carcinogenic attributes and residual toxicity as compared to the safer natural antimicrobial agents (N. Shehab et al., 2009). Chemical preservatives are dangerous to use in pediatric formulations for their toxicity and have many cause side effects on children. Therefore, recent research has been focused on natural antimicrobial agents to investigate their preservation abilities and identify the specific effective substance to expand the spectrum of antimicrobial activity over that of the regulatory approved substances. Naturally derived compounds and other natural products may have applications in controlling bacteria in Pharmaceutical dosage form (V.W.C. Sutton et al., 2002).

One group of naturally derived antimicrobial compounds is medicinal plants and their essential oils. These compounds are safe, have a certain degree of antimicrobial activity, and could inhibit the growth of pathogens and spoilage microorganisms thereby improving the shelf life of pharmaceutical products (M. Mari et al., 2003; J. Obagwuet al., 2003). The ability of medicinal plant extracts to control the growth of pathogens and spoilage bacteria and their use as alternatives to conventional natural preservatives is emerging because they are generally safe to humans, and friendly to the environment (R. Thangavelu et al., 2004).

There is an increasing interest in phytochemicals as new sources of natural antioxidant and antimicrobial agents. The use of synthetic antioxidants in the food industry is severely restricted as to both application and level (K. TAWAHA et al., 2007; Y. PENG et al., 2005). Currently, there is a strong debate about the safety aspects of chemical preservatives, since they are considered responsible for many carcinogenic and teratogenic attributes, as well as residual toxicity (M.R. MOREIRA et al., 2005).

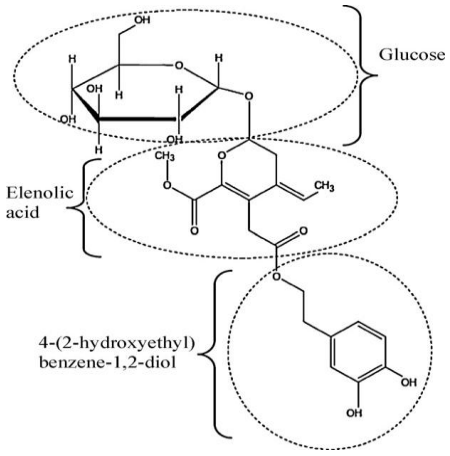
The effectiveness of natural plant extracts need to be evaluated to specify their antimicrobial activity and potential side effects in packaged pharmaceutical products.

Therefore, the study of the antimicrobial activity of some medicinal plants extract has become an important research interest and a big challenge. (H. Shtaya, 2015).

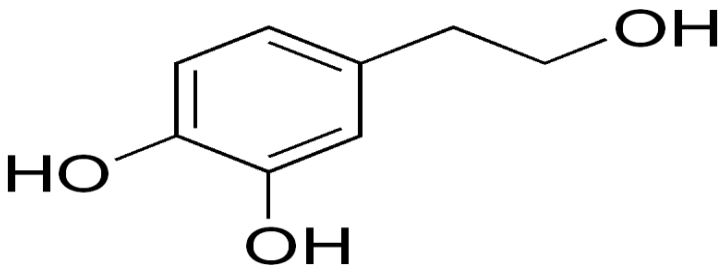
1.2 Olive leaves extract

The olive tree, botanically-classified as Olea europaeaL.is one of the most important fruit trees in Mediterranean countries. The characteristic green to blue-black fruit of this shrub yields useful edible oil. Both the oil and the dried green-grayish colored leaves are used medicinally (Wernet al., 1985). The olive tree has been held in high esteem throughout history. The oil is symbolic of purity and goodness, while the olive branch represents peace and prosperity. Historically, the knowledge of the medicinal properties of the olive tree date back to the early 1800s, when it was used in liquid form for malaria treatment (Wernet al., 1985). In the early 1900s a bitter compound was found in the leaves of certain olive trees called "oleuropein," which was thought to be part of olive tree's potent disease-resistant structure (Walker et al., 1997). The olive tree has been the source of natural healing agents down through the ages including the olive oil produced from its fruit. For centuries, teas and other preparations made from olive leaves have been used successfully to treat fevers and gastrointestinal complaints, including parasites in human patients. Olive leaf extract is effective against a broad spectrum of microbial agents, including viruses, bacteria and even parasites, and can considered one of the most useful and safe natural anti- microbial herbal extracts yet discovered (Pooleyet al., 1997). It can inhibit and kill over 100 microorganisms which can cause disease, and death on a broad scale, thus it can be considered nature antibiotic remedy to be used to prevent and treat numerous animal infectious conditions and health problems related to: viruses, bacteria, parasites, allergy conditions, skin problems (psoriasis), inflammation (arthritis, sinusitis, bursitis, etc.), gastrointestinal problems, ulcers, free radical overload, overburdened immune system, and wound healing.

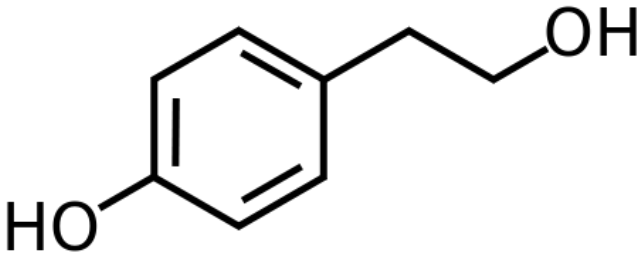
Olive leaf extract was derived from the leaves of the olive tree. It is a source of many phytochemicals. It was found to be part of a compound produced by olive trees that make them particularly vigorous and resistant to insect and bacterial damage (Pooley et al., 1997) The OleaeuropaeaL. leaves represent a typical herbal drug of the Mediterranean area, commonly used in traditional medicine as vasodilatory, hypotensive, anti-inflammatory, antirheumatic, diuretic, antipyretic, and hypoglycemic agents (Somovaet al., 2000). The active constituents of olive leaf have a wide number of ingredients, including the chief constituent oleuropein (60-90 mg/g) and several types of polyphenolic compounds. The following polyphenols were detected in olive leaf tissue: oleuropein, hydroxytyrosol, tyrosol (figure (1.1), elenolic acid derivatives, caffeic acid, oleuropein, verbascoside, rutin, luteolin 7-O-glucoside, luteolin 4-O-glucoside, apigenin-7-O-rutinoside and apigenin 7-O-glucoside (Benavatet al., 2000). There are at least six active substances (oleuropein, hydroxytyrosol, caffeicacid, vanillin, luteolin-7-glucoside, and verbascoside) in the extract. These six substances work together synergistically to prevent resistance by pathogen microorganisms. While oleuropein is the ingredient most studied, there are in fact 95 different chemicals in the leaf and a balance of ingredients seems to work the best. Oleuropein content varies from17% to 23% depending upon the time of year the leaves are harvested (Le toutouret al., 1992).



Oleuropein



Hydroxytyrosol



Tyrosol

Figure (1.1): Chemical structure of oleuropein, hydroxytyrosol and tyrosol.

1.2.1 Oleuropein

Oleuropein (figure (1.1) is a natural product of secoiriodoid group; hetrosidic ester of elenolic deteracid and 3,4-dihydroxyphenyl ethanol, containing a molecule of glucose, the hydrolysis of which yields elenolic acid glucoside and hydroxytyrosol. Many molecules isolated from Oleaeuropea fruits or leaves are thought to have been originated from Oleuropein via aglycon, by the opening of elenolic acid ring with a final rearrangement into the secoiridoid compound, such as hydroxytyrosol(Syedet al., 2010).

The amount of oleuropein in olive leaves depends on several factors, including oleaeuropea variety, time collection, possible infestation by olive fly DacusOlea, climate, conditions of storage, and the methodology of extraction. Oleuropein content varies from 17%-23% depending on the time of year the leaves are harvested.

1.2.1.1Pharmacological properties of Oleuropein

Oleuropein has several pharmacological properties, including antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer, antimicrobial, and antiviral, and for these reasons, it is commercially available as food supplement in Mediterranean countries. In addition, Oleuropein has been shown to be cardioprotective against acute adriamycin cardiotoxicity and has been shown to exhibit anti-ischemic and hypolipidemic activities (Syed et al., 2010), figure 2.

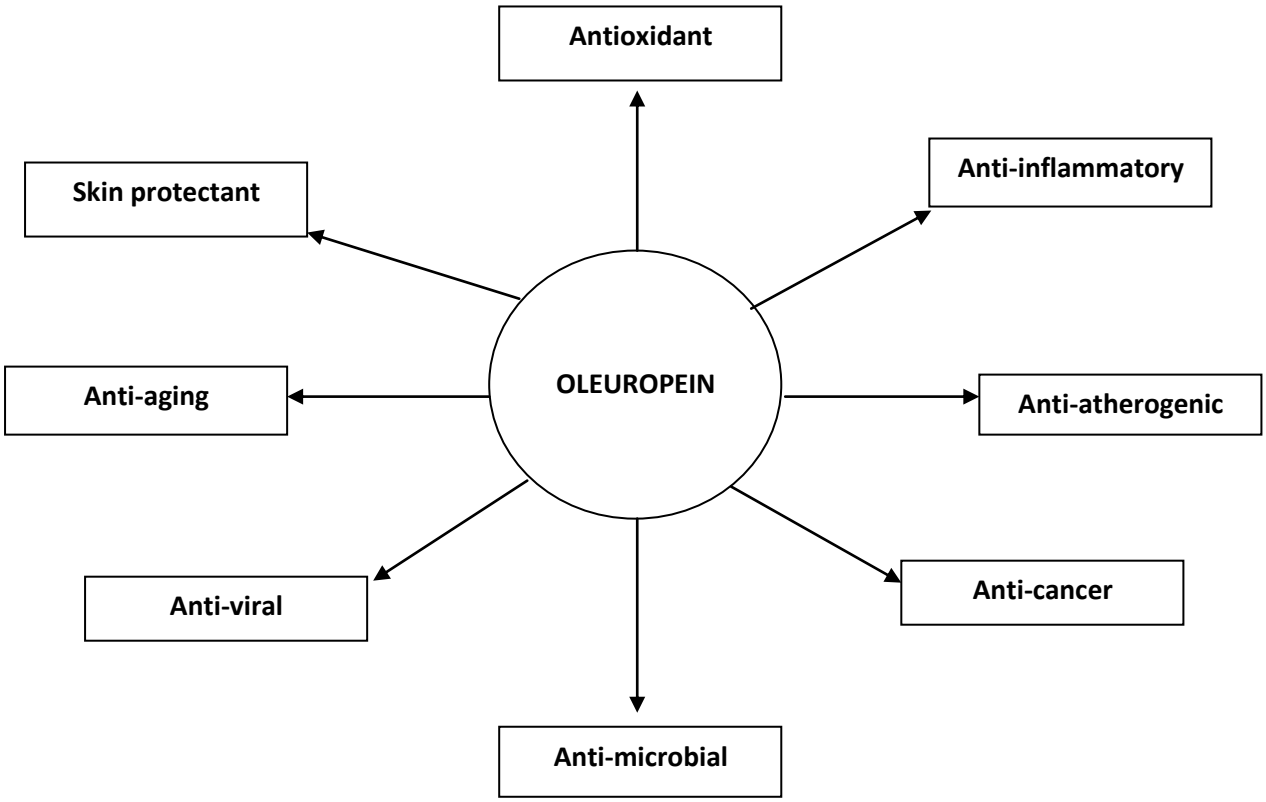


Figure (1.2): Pharmacological effects of Oleuropein

1.2.1.2 Antioxidant activity:

Oleuropein has high antioxidant activity in vitro, comparable to a hydro-soluble analog of Tocopherol. Oleuropein scavenges superoxide anions and hydroxyl radicals, and inhibits the respiratory burst of neutrophils and hydrochlorous acid-derived radicals (Visioli et al., 2002).

1.2.1.3 Anti-inflammatory effect:

Recent studies showed that Oleuropein increases nitric oxide (NO) production in macrophages challenged with lipopolysaccharide through induction of the inducible form of the enzyme nitric oxide synthase, thus increasing the functional activity of these immunocompetent cells. It is well known that Oleuropein elicits anti-inflammatory effects by inhibiting lypoxygenase activity and the production of leukotriene B4 (Visioliet al., 1998).

1.2.1.4 Antimicrobial effect:

Oleuropein has been shown to have strong antimicrobial activity against both Gram-negative and Gram-positive bacteria as well as mycoplasma. Phenolic structures similar to Oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans. The exact mechanism of the antimicrobial activity of Oleuropein is still not completely established, although some authors have proposed that it is due to the presence of the ortho-diphenolic system (catechol)(Owenet al.,2000).

1.2.1.5 Skin protectant:

Recent studies have shown that the phenol components of olive leave have a direct antioxidant action on skin, especially Oleuropein, which acts as a free radical scavenger at the skin level (Ancoraet al.,2004).

1.2.1.6 Anti-aging:

Normal human fibroblasts undergo replicative senescence due to both genetic and environmental factors. The proteasome, a multicataly ticnonly sosomal protease, has impaired function during aging, while its increased expression delays senescence in human fibroblasts. Oleuropein enhances proteasome activities in vitro more effectively than other known chemical activators, possibly through conformational changes of the proteasome. Moreover, continuous treatment of early passage human embryonic fibroblasts with

Oleuropein decreases the intracellular levels of reactive oxygen species (ROS), reduces the amount of oxidized proteins through increased proteasome-mediated degradation rates and retains proteasome function during replicative senescence. Importantly, Oleuropein-treated cultures exhibit a delay in the appearance of senescence morphology, and their lifespan extended by approximately 15 % (Katsikiet al., 2007).

1.2.1.7 Other activities:

Further pharmacological activities of Oleuropein includes diverse healing properties due to its vasodilatory, anti-platelet aggregation, hypertensive, anti-rheumatic, diuretic and antipyretic effects. Prevention of free radical formation by Oleuropein occurs through its ability to chelate metal ions, such as Cu and Fe, which catalyze free radical generation reactions, and through its inhibitory effect on several inflammatory enzymes like lipoxygenases. Previously, Oleuropein was reported to have an anti-hyperglycemic effect in diabetic rats. Oleuropein inhibits hyperglycemia and oxidative stress induced by diabetes, which suggests that administration of Oleuropein is helpful in the prevention of diabetic complications associated with oxidative stress (Syedet al., 2010).

Figure (1.3): Olive leaves extract

1.3 Thyme (Thymus Vulgaris)

Are aromatic plants of the Mediterranean region and common herb in North Africa, and is commercially cultivated in large scale in many countries. It belongs to the Mint family, Labiatae. Thyme is herbaceous perennials subshrubs with a bushy, woody-based evergreen plant, rarely grows more than 40cm tall, with highly aromatic, tiny, gray-green leaves which is responsible for its characteristic flavor and fragrance. beautiful purple flowers bloom in the early summer. Thyme plant is an easy to grow on rocky soil and hot, dry condition, which requires little or no care (M.Coisin, et al 2012) are showed in figure (1.4).

Thyme species are used as a traditional medicine as herbal tea to treat cold, flu and cough. It has been reported that thyme, in general, contain various medicinal benefits and used as carminative, digestive, antispasmodic, anti-inflammatory and expectorant.

The essential oils whit in thyme species contains large amounts of thymol, which is strong antibacterial agent (E.Basch,et al 2004)as well as a strong antiseptic and antioxidant (B.Imelouane, et al 2009).

Thymus vulgaris essential oil is a mixture of monoterpenes. The two main compounds of this oil are the natural terpenoidthymol and its phenol isomer carvacrol which are showed in figure (1.5) (A.Grigore et al 2010).

Figure (1.4): Thyme leaves

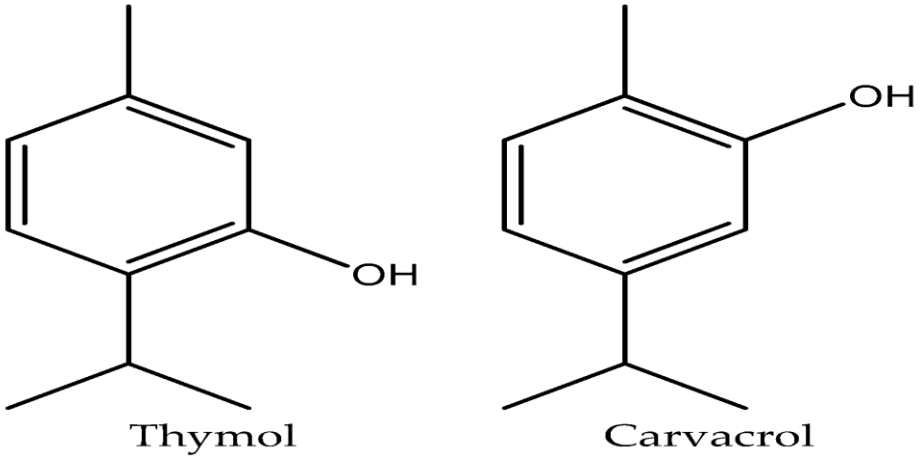


Figure (1.5): Chemical structure of Thymol and Carvacrol

1.3.1 Chemical Composition of the Essential Oil of thyme

The essential oil from *T. vulgaris* showed a high content of oxygenated monoterpenes (56.53%) and low contents of monoterpene hydrocarbons (28.69%), sesquiterpene hydrocarbons (5.04%) and oxygenated sesquiterpenes (1.84%) (Almaqtari M et al.,2011) . The predominant compound among the essential oil components was thymol (51.34%) while the amount of all other components of the oil was less than 19%.

1.3.2 Pharmacological properties of thyme oil

1.3.2.1 Anti Inflammattory Property

Thymus vulgaris oil is a mixture of monoterpenes. the most compounds of this oil are the natural terpenoid thymol, phenol chemical compound carvacrol(Amiri H et al 2012 and Nickavar B et al 2005) that have antioxidative, antimicrobial, used in medicinal drug, antitussive, antispasmodic, and antibacterial effects (Hoferl M et al 2009 and ESCOP (2007)).

1.3.2.2 anti bacterial activity

The efficacy of aromatic oils obtained from Thymus vulgaris L. was tested against nine strains of gram-negative bacteria and six strains of gram-positive bacteria. To detect the antibacterial activity of the selected essential oils and the parameter to illustrate

and measure the antibacterial activity of thyme oils was the detection time. Plate count technique was used to study the inhibitory effect by direct exposure. The result showed that thyme oil had a significant effect on microorganisms tested. A number of species were able to recover at least 50% of the metabolic function once they were connected to the inhibitor, while most strains were found to be almost completely inhibited (Marino et al., 1999).

1.3.2.3 Antioxidant properties

The leafy parts of thyme and its oil are utilized in foods for the flavor, aroma and preservation and additionally in folk medicines. El-Nekeety conducted an experiment to work out the elements of Thymus vulgaris L. oil and to evaluate the protecting effects of this oil against aflatoxininduce oxidative stress in rats. The results indicated that the oil contains Carvacrol (45 mg/g), Thymol (24.7 mg/g), β-Phellandrene (9.7 mg/g), essential oil (4.1 mg/g), Humuline (3.1 mg/g), α-Phellandrene (2.3 mg/g) and Myrcene (2.1 mg/g) [10]. However, α and β-pinene, Myrcene, α-thyjone, Tricyclene, 1, 8-cineole, and β-sabinene were found in very lower concentrations. Treatment with aflatoxins alone disturbs lipid profile in blood serum, decreases total antioxidant capability, increased creatinine, uric acid and nitric oxide in blood serum and lipid peroxidation in liver and excretory organ attended with a sever histological changes within the liver tissues. The oil alone at the 2 tested doses didn't induce any important changes within the biochemical parameters or the histological image. The combined treatment showed important enhancements altogether tested parameters and histological footage within the liver tissues. Moreover, this improvement was additional pronounced within the cluster received the high dose of the oil (Prasanth Reddy V et al., 2014).

1.4 objectives

The objectives of this study are:

1. To extract olive leaves using ethanol (80%).
2. To extract thyme oil from thyme plant using Steam distillation.
3. To determine the antimicrobial activity of pure oleuropein (pharmacopeial grade) on clinical bacterial and fungal isolates and compare that with routinely used preservatives.
4. To determine the antimicrobial activity of Olive leaves extract (OLE) on clinical bacterial and fungal isolates and compares that with routinely used preservatives.

The Antimicrobial Effectiveness Test or efficacy test will be done on the following standard reference organisms:

- *Candida albicans*(ATCC No. 10231)
- *Aspergillus niger*(ATCC No. 16404)
- *Escherichia coli* (ATCC No. 8739)
- *Pseudomonas aeruginosa* (ATCC No. 9027)
- *Staphylococcus aureus* (ATCC No. 6538).

5. Incorporate oleuropein and essential of thyme oil and OLE into pharmaceutical preparations (containing dexamethasone sodium phosphate as active ingredient and methyl and propylparaben as chemical preservative and Sodium chloride Nasal Spray containing Sodium chloride (NaCl) as active ingredient and benzalkonium as chemical preservative and vitamin D3 Oral drops containing vitamin D3 as active ingredient and Butylhydroxytoluene as a chemical antioxidant.
6. Investigate the optimum concentration of oleuropein, and OLE and thyme oil in the syrup as natural preservative/antioxidant and compare it with methyl and

propylparaben and benzalkonium chloride and Butylhydroxytoluene as the antioxidant.

7. Evaluate the antimicrobial effectiveness of the pharmaceutical preparations containing oleuropein or OLE or a mixture of oleuropein and thyme oil, and compare it with pharmaceutical syrups containing chemical preservatives as (positive control product).
8. study the stability of the pharmaceutical preparations in terms of (% of the active ingredient: dexamethasone sodium phosphate, Sodium chloride (NaCl), vitamin D3) and % of oleuropein.

Chapter Two

Literature Review

2.1 Literature Review

On reviewing available literature, it is evident that extensive research has been carried out to address various aspects of evaluating the effectiveness of natural plant extracts to determine their antimicrobial activity and potential side effects in pharmaceutical products. With this in mind, in an attempt to simplify the topic and delimit the subject to some extent, this chapter will focus primarily on the work that has been carried out the approximation of antimicrobial activity systems.

The main objective of this chapter is to provide a look at the theoretical and practical aspects concerning OLE antimicrobial activities and compare their effectiveness. This includes examining OLE and thyme oil as preservatives for pharmaceuticals. Therefore,

studying the antimicrobial activity of some medicinal plant extracts has become an important research concern and a major challenge.

2.2 Antimicrobial activity of OLE and thyme oil

Nature is full of miracles. There are herbal properties for incredible and countless herbs. Currently patients are trending to use natural therapy (natural medicine) because of the significant side effects of synthetic drugs (Atia z et al 2007).

The olive tree contains a high percentage of the biophenols such as oleuropein, verascascide, ligstrosides, tyrosol or hydroxyl tyrosol (Keskin D et al 2012). We will discuss some studies on activity against microbes, olive leaves and thyme oil

(Furneri et al. 2002), In this study activity of OLE was studied against gram positive bacteria and gram negative. The minimum inhibitory concentration was determined in the laboratory by a broth microdilution assay. Water was used as a solvent and the concentration used were (20-320 mg / ml) Microorganisms. The result of this study was that Oleuropein is more toxic for gram positive bacteria than gram negative bacteria, where the conclusion of the study was that Oleuropein was found to be effective against mycoplasmas. There is a consistency with (Aliabadi et al 2012) in a study against microbes where the bacteria were used mainly (Staphylococcus aureus PTCC 1431, Salmonella typhimurium PTCC 1639, and Escherichia coli PTCC 1399, Klebsiella pneumonia PTCC 1053, Bacillus cereus PTCC 1274). Water was used as a solvent and different concentrations were used (10-15-25-30-50) mg / ml Olive leaf aqueous extract) as a result of the research Olive Leaf aqueous extract showed good antimicrobial abilities and highest inhibitory of 11.5 against salmonella typhi where there was a similarity in that the extract of olive leaves had the ability against microbes. The results were also consistent with (gumgumjee et al 2014),when three types of gram negative bacteria and three gram positive bacteria were used against agar assay method and antimicrobial activity was

determined by measuring the inhibitory zone by using petroleum ether , ethanol, ethyl acetate as in solvent and concentration of (200 mg / ml), The results of this study (Ethanol extract is the most effective against all bacteria, while Petroleum ether extract of the leaves and stem showed no activity against B. subtilis MRSA & S. aureus, as well as low activity against E. coli, Kpneumonia and P. aeruginosa. Ethyl acetate extract of leaves showed a moderate effectiveness against all bacteria.

(Gokman et al., 2015), The effectiveness of the OLE may not be due to one main active constituent but may be due to the combine action of different compounds originally present in the plant. It was also consistent with the study where five gram-positive bacteria were evaluated for and five gram- negative using zone of inhibitory (ZOI) and MIC was determined by the concentration (50 mg / ml) and the result of this study was presented the highest antibacterial activity against Bcereus & the lowest antibacterial against S.typhy. It was also agreed with the (Salma malik et al 2015); two types of Gram positive bacteria (S aureus & Bcereus) were evaluated using the zone of inhibitory (ZOI) and MIC was determined by using (methanol) as a solvent and concentration (500,1000,1500,2000 mg / ml) and the result of this study showed that OLE was effective against two gram positive strains.

Markin et al. (2003).In this study, the method of In vitro scanning electron microscopic observation for candidia albicans was used. . The result of this study was (Deramatophytes were inhibited by 1.25 % (w / v) OLE following after three day, Candida albicans was killed following after 24 h in the presence of 15% (w / v) OLE), where the conclusion of this study was that "Olive leaves extract" shows antibacterial and antifungal properties. In addition to this study which was in 2014,(Lamprini et al 2014) using the MIC assay and MBC method (acetone, water) as solvent concentration of olive leaf extract (10 mg / ml) and the microorganisms used were bacteria and fungi The result of this research showed a

milder inhibitory effect against oral pathogen, OLE were active against the tested pathogens specifically gram- negative anaerobic bacteria.

Battinelli et al., (2005), in this study OLE was tested against bacteria and fungi where the method was used MIC and MFC (minimum fungicidal concentration), The organisms were used are bacteria and fungus.

The result of this study showed a different spectrum of antimicrobial activity and MFC values were the same of the MIC ones). The conclusion of this study was that OLE is useful material in the topical treatment of fungal cutaneous infection.

The effectiveness of OLE against fungus was studied using the disc diffusion, method where it has been used over solvent (water, acetone, methanol, ethyl acetate) korukluoglu et al., (2007), the concentration used was used 30 mg / ml of extract of olive leaves and microorganisms. The conclusion of this study was that aqueous extract showed the most prominent activity. diethyl ether extracts of the olive leaves showed poor antifungal activity.

Pereira et al., (2007) This research studied the effectiveness of OLE against microbes, the method used was in vitro MIC for the microbial inhibition of microbial, where water was used as a solvent and concentration 0.05-5 mg / ml, Gram positive (Bacillus cereus, B. subtilis and Staphylococcus aureus), Gram negative bacteria (Pseudomonas aeruginosa, Escherichia coli and Klebsiellapneumoniae), and fungi (Candida albicans and Cryptococcus neoformans). The result of this study showed that at low concentration OLE showed an unusual combined antibacterial and antifungal activity. The conclusion of this study was that extracts of olive leaves and oleuropein, have a potential effect against microbes.

Sudjana et al (2009) had studied the effectiveness of OLE against the intestinal bacteria using agar dilution and broth microdilution techniques; MIC, MBC & MFC were

determined, by using of the extract of olive leaves (4.4mg/ml) and micro organisms (H. pylory and C. jejuni and Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis, E. coli, Shigellasonnei and Yersinia sp). The result of this study was that OLE may have a role in regulatory the composition of gastric flora by selectively reducing level of (H.pylory and C.jejuni); while the result of this study was (Olive leaf extract has not broad spectrum in action showing appreciable activity only against (H.pylory, S.aureus, C.jejuni and Methicillin-resistant Staphylococcus aureus).

Erdohan et al., (2011); in this study, the extract of olive leaves was evaluated on agar diffusion method, chloroform (methanol) was used as a solvent. The concentration of olive leaf extract was (3 gr / 100ml), the result of this study was (OLE concentrate in the disks changed within range of 0.6 -3.6 mg and increasing OLE has a great potential in antimicrobial food package to reduce post-process growth of bacteria.

Faiza et al.,(2011); In this study antimicrobial activity of OLE was measured, using bacteria and fungus on agar disk diffusion method and measurement of diameters of inhibition zone. Ethyl acetate acetone was used as solvent and in extract concentrations of (10-15-20-30-50) mg / ml); the microorganisms used were Staphylococcus aureus ATCC 25923, Enterococcus feacalis ATCC 29212, Bacillus cereus ATCC 10876, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Listeria monocytogenes ATCC 15313, Klebsiella pneumonia ATCC 700603, Enterobacter cloacae ATCC 13047, Citrobacterfreundii ATCC 8090, Proteus mirabilis ATCC 35659 and Salmonella typhemurium ATCC 13311) and fungi (Cladosporiumherbarum MNHN 3369, Alternariaalternaria MNHN 843390, Aspergillus fumigates MNHN 566, Aspergillus flavus MNHN 994294). The result of this study was that all extract concentrations showed good inhibitory effects toward E.coli & bacillus cereus as compared to other bacteria. Ethyl acetate and acetone extracts were more effective against fungi. The conclusion of

this study indicates that olive leaves are a potent source of antibacterial and antifungal against.

Altaf Hussain et al.(2014); Activity of OLE has been studied. Agar disc diffusion method was used and zone of inhibition (ZOI) was measured. The results showed that OLE had potential antimicrobial activities against some of gram positive and gram negative bacterial strain. The findings of this study suggested that Leaf Extract is a cheap and effective antibacterial agent that can be used as alternative to purified oil.

E.O. Dada (2013);in this study, Activity of OLE was evaluated against six bacterial pathogens: (Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa and Salmonella enterica). Water and methanol was used as a solvent.

The result of this study was (The methanolic extract, at a concentration of 50 mg / mL, exhibited the highest inhibitory potential on Salmonella enterica and Escherichia coli with a zone of inhibitory of 13.0 mm and 10.5 mm respectively while the aqueous extract had the highest inhibitory potential on Enterococcus faecalis and Escherichia coli with an inhibitory zone of 5.0 mm) obtained with the methanolic leaf extracts of the plant was from 3.125 to 12.5 mg / mL and 12.5 to 25 mg / mL for the aqueous extract.

(Atai et al., 2016) in this review article evaluated the ability of extract olive leaves as antimicrobial; the study contained 26 related scientific articles, including 21 evaluation of the effectiveness of (OLE) against bacteria and 3 studies of its effectiveness against viruses and 7 studies of the effectiveness against fungus (Figure 2.1).

Majority of studies reported a great antimicrobial activity (figure 2.2) of OLE .most of them demonstrated that different solvent and concentration presents a significant effectiveness against microorganisms.

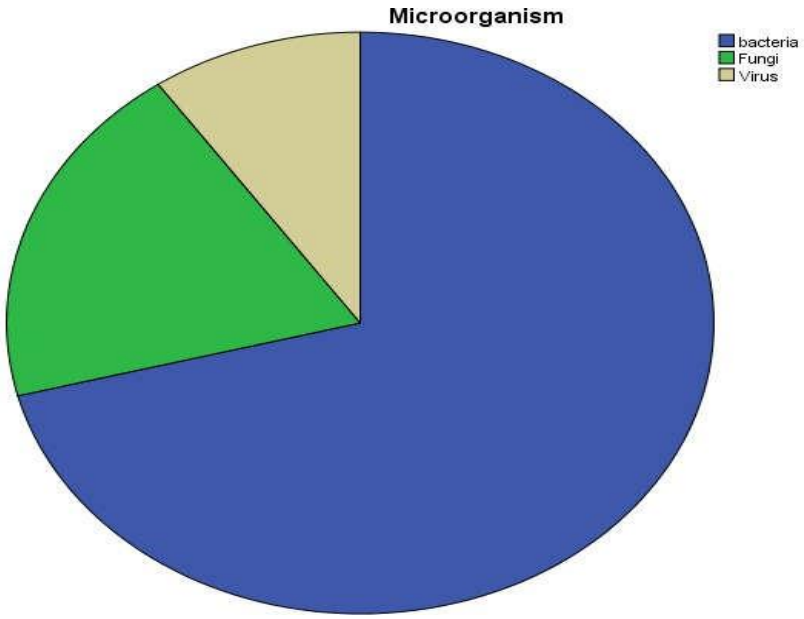


Figure (2.1): Type of microorganisms used in antimicrobial assessment of OLE

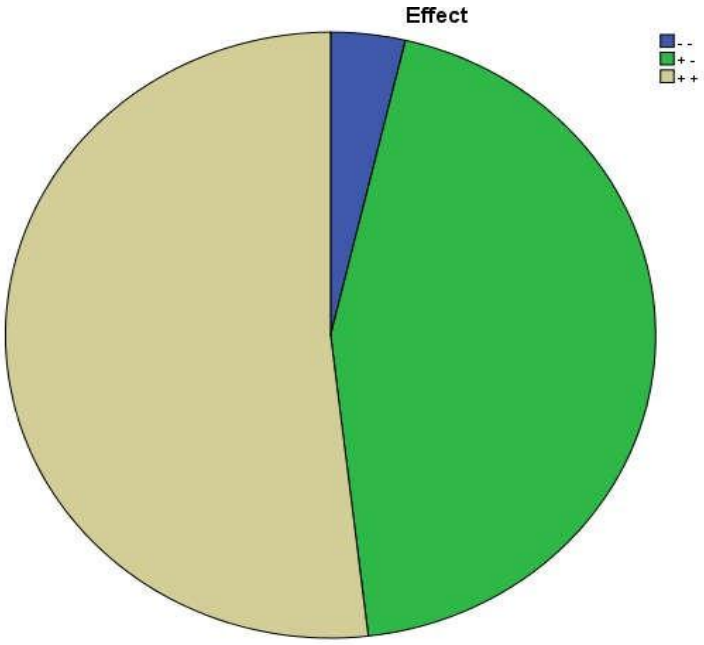


Figure (2.2):Type of antimicrobial effect of OLE . ++: studies which are effective against all tested microorganisms.+-:studies which are effective against some strains of tested microorganism .--: studies which had no effect on tested microorganism.(Atai et al., 2016)

Marija Bo skovic et al., (2015); this study had been evaluated the activity of thyme oil against microbes. MICs were determined by broth microdilution method, the investigation of the antibacterial effects of thyme oil was performed on Staphylococcus aureus, and, Escherichia coli and Bacillus cereus, Salmonella Enteritidis, Salmonella Thyphimurium,

methicillin resistant Staphylococcus aureus, Salmonella Thyphimurium, Staphylococcus aureus. The result of this study was thyme oil exhibited antibacterial activity against all tested microorganisms.

(Bulent Cetin et al., 2011); in this study, an effective evaluation was performed against microbes effects of the essential oils from thyme and oregano. The test method used was DD and MIC methods. A total of 43 microorganisms were studied including 26 bacteria, 14 fungal species and 3 types of yeasts. The result of this study was: mean inhibitions and MIC values of bacterial strains varied from (8 and 72) mm to 7.8 and 500 μg mL^{-1}, respectively. The maximal inhibiting zones and MIC values of the yeast and fungi species sensitive to the essential oils were 8-74 mm and 7.8-500 μg mL^{-1}, respectively. The susceptibility of the tested microorganisms varied depending on the essentialoils of oregano and thyme may be considered a potential source of a natural antimicrobial for the food industry after testing the toxic and irritating effects on humans.

Gavaric, N et al., (2015) inthis study, antioxidant and antibacterial activities of thyme and oregano essential oils, thymol and carvacrol, as well as their possible synergism were investigated. Antioxidant capacity was assessed throughout neutralization of DPPH and OH radicals. For determination of antibacterial activity against selected gram-positive (Staphylococcus aureus and Bacillus cereus) and gram-negative bacteria (Salmonella Infantis, Escherichia coli) by broth micro-dilution method was used, the combination testing for potential synergy of thyme and oregano essential oils, thymol and carvacrol was performed and evaluated with fractional inhibitory concentration. Thymol and carvacrol were found to be dominant component of investigated essential oils.

All studied samples expressed strong antioxidant and antibacterial potential. In synergy testing, both combinations (thyme/oregano essential oils and thymol / carvacrol) were found to have additive effect (FIC=0.75). These results confirm thyme and oregano

essential oils, thymol and carvacrol as well as their combinations for possible application as natural additives in different products (food, cosmetics and nutriceuticals).

Chapter Thre

Materials and methods

3.Materials and methods

3.1 Materials and reagents:

Materials and reagents used in this study are illustrated in Tables (3.1) and (3.2). All materials used in the formulation of pharmaceutical preparations are of pharmaceutical grade.

Table (3.1): List of materials used in the formulation of pharmaceutical preparations

No.	Materials of pharmaceutical preparations		
1	Dexamethasone sodium phosphate	12	cremophor RH40 (polyoxyl40 hydrogented castor oil)
2	Cherry flavor	13	EDTA
3	Red color	14	Sodium dihydrogen phosphate
4	Titreplex	15	dibasic sodium phosphate
5	Saccharin Sodium	16	vitamin D3 crystals
6	Sucrose	17	Tween 80
7	OLE	18	Citric acid anhydrous
8	Glycerin	19	Orange oil flavor
9	Distilled water	20	Oleuropein
10	Sodium chloride (NaCl)		
11	Thyme oil		

- All reagents were of analytical grade table (3.2) these materials and reagents were purchased from reliable sources and donated by Beit Jala Pharmaceutical Co, Ltd / Bethlehem - Palestine.

Table (3.2): List of materials used for extraction, formulation, chemical- physical-microbiological analysis, and stability testing

No.	list all materials and reagents of analytical grade		
1	Olive leaves	12	Letheen Broth, Letheen Agar
2	Oleuropein 40%	13	Broth media
3	Distilled water Thyme leaves	14	**Sterile saline**
4	Acetic acid	15	**Poly sorbate 80**
5	Ethanol	16	Fluid Lactose Medium
6	Methanol	17	**Phosphate buffer stock solution**
7	Ethylacetate	18	**Eosin Y**
8	Distilled water	19	**Glacial acetic acid**
9	Soybean-Casein Digest Medium	20	**Silver nitrate**
10	Soybean-Casein Digest Broth,		HDPE bottles, LDPE Caps, Amber Glass Bottles type III
11	Casein Digest- soy Lecithin Polysorbate		

3.2 Equipment and Tools

Equipment and tools used for extraction, formulation, chemical- physical-microbiological analysis, and stability testing are illustrated in Table (3.3).

All Syringes, vials, pipettes, glass ware, stands and tubes were supplied by Beit Jala Pharmaceuticals Company.

Table (3.3): list all Tools and Equipment used in the study

No.	Equipment and Tools
1	Laminar Flow Cabinet
2	Steam Autoclave
3	Vortex
4	UNIMAX 1010 shaker
5	UV- visible Spectrophotometer
6	pH meter 211R Hanna
7	Mixer tank
8	Sterile petri dishes (diameter of 9 cm)
9	Plates
10	Steam distillation Device
11	Thermometer
12	Extraction Tools
13	Filters: Whatman No.1 filter
14	HPLC Apparatus

1.3 Sample Collection and preparation

3.3.1 Olive leaves

Olive leaves samples were obtained from trees localized in Bethlehem in West Bank/ Palestine in November 2017. The leaves were dried at ambient temperature. Then the dried

samples were grinded and passed through sieve mesh # 120 mm to obtain powder which was stored at room temperature in dark until extraction.

3.3.2 Thyme leaves

Thyme leaves samples were obtained from plants localized in Bethlehem in West Bank/ Palestine in December 2017, until oil is extracted from the leaves by Steam distillation.

3.4 Extraction of Olive leaves

10 grams of olive leaves powder were macerated in 100 ml of 80% ethanol for 4 hours at 40^{o}C. The extracts were then filtered through a Whatman No.1 filter (Whatman, UK) to separate the coarse particles from the solution. The filtrate was then evaporated at room temperature under vacuum by using rotary evaporator. The concentrated extract was stored in a refrigerator at (2-4°C) until used.

3.5 Extraction of Thyme oil by steam distillation

3.5.1 Water Distillation

This is the simplest and the most cost - effective distillation method. The plant material is immersed in water and boiled. The steam and oil vapor are condensed and the oil is separated from the water. This method is suitable for flower blossoms and finely powdered plant material.

The distillation temperature should be about 100°C. Care needs to be taken to prevent the plant material being damaged by contacting the overheated still walls. The pressure in the still should be atmospheric. The distillation time depends on the plant material being processed. Prolonged distillation produces only a small amount of extra oil, but does add unwanted high boiling compounds and oxidation products.

3.5.2 Procedure

A specific amount of dried or fresh plant material: thyme (500 g) was placed in the

steam distillation apparatus, and then the instrument is turned on where steam of boiling water is used for extraction of essential oils from the sacs of the plant materials. The steam which contains the essential oil is passed through a cooling system to be condensed into a liquid from where the essential oil and water are then separated.

The hydrosol (mixture of oil and water) will be tested for its antibacterial effectiveness and for its use as preservative (preservative effectiveness test).

3.6 Preparation of some pharmaceutical dosage forms containing Oleuropein, OLE and thyme oil:

3.6.1 Preparation of Dexamethasone sodium phosphate oral syrup

Label claim: Each 5 ml contains 0.658 mg of Dexamethasone sodium phosphate / 5ml.

Table (3.4) Illustrates the general formula for Dexamethasone sodium phosphate oral syrup where the function of each component is indicated.

No.	Component	Qty. /100 ml	Function
1	Dexamethasone sodium phosphate	0.013g	Active ingredient
2	Cherry flavor	0.1025g	Flavor
3	Red color	5.25 mg	Coloring agent
4	Titriplex	0.1g	Chelating agent
5	Saccharin Sodium	0.033g	Sweetening agent
6	Sucrose	50 g	Sweetening agent
7	Oleuropein or OLE	0.6 g or 0.4 g	Preservative
8	Glycerin	2.6 g	Co solvent
9	Distilled water	q.s 100 ml	Solvent

Method of preparation

1. Transfer 2 liter of purified water at 50^{o}C to the mixing tank.
2. Mix at 1200 rpm for 4 minutes with manual circulation.
3. Transfer the sugar to the mixing tank gradually with mixing at 1200 rpm for 15 min till complete dissolving
4. Transfer the glycerin to the mixing tank .then mix for 2 min
5. Add oleuropein to the mixing tank
6. Dissolve Titriplex in 50ml purified water transfer to the mixing tank and mix for 5 minutes.
7. Dissolve *red color in 50ml* purified water transfer to the mixing tank and mix for 5 minutes
8. Dissolve *Saccharin Sodium in 50ml* purified water transfer to the mixing tank and mix for 5 minutes
9. Make up to volume (3.8 liter) with purified water and mix for 5 minutes.
10. Cool the preparation to be less than 35^{o}C
11. Add cherry flavor and mix for 1 minute
12. Dissolve *Dexamethasone sodium phosphate* in 200ml cold purified water
13. Transfer to the mixing tank and mix for 5 minutes, with circulation.

3.6.2 Preparation of Sodium chloride Nasal spray

Label claim: Each 10 ml contains 0.074mg of Sodium chloride / 10 ml

Table (3.5) illustrates the general formula for Sodium chloride *Nasal spra*y where the function of each component is indicated.

No.	Component	Qty. /100 ml	.Function
1	Sodium chloride	0.74 g	Active ingredient
2	Oleuropein	0.4 g	Preservative
3	Thyme oil	0.1g	Preservative
4	Cremophor RH40 (Polyoxyl 40 hydrogenated castor oil)	1g	Surfactant
5	EDTA	0.06 g	Chelating agent
6	Sodium dihydrogen phosphate	0.22 g	Buffering agent
7	dibasic sodium phosphate	0.72 g	Buffering agent
8	purified water	100 ml	Solvent

Method of preparation

1. Disinfectant the preparation area with suitable disinfecting agent before preparation.
2. Transfer 2liter of purified water to main mixing vessel then heat to about 70^{o}C.
3. Add oleuropein to main mixing vessel and mix for 5 minutes using fast agitator at speed=1500rpm
4. Add Cremophor RH40 (polyoxyl40 hydrogenated castor oil) to main mixing vessel and mix for 5 minutes using fas
5. t agitator at speed=1500rpm

Dissolve EDTA in about 200ml of purified water at about 70 °C, then incorporate the solution to main mixing vessel and mix for two minutes using fast agitator at 1500 rpm speed.

6. Add Sodium chloride to main mixing vessel and mix using fast agitator at speed = 1500rpm until dissolved (about 5 minutes).
7. Add Sodium dihydrogen phosphate to main mixing vessel and mix for 5 minutes using fast agitator at speed=1500rpm.
8. Add dibasic sodium phosphate to main mixing vessel and mix for 5 minutes using fast agitator at speed=1500rpm.
9. Cool the preparation to less than 35^{o}C.
10. Make up to volume (4 liter) with purified water and mix for 5 minutes using fast agitator at speed=1500rpm.

3.6.3 Preparation of vitamin D3 Oral drops

Label claim: Each 10 ml contains 3.485 mg of vitamin D3 crystals / 10 ml

Table (3.6) illustrates the general formula for vitamin D3 oral drops where the function of each component is indicated.

No.	Component	Qty. /100 ml	Function
1	vitamin D3 crystals	0.03485 g	Active ingredient
2	Tween 80	2.5 g	Emulsifier
3	Glycerin	65 g	Preservative
4	citric acid andydrous	0.1 g	pH adjustment
5	Oleuropein	0.4 g	Antioxidant and preservative
6	Thyme oil	0.2 g	Antioxidant and preservative
7	Orange oil flavor	3.325 mg	Flavor
8	Purified water	100 ml	Vehicle

Method of preparation

1. Turn off fluorescent light and use the sodium lamp
2. Transfer tween 80 to mixing tank
3. Add vitamin D3crystals and mix until completely dissolved for about 45 minutes
4. Transfer vitamin D3 crystals and Tween 80 mixture to the mixing tank.
5. Add glycerin to the above mixture and mix for about 15 minutes using mixing heat.
6. Dissolve oleuropein in orange oil flavor, transfer the mixture to the mixing tank and mix for 5 minutes
7. Dissolve citric acid anhydrous in 200ml purified water.
8. Add citric acid solution to the mixing tank, and mix for 5 minutes.
9. Make up to volume (4 liter) with purified water.
10. Mix the whole tank for 10 minutes by using mixing heater

3.7 Chemical and physical analytical Test Methods:

3.7.1 Determination of oleuropein

Determination of oleuropein in pharmaceutical preparations, reversed - phase HPLC method was used with silica-based C18 bonded phase column (C18, 250mm × 4.6 ID) with mobile phase consisting of a mixture of water and acetonitrile (80/20 volume ratio) containing 1% acetic acid at a flow rate of 1.0 mL/min. UV detector at 237 nm was used for oleuropein determination. The injection volume used is 20.0 µl for both standard and sample solutions. Identification of oleuropein in olive leaves extracts was based on retention times in comparison with standard of oleuropein. The quantitation was carried out using the external standard method. The concentration of oleuropein was calculated using peak area and the calibration curves obtained from oleuropein standard solution.

3.7.2 Determination of Dexamethasone sodium phosphate

For determination of Dexamethasone sodium phosphate from formula syrup, reversed phase HPLC method was used with column: (RP- select B,125Xmm,5um) mobile phase consisting of a mixture of methanol and acetonitrile (40/60 volume ratio) containing 1% acetic acid at a flow rate of 1.2 mL/min. UV detector at 240 nm was used for Dexamethasone sodium phosphate determination. The injection volume used is 20.0 µl for both standard and sample solutions. Identification of Dexamethasone sodium phosphate from formula syrup was based on retention times in comparison with standard of Dexamethasone sodium phosphate. The quantitation was carried out using external standard method. The concentration of Dexamethasone sodium phosphate was calculated using peak area and the calibration curves obtained from standard solution. The amount of Dexamethasone sodium phosphate was expressed as milligram per ~~gram~~ 5 ml of formula syrup.

3.7.3 Determination of Sodium chloride

Reagent:

a) adsorption indicator - dissolve 50mg of eosin y in 10 ml of water
b) glacial acetic acid and methanol,
c) 0.05 N silver nitrate: dissolve about 2.124 g of silver nitrate in 250 ml of water
d) standardize the solution as follows:

Transfer about 100 mg, accurately weighed, of reagent grade Sodium chloride, previously dried at 110 C° for 2 hours to a 100-ml beaker and dissolve in 5 ml of water. Add 5 ml of acetic acid, 50 ml of methanol, and about 0.5 ml of Eosin and stirr, preferably with a magnetic stirrer, and titrate with the silver nitrate solution.

The concentration of sodium chloride in the pharmaceutical product was determined by titration using standardized silver nitrate solution.

3.7.4 Determination of vitamin D3

Determination of vitamin D3 from formula, reversed phase HPLC method was used with column (RP18e, 150 - 4.6 mm,5µm) with mobile phase consisting of a mixture of methanol and acetonitrile (25/75 volume ratio) containing 1% acetic acid at a flow rate of 1.2 mL/min. UV detector at 268 nm was used for vitamin D3 crystals determination. The injection volume used is 20.0 µl for both standard and sample solutions. Identification of vitamin D3 from formula was based on retention times in comparison with standard of vitamin D3 crystals. The quantitation was carried out using external standard method. The concentration of vitamin D3 crystals was calculated using peak area and the calibration curves obtained from vitamin D3 crystals standard solution. The amount of vitamin D3 crystals was expressed as milligram per ml of formula.

3.7.5 Determination of PH

Determination of the prepared formula was measured using pH meter (pH meter 211R Hanna). Calibrate the pH meter electrode between pH 4.0 and 7.0 in case the product pH is below 7.0, or calibrate the pH meter electrode between pH 7.0 and 9.0 in case the product pH is above 7.0. Immerse the electrode into the product and document the reading after stabilization. Repeat the measurement three times and calculate the average of readings. .

3.8 Microbiological Test methods

3.8.1 Antimicrobial Effectiveness Testing

This procedure is designed to evaluate the antimicrobial effectiveness of antimicrobial preservatives used in ~~Ora~~l products made with aqueous base or vehicles

3.8.1.1 General instructions

- Prepare the agar media to be ready for use, in a liquid form at 45^{o}C.

- The bacterial and yeast suspensions are to be used within 2 hours of harvest otherwise refrigerate the suspension for 24 hrs. , while the fungal preparation may be stored under refrigeration for up to 7 days.
- Work aseptically under laminar flow for all the preparations.

3.8.1.2 Media:

For the cultivation of the test organisms, select agar medium that is favorable to the rigorous growth of the respective stock culture. The recommended media are Soybean Casein Digest Agar/Broth and Sabouraud's Dextrose Agar/Broth. Add a suitable inactivator (neutralizer) for the specific antimicrobial properties in the product to the broth and/or agar media used for the test procedure if required.

3.8.1.3 Growth promotion of the media

Media used for testing needs to be tested for growth promotion by inoculating the medium with appropriate microorganisms. It is preferable that test microorganisms be chosen for growth promotion testing (Section 3.8.1.5).

Solid media tested for growth promotion is to be set up using pour plate method in order to determine a microbial plate count (CFU) which must be ≥ 70% of the microorganism inoculum's calculated value.

3.8.1.4 Test organisms:

All cultures must be no more than 5 passages removed from the original stock culture.

a. Candida albicans (ATCC # 10231)

b. Aspergillus niger (ATCC # 16404)

c .Escherichia coli (ATCC # 8739)

d. Pseudomonas aeruginosa (ATCC # 9027)

e .Staphylococcus aureus (ATCC # 6538)

3.8.1.5 Preparation of inoculums

1. Preparatory to the test:

- The surface of a suitable volume of solid agar medium is inoculated from a recently revived stock culture of each of the specified microorganisms.
- Culture conditions for the inoculum preparations are described in Table (3.7).

Table (3.7) Culture Conditions for Inoculum Preparation:

Organism	Suitable Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
Escherichia coli *ATCC No. 8739*	SCD, SCDA	32.5 ± 2.5°C	18 – 24 hours	3 – 5 days
Pseudomonas aeruginosa *ATCC No.* 9027	SCD, SCDA	32.5 ± 2.5°C	18 – 24 hours	3 – 5 days
Staphylococcus aureus *ATCC No. 6538*	SCD, SCDA	32.5 ± 2.5°C	18 – 24 hours	3 – 5 days
Candida albicans *ATCC No. 10231*	SAB, SABDA	22.5 ± 2.5°C	44 – 52 hours	3 – 5 days
Aspergillus niger *ATCC No. 16404*	SAB, SABDA	22.5 ± 2.5°C	6 – 10 days	3 – 7 days

***2. Harvesting the bacterial and Candida albicans cultures*:**

2.1 The surface growth is washed using sterile saline TS.

2.2 The suspension is collected in a suitable vessel

2.3 Sufficient sterile saline TS was added to obtain a microbial count of about $1x10^8$ CFU per mL.

2.4 The number of cells was counted by measuring the turbidity using a spectrophotometer at 650 nm to obtain an optical density (O.D.) of:

A. 0.3-0.45 for *S. aureus* (~1-3 $X10^8$ CFU/ ml).

B. 0.2-0.3 for *P. aeruginosa* and *E. coli* (~1-3 $X10^8$ CFU/ ml).

C. $\leq$ 1.0 for *C. albicans* ($\sim$ 1-3 x 10^8 CFU / ml)

2.5. Several dilutions were made ($10^{-3} - 10^{-6}$) and cultivated by pour plate method (1ml from each dilution in Sabouraud Dextrose Agar, in duplicates)

2.6. Bacteria was inoculated for 24 hours at $35^oC\pm$ 2, and *C. albicans* at $23^oC\pm$ 2 for 2-3 days and the CFUs were counted (count plates having between 30-100 CFU).

***3. Harvesting the Aspergillus Niger cultures*:**

3.1 The surface growth was washed using a sterile saline TS containing 0.05% of polysorbate 80 & Collected in a suitable vessel

3.2 A sufficient sterile saline TS was added to obtain a microbial count of about $1x10^8$ CFU per mL.

3.3 Several dilutions were made ($10^{-3} - 10^{-8}$) and seeded by pour plate method (1ml from each dilution in Sabouraud Dextose Agar, in duplicates)

3.4Cultures were incubated between 2-4 days at 23 ± 2^oC and the CFUs were counted (count plates having between 10-100 CFU).

4. To determine the number of CFU per mL in each suspension, use the condition of media and microbial recovery incubation times listed in Table (3.2) to confirm the initial CFU per mL estimate. (This value serves to calibrate the size of inoculum used in the test)

3.8.2 Procedure

The procedure requires that the test be conducted with a suitable volume of product. It is advisable to begin with at least 20 mL of product. Use the original product containers whenever possible or five sterile, capped bacteriological containers of suitable size into which a suitable volume of product has been transferred. If the diluted product exhibits antimicrobial properties, specific neutralizers may need to be incorporated into the

diluents or the recovery media. For purposes of testing, products have been divided into two categories:

b) **Category 1 products**: Dexamethasone Sodium Phosphate Oral syrup and Vitamin D3 Oral Drops

Test Procedure for Category Oral Products:

1. The tested reagent was conducted in 5 original containers (if sufficient volume of product is available in each container and the product container can be entered aseptically- (i.e. needle and syringe through an elastomeric rubber stopper) or in five sterile, capped bacteriological containers of suitable size into which a sufficient volume of product has been transferred.
2. Each container was inoculated with one of the prepared and standardized inocula, and mixed:
    - The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.
    - The volume of the suspension inoculum used is between 0.5% and 1% of the volume of the product (for example: if volume tested is 20 ml, then inoculated volume shall be 100 to 200 µl).
    - The concentration of test microorganisms that is added to the product shall reach a final concentration in the test preparation after inoculation between $1x10^5$ and$1x10^6$ CFU per mL of the product (For example, when 100 to 200µl inoculum of $1x10^8$ CFU/ ml are inoculated in 20ml sample, the final concentration will be 0.5 to $1x10^6$ CFU/ ml respectively).
3. The inoculated containers were incubated at 22.5 $\pm$ 2.5^{o}C by UNIMAX 1010 shaker / incubator 1000 and of 32.5 $\pm$ 2.5^{o}C by UNIMAX 1010 shaker / incubator 1000.

4. A sample was obtained from each container at the appropriate intervals.
5. Any changes observed in appearance at these intervals were recorded.
6. The numbers of CFU present in each test preparation for the applicable intervals were determined by the plate-count method.
7. An inactivator (neutralizer) of the specific antimicrobial was incorporated or the product was diluted 1:10, 1:20. . . . 1:100 using sterile purified water in order to determine the effective point of preservative in the plate count or in the appropriate dilution prepared for plating. These conditions are determined in the validation study of the sample based upon the conditions of media and microbial recovery incubation times listed in Table 2 *(7, 14 and 28 days-refer to Table (3.2) in attachments)*.
8. Using the calculated concentrations of CFU per mL present at the start of the test, the change in $\log_{10}$ values of the concentration of CFU per mL for each microorganism at the applicable test intervals was calculated, and the changes in terms of log reductions was expressed (The log reduction is defined as the difference between the log 10 unit value of the starting concentration of CFU/ml in the suspension and the log 10 unit value of CFU/ml of the survivors at that time point).

c) **Category 2 products**: Sodium Chloride Nasal Spray Proceed as directed in Category Dexamethasone Sodium Phosphate Oral syrup and Vitamin D3 Oral Drops products to obtain a concentration of microorganisms in the inoculum between $1X10^{-5}$ and $1X10^{-6}$ CFU per mL of the product.

3.8.3 Interpretation

The criteria for microbial effectiveness are met if the specified criteria are met, see table below. No increase is defined as not more than 0.5 log10 unit higher than the previous value measured

Category 1 products:

Bacteria	Not less than a 1.0 log reduction from the initial count at 14 days, and no increase from the 14day count at 28 days
Yeast and Molds	No increase from the initial calculated count at 14, and 28 days.

Category 2 products:

Bacteria	Not less than a 2.0 log reduction from the initial count at 14 days, and no increase from the 14day count at 28 days
Yeast and Molds	No increase from the initial calculated count at 14, and 28 days.

3.8.4 Total Aerobic Microbial Count for Liquid Dosage form of Finished Products:

The purpose of these tests is to test the qualitative and quantitative estimation of total viable aerobic count of microorganisms in finished liquid dosage forms .

3.8.4.1 these tests are applicable to:

- Non-sterile liquid pharmaceutical preparations as syrups.
- All liquid forms requiring testing for *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella*, *Staphylococcus aureus*, as well as yeasts and molds.

3.8.4.2 General instructions

a) *For Liquids*: Perform the test directly.

b) Test controls: At the time of the test, perform, buffer/broth negative controls, and media negative controls.

c) *Samples:* Use 10 bottles per product sample tested (if volume > 100 ml), or 20 bottles (if volume < 100 ml)/ batch.

d) Physical inspection should be performed for liquid product:

- Seeking for solid particulate contamination (fiber, crystals, turbidity ….. etc)

- Broken bottles or loose (unscrew) caps..

3.8.4.3 Sampling Plan:

1. Sampling from finished products:

These products are sampled by the samplers in their final form, in closed bottles,

2. Procedure:

2.1 Plate Method For Bacterial Count

Samples were transferred aseptically to sterile bottles as follows:

a) About 10 ml of specimen were transferred accurately to a sterile bottle & was repeated with another sterile bottle.

b) 90 ml of SCDM or CDSLP (or phosphate buffer of pH 7.2) were added in 1 bottle, and 90 ml of FLM in the other bottle, so that the final dilution is 1:10. The bottles were closed and mixed by swirling the bottle gently.

c) The SCDM bottle (or CDSLP or phosphate buffer bottle) was opened, and with a sterile pipette 1 ml of the sample was drawn and transferred to a petri dish contains already solidified SCDA then the petri dish was covered. This step was repeated with another plate

d) Each plate was rotated gently so that the 1 mL of sample covers all the surface of the agar in the plate.

e) The plates were allowed to settle for at least 10 minutes.

f) After 10 minutes, using a sterile pipette, the remaining liquid on the agar surface was drawn.

g) The dishes were inverted , and incubated at 35^{o}C for 48-72 hours.

h) Results were observed and average number of microorganisms was expressed from the 2 plates in each case (sample or control) as follows:

- **If growth**: Average number of microorganism in each plate/ml wass registered (taking into account the initial dilution 1:10).
- **If no growth**: Result were expressed $\leq$ 10 CFU/ml for a negative result.

2.2 Plate Method for Total Aerobic Yeast and Molds Count

a) The same procedure as for bacterial count (section **3.4.5**.1) but Sabouraud Dextrose Broth/Agar (Sab.D / SDA) were used instead of SCDM/ SCDA.

b) The Sab.D bottle and SDA plates were incubated at 20-25^{o}C for 5-7 days.

2.3 The Membrane Filtration Method

A sterile pipette was used draw 10 mL from each sample bottle (10 bottles/ test) and transfer into a sterile 100 ml bottle.

3.8.2.4.A. Filtration:

a. Filter the sample preparations through a 0.45 micrometer membrane filter about 50mm in diameter.
b. Rinse 3 times with 100 mL portions of the chosen diluent.

3.8.4.4.B. Total Aerobic Bacterial Count (TABC) and Total Combined Yeasts and Molds Count (TYMC)

1. Sterile blunt-tipped forceps, aseptically remove filter and place it were used on the surface of solidified SCDA contained in a petri dish, for the TABC test.
2. Repeat the procedure and place the other filter was used on the surface of solidified SDA contained in a petri dish for the TYMC test.
3. Repeat the same procedure for a control filter used.
4. Incubate the SCD plates at 30-35^{o}C for 48-72 hours and the SDA plates at 20-25^{o}C for 5-7 days.
5. At the end of the incubation period, count the colonies.

6. If no colonies are present, report results as 0 CFU per 10g or 10 mL of the sample. (or < 10 CFu 1 g or ml).

3.8.4.4.C. Acceptance criteria for nonsterile finished products (USP/BP)

Table (3.8): Acceptance criteria for nonsterile finished products (USP/BP)

Route of Administration	TABC (CFU/g or ml)	TYMC (CFU/g or ml)	Absence of*
Nasal/Otic/	**<200 (10^2)**	**<20 (10^1)** **<10**	*P. aeruginosa, and S. aureus*
Oral Liquids	**<200 (10^2)**	**<20 (10^1)**	*E.coli*

** USP/ BP acceptance criteria interpretation

- 10^1 CFu : maximum acceptable count =20
- 10^2CFu : maximum acceptable count =200
- 10^3CFu : maximum acceptable count =2000

3.9 Stability study

The stability study was conducted for the pharmaceutical formulations (Dexamethasone Sodium Phosphate Oral Syrup and Vitamin D3 Oral Drops and Sodium Chloride Nasal Spray), which contain natural substances (oleuropein and thyme oil) as well as for the positive control formulation.

Further experiments were conducted at end of each month for three months, antimicrobial tests and Physical parameters such as color, pH, and Precipitation, drug content, determination of the content of the natural substance oleuropein by HPLC at the end of each month for three months on accelerated conditions(40 ± 2 ° C / 75% ± 5% RH)

Chapter Four

Results & Discussion

4.1 Growth promotion of the media

The results of the growth promotion test showed that all the materials (Sabouraud Dextrose Agar and Soybean Casein Digest and Sabouraud Medium and Soybean Casein Digest Medium) effective on microorganism used in the study and correspond to the sop system see table (4.1 - 4.4)

Table (4.1) Growth promotion of the Sabouraud Dextrose Agar

Medium : Sabouraud Dextrose Agar				
Test Done	Manufacturer Specifiations	Test Result		
Appearance of Dehydrated medium	Light beige, free flowing and homogenous.	Complies		
Solubility/color of solution	6.5% solution, soluble in distilled water or deionized water on boiling .solution is light to medium amber without precipitate	Complies		
Appearance of prepared Medium	light to medium amber, slightly Opalescent without precipitate	Complies		
PH : at 25 ^{o}C	5.6 $\pm$ 0.2	5.8 (pass)		
Culture Response/ growth promotion test (Microorganism/ATCC/Incubation time)		10^1	10^2	10^3
	1) C. albicans 10231/3 day	490	>1000	>1000

	2) As. Niger 16404/ 5 days	0	3	50

Table (4.2) growth promotion of the Soybean Casein Digest Agar

Medium : Soybean Casein Digest Agar				
Test Done	Manufacturer Specifications	Test Result		
Appearance of Dehydrated medium	Light beige, free flowing and homogenous.	Complies		
Solubility/color of solution	4% solution, soluble in distilled water or deionized water on boiling .solution is light to medium amber slightly Opalescent.	Complies		
Appearance of prepared Medium	Light amber, slightly Opalescent with no significant precipitate.	Complies		
PH : at 25 ^{o}C	7.3 $\pm$ 0.2	7.2 (pass)		
Culture Response/ growth promotion test (Microorganism/ATCC/Incubation time)		10^{1}	10^{2}	10^{3}
	S. aureus / 18-24 hrs	30	394	>1000
	E.coli / 18-24 hrs	10	137	>1000

Table (4.3) growth promotion of the Sabouraud Medium

Sabouraud Medium				
Test Done	Manufacturer Specifications	Test Result		
Appearance of Dehydrated medium	Light beige, free flowing and homogenous.	Complies		
Solubility/color of solution	6.5% solution, soluble in distilled water or deionized water on boiling .solution is light to medium amber without precipitate	Complies		
Appearance of prepared Medium	Very light amber,slightiy Opalescent without precipitate	Complies		
PH : at 25 ^{o}C	5.6 $\pm$ 0.2	5.7 (pass)		
Culture Response/ growth promotion test (Microorganism/ATCC/Incubation time)		10^{1}	10^{2}	10^{3}
	As. Niger / 18-24 hrs	turbid	turbid	Turbid
	C. albicans /18 -24 hrs	turbid	turbid	turbid

Table (4.4) growth promotion of the Soybean Casein Digest Medium

Medium : Soybean Casein Digest Medium				
Test Done	Manufacturer Specifications	Test Result		
Appearance of Dehydrated medium	Light beige, free flowing and homogenous.	Complies		
Solubility/color of solution	3% solution, soluble in distilled water or deionized water on boiling .solution is light amber , clear	Complies		
Appearance of prepared Medium	light amber , clear	Complies		
PH : at 25 ^{o}C	7.3$\pm$ 0.2	7.5 (pass)		
Culture Response/ growth promotion test (Microorganism/ATCC/Incubation time)		10^1	10^2	10^3
	S. aureus / 48 hrs	turbid	turbid	Turbid
	C. albicans/ 48 hrs	turbid	turbid	turbid

4.2 Antimicrobial Effectiveness Testing:

To use oleuropein and olive leaves extract (OLE) as preservative in pharmaceutical syrup, first antimicrobial activity of samples of oleuropein and OLE was tested at different concentrations using distilled water as solvent. (0.2, 0.4 and 0.6% w/v) of oleuropein was tested while (0.2, 0.3 and 0.4% w/v) of OLE was used. Tables 1-6 show antimicrobial activities of oleuropein and OLE samples at different concentrations.

In this test, three types of bacteria, one yeast and one fungus (mold) were tested for antimicrobial activity of oleuropein and olive leaves extract. Bacteria Includes one gram positive *(Staphyloccus aureus*,(ATCC NO. 6538)) and two gram negative *(Pseudomonas aeruginosa*(ATCC 9027)), and (*Escherichia coli* , (ATCC NO. 3739) while *Candida albicans* (yeast) (ATCC NO.10231) and *Aspergillus niger (*mold) (ATCC NO. 16404) were used in this test which are examples of fungi that can cause infections in human body.

4.3 Oleuropein

Using 0.2% w/v oleuropein, results showed that there is more than 1 log reduction (10 folds) in the counts (CFU) of the three bacteria tested from the initial count at 14 days (for *Staphyloccus aureus* there is complete inhibition after 7, and 14 days of incubation at all dilution used, the same for *Pseudomonas aeruginosa* where more than 10 fold (1. Log) reduction in counts after 7 and 14 days using all dilutions, same results (as for *Pseudomonas aeruginosa*) were obtained for *Escherichia coli*, and there is complete inhibition of these three bacterial strains after 28 days (i.e. no increase from the 14 days count to 28 days (see Table 4.1). So this implies that 0.2% w/v oleuropein can be used as antimicrobial agent against these three types of bacteria. But to be used as preservative, the compound (potential preservative) should be also effective against yeast and mold in addition to those three types of bacteria mentioned above. However at 0.2% w/v oleuropein, there is increase in the count of yeast (*Candida albicans*) and fungus*(Aspergillus niger*) from the initial (time zero) at 14 days, which indicates that oleuropein at this concentration cannot be used as preservative since it is not effective against yeast and mold (see table 4.1) but it can be used as antimicrobial against the three types of bacteria mentioned above. So this leads us to increase the concentration to (0.4% w/v) At this concentration, oleuropein was found to be effective against the three bacteria (as 0.2% concentration) where more than 10 fold reduction in the counts were obtained at 14 days and no increase in the counts after 28 days. Regarding its activity against yeast and mold, results showed that it is (as 0.2% w/v) not effective against yeast and mold, (Table 4.2). Increasing the concentration of oleuropein to 0.6% resulted in complete inhibition of all three bacteria strains after 7, 14 and 28 days, and no increase in the yeastand mold counts from initial time at 14 and 28 days, (see Table 4.3). This result indicates that (0.6% w/v) oleuropein is a good concentration to be used as preservative.

Table (4.5). Antimicrobial activity of oleuropein (0.2% w/v) in distilled water against three types of bacteria, one yeast and one mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	$>>10^3$ ..	$>10^3$	$>10^3$	1201	406				$>10^3$	
		7	0	0	0	0	0				$>10^3$	1748
		14	0	0	0	0	0				$>10^3$	838
		28	0	0	0	0	0				770	68
P. aeroginosa	9027	0	$>>10^3$	$>10^3$	$>10^3$	546	133			180	260	
		7	$>10^3$	717	251	61	0				$>10^3$	1270
		14	103	29	10	0	0				$>10^3$	440
		28	0	0	0	0	0			$>10^3$	375	
E.coli	8739	0	$>>10^3$	$>10^3$	$>10^3$	1118	511			449	32	
		7	$>10^3$	870	291	76	12				$>10^3$	$>10^3$
		14	100	29	0	0	0				$>10^3$	597
		28	0	0	0	0	0				912	100
C. albicans	10231	0	$>10^3$	$>10^3$	$>10^3$	1301	509		928	115	28	
		7	$>10^3$	$>10^3$	$>10^3$	1533	477			$>10^3$	1450	
		14	$>10^3$	$>10^3$	$>10^3$	1023	142			$>10^3$	$>10^3$	
		28	$>10^3$	$>10^3$	1230	208	181				1269	
A. Niger	16404	0	$>>10^3$	$>10^3$	660	3	9	$>10^3$	484	69	5	
		7	$>10^3$	511	129	51	5		90	6	1	
		14	$>10^3$	1515	316	39	4		28	4	1	
		28	$>10^3$	380	41	12	0		66	10	2	

Table (4.6). Antimicrobial activity of oleuropein (0.4% w/v) in distilled water against three types of bacteria, one yeast and one mold.

Microorganism 10^6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	>>10^{3}..	>10^3	>10^3	1175	386			577	45	
		7	0	0	0	0	0				>10^3	1748
		14	0	0	0	0	0				>10^3	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	>>10^3	>10^3	>10^3	348	98			180	16	
		7	1416	280	41	7	0				>10^3	1270
		14	0	0	0	0	0				>10^3	440
		28	0	0	0	0	0			>10^3	375	
E.coli	8739	0	>>10^3	>10^3	>10^3	1029	336			449	32	
		7	>10^3	636	187	30	4				>10^3	>10^3
		14	0	0	0	0	0				>10^3	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	>>10^3	>10^3	820	111	11		928	115	28	
		7	>>10^3	>10^3	>10^3	1168	318			>10^3	1450	
		14	>10^3	>10^3	>10^3	921	63			>10^3	>10^3	
		28	>10^3	>10^3	1056	176	75				1269	
A.niger	16404	0	>>10^3	>10^3	529	77	4	>10^3	484	69	5	
		7	>10^3	300	104	28	2		90	6	1	
		14	>10^3	1220	208	18	3		28	4	1	
		28	577	233	13	1	0		66	10	2	

Table (4.7) Antimicrobial activity of oleuropein (0.6% w/v) in distilled water against three types of bacteria, one yeast and one mold.

Microorganism 10^6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	$>10^{3..}$	$>10^3$	$>10^3$	541	87			577	45	
		7	0	0	0	0	0				$>10^3$	1748
		14	0	0	0	0	0				$>10^3$	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	$>10^3$	$>10^3$	291	28	3			180	16	
		7	779	62	8	2	0				$>10^3$	1270
		14	0	0	0	0	0				$>10^3$	440
		28	0	0	0	0	0			$>10^3$	375	
E.coli	8739	0	$>10^3$	$>10^3$	$>10^3$	577	51			449	32	
		7	$>10^3$	416	94	13	6				$>10^3$	$>10^3$
		14	0	0	0	0	0				$>10^3$	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	$>>10^3$	$>10^3$	633	100	12		928	115	28	
		7	$>10^3$	$>10^3$	$>10^3$	1114	327			$>10^3$	1450	
		14	770	640	514	155	12			$>10^3$	$>10^3$	
		28	440	539	70	2	0				1269	
A. Niger	16404	0	$>10^3$	$>10^3$	270	34	3	$>10^3$	484	69	5	
		7	324	45	3	0	0		90	6	1	
		14	150	21	2	0	0		28	4	1	
		28	230	16	6	0	0		66	10	2	

4.4 Olive leaves extract

The same tests were repeated using OLE instead of oleuropein at three concentrations (0.2, 0.3, and 0.4% w/v). At (0.2% w/v) OLE, results showed that it is effective against the three bacteria tested but not effective against yeast and mold (Table 4.4). Increasing the concentration to (0.3% w/v) OLE did not give the desired activity against yeast and mold (Table 4.5), which guides us to increase the concentration to (0.4% w/v) which resulted in a good activity against the three bacteria as well as yeast and mold (Table 4.6). So this implies that OLE at this concentration can be used as the preservative in pharmaceutical syrup.

Comparison between oleuropein and OLE

It is interesting to compare oleuropein with OLE in terms of antimicrobial activity. Referring to tables 1- 6, it is clear that OLE is more effective compared to oleuropein till (0.4% w/v) of OLE is sufficient to be used as preservative compared to (0.6% w/v) oleuropein. This may be attributed to additional active compounds (e.g. polyphenolic and flavonoids) in OLE which may have such activity or have synergistic activity.

Table (4.8). Antimicrobial activity of OLE (0.2% w/v) in distilled water against three types of bacteria, one yeast and one mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	>>10^{3}..	>10^{3}	>10^{3}	1315	481			577	45	
		7	0	0	0	0	0				>10^{3}	1748
		14	0	0	0	0	0				>10^{3}	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	>>10^{3}	>10^{3}	>10^{3}	611	213			180	16	
		7	1515	491	101	21	0				>10^{3}	1270
		14	0	0	0	0	0				>10^{3}	440
		28	0	0	0	0	0			>10^{3}	375	
E.coli	8739	0	>>10^{3}	>10^{3}	>10^{3}	1316	512			449	32	
		7	>10^{3}	821	317	81	22				>10^{3}	>10^{3}
		14	90	42	9	0	0				>10^{3}	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	>10^{3}	>10^{3}	1117	355	109		928	115	28	
		7	>10^{3}	>10^{3}	>10^{3}	1490	501			>10^{3}	1450	
		14	>10^{3}	>10^{3}	>10^{3}	1115	282			>10^{3}	>10^{3}	
		28	>10^{3}	>10^{3}	>10^{3}	251	301				1269	
A.niger	16404	0	>10^{3}	>10^{3}	833	196	30	>10^{3}	484	69	5	
		7	>10^{3}	517	216	55	12		90	6	1	
		14	>10^{3}	1515	409	56	22		28	4	1	
		28	617	455	31	5	0		66	10	2	

Table (4.9). Antimicrobial activity of OLE (0.3% w/v) in distilled water against three types of bacteria, one yeast and one mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	$>10^{3}$..	$>10^{3}$	$>10^{3}$	1466	491			577	45	
		7	0	0	0	0	0				$>10^{3}$	1748
		14	0	0	0	0	0				$>10^{3}$	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	$>10^{3}$	$>10^{3}$	$>10^{3}$	531	221			180	16	
		7	1516	741	312	76	15				$>10^{3}$	1270
		14	93	30	6	0	0				$>10^{3}$	440
		28	0	0	0	0	0			$>10^{3}$	375	
E.coli	8739	0	$>10^{3}$	$>10^{3}$	$>10^{3}$	1312	512			449	32	
		7	$>10^{3}$	821	310	90	16				$>10^{3}$	$>10^{3}$
		14	135	24	5	0	0				$>10^{3}$	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	$>10^{3}$	>100	1087	152	33		928	115	28	
		7	$>10^{3}$	$>10^{3}$	$>10^{3}$	1461	519			$>10^{3}$	1450	
		14	$>10^{3}$	$>10^{3}$	$>10^{3}$	1201	155			$>10^{3}$	$>10^{3}$	
		28	$>10^{3}$	$>10^{3}$	$>10^{3}$	551	175				1269	
A.niger	16404	0	$>>10^{3}$	$>10^{3}$	711	122	12	$>10^{3}$	484	69	5	
		7	$>10^{3}$	633	188	47	6		90	6	1	
		14	$>10^{3}$	1420	325	45	22		28	4	1	
		28	716	341	22	5	0		66	10	2	

Table (4.10). Antimicrobial activity of OLE (0.4% w/v) in distilled water against three types of bacteria, one yeast and One mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	$>10^{3}$..	$>10^{3}$	$>10^{3}$	491	79			577	45	
		7	0	0	0	0	0				$>10^{3}$	1748
		14	0	0	0	0	0				$>10^{3}$	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	$>10^{3}$	$>10^{3}$	267	21	2			180	16	
		7	519	43	3	0	0				$>10^{3}$	1270
		14	0	0	0	0	0				$>10^{3}$	440
		28	0	0	0	0	0			$>10^{3}$	375	
E.coli	8739	0	$>10^{3}$	$>10^{3}$	$>10^{3}$	435	32			449	32	
		7	$>10^{3}$	218	79	11	3				$>10^{3}$	$>10^{3}$
		14	0	0	0	0	0				$>10^{3}$	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	$>10^{3}$	$>10^{3}$	510	78	9		928	115	28	
		7	1150	880	760	445	246			$>10^{3}$	1450	
		14	880	776	446	109	2			$>10^{3}$	$>10^{3}$	
		28	411	408	42	0	0				1269	
A. Niger	16404	0	$>10^{3}$	$>10^{3}$	185	21	0	$>10^{3}$	484	69	5	
		7	411	32	2	0	0		90	6	1	
		14	132	15	5	0	0		28	4	1	
		28	21	8	0	0	0		66	10	2	

4.4 Thyme oil

The same tests were repeated using Thyme oil at (0.1% v/v)concentration. At this concentration, results showed that it is effective against the three bacteria only (Table 4.7). Mixture of thyme oil at this concentration and oleuropein (0.4% w/v) showed also activity against the three bacteria tested and effective against yeast and mold (Table 4.8). This implies that thyme oil/oleuropein mixture can be used as natural preservatives in pharmaceutical preparations.

Table (4.11). Antimicrobial activity of thyme oil (0.1% w/v) in distilled water against three types of bacteria, one yeast and One mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	$>10^{3..}$	$>10^{3}$	$>10^{3}$	491	79			577	45	
		7	821	617	0	0	0				$>10^{3}$	1748
		14	0	0	0	0	0				$>10^{3}$	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	$>10^{3}$	$>10^{3}$	267	21	2			180	16	
		7	519	43	3	0	0				$>10^{3}$	1270
		14	0	0	0	0	0				$>10^{3}$	440
		28	0	0	0	0	0			$>10^{3}$	375	
E.coli	8739	0	$>10^{3}$	$>10^{3}$	$>10^{3}$	435	32			449	32	
		7	$>10^{3}$	218	79	11	3				$>10^{3}$	$>10^{3}$
		14	0	0	0	0	0				$>10^{3}$	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	$>10^{3}$	$>10^{3}$	910	401	290		928	115	28	
		7	$>10^{3}$	$>10^{3}$	860	445	246			$>10^{3}$	1450	
		14	880	776	516	109	62			$>10^{3}$	$>10^{3}$	
		28	901	808	550	202	75				1269	
A. niger	16404	0	$>10^{3}$	$>10^{3}$	$>10^{3}$	811	623	$>10^{3}$	484	69	5	
		7	$>10^{3}$	$>10^{3}$	921	732	435		90	6	1	
		14	486	355	513	420	390		28	4	1	
		28	521	301	500	450	399		66	10	2	

Table (4.12). Antimicrobial activity of oleuropein (0.4% w/v) and thyme oil (0.1% v/v) in distilled water against three types of bacteria, one yeast and One mold.

Microorganism 10*6 CFU/ml	ATCC NO.	Day	Test dilution					Control dilution (without preservative)				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10-6
S. aureus	6538	0	>10^{3}..	>10^{3}	>10^{3}	311	61			577	45	
		7	0	0	0	0	0				>10^{3}	1748
		14	0	0	0	0	0				>10^{3}	838
		28	0	0	0	0	0				770	68
P. aeruginosa	9027	0	>10^{3}	>10^{3}	215	31	5			180	16	
		7	419	23	9	0	0				>10^{3}	1270
		14	0	0	0	0	0				>10^{3}	440
		28	0	0	0	0	0			>10^{3}	375	
E.coli	8739	0	>10^{3}	>10^{3}	>10^{3}	255	52			449	32	
		7	>10^{3}	202	99	21	8				>10^{3}	>10^{3}
		14	0	0	0	0	0				>10^{3}	597
		28	0	0	0	0	0				912	100
C. albican	10231	0	>10^{3}	>10^{3}	703	58	15		928	115	28	
		7	1150	820	560	245	346			>10^{3}	1450	
		14	810	476	446	109	8			>10^{3}	>10^{3}	
		28	411	455	31	0	0				1269	
A. niger	16404	0	>10^{3}	>10^{3}	185	21	0	>10^{3}	484	69	5	
		7	317	42	2	0	0		90	6	1	
		14	192	16	18	0	0		28	4	1	
		28	131	9	0	0	0		66	10	2	

In order to test the effectiveness of natural substances (oleuropein and OLE) and (thyme oil) as natural preservatives and natural antioxidants in pharmaceuticals, three types of drugs were used primarily for children to study the possibility of replacing chemical preservatives and antioxidants (which are unsafe) especially for children by natural substances which are more safe. The pharmaceutical drugs used were Dexamethasone Sodium Phosphate Oral Syrup, Sodium Chloride Nasal Spray, and Vitamin D3 Oral drops. The natural materials were introduced in accordance with the results of the antimicrobial tests. The pharmaceutical syrups were then followed up when the natural materials were tested for their effectiveness as preservatives through several tests in the microbiology labs. The preparations were also followed up in terms of the proportion and consistency of natural materials as well as the stability and proportion of the active materials through analysis by HPLC.

4.5 Use of natural preservatives in pharmaceutical preparations

In this work, oleuropein and OLE were used in the Dexamethasone Sodium Phosphate Oral Syrup pharmaceutical preparation, while oleuropein and thyme oil were used in

Sodium Chloride Nasal Spray, and oleuropein and thyme oil were used in Vitamin D3 Oral Drops.

4.5.1 Dexamethasone Sodium Phosphate Oral Syrup with natural preservative (oleuropein and OLE)

The formulation and follow-up of the preparation were done by analyzing and testing the ratio of the natural substance oleuropein as a natural preservative. Also, active substances (Dexamethasone phosphate) were determined by HPLC and the natural material was followed up in the preparation in terms of its effectiveness as antimicrobial for three months. The three pharmaceutical drugs were stored at accelerated conditions(40 ± 2 ° C /

75% ± 5% RH) to test the stability of the product (concentration of active and natural preservative and stability of the drug itself: pH, precipitation, appearance, color).

By testing the % of oleuropein by (HPLC) in Dexamethasone Sodium Phosphate Oral Syrup, results showed that oleuropein is stable during three months of storage at Accelerated Conditions while the % of oleuropein was 98% at day zero decreased to 97%, 95%, 94% at 1,2,3 months of storage respectively. The results showed that oleuropein is stable in this formula. Chromatogram of Oleuropein standard and sample is shown in figure 4.1, 4.2.

The percentage of the active ingredient (Dexamethasone sodium phosphate) for three months was 96% at zero days decreased to 94%, 94%, 92% in 1,2,3 months of storage respectively (Figure 4.3),where the results indicate that the active substance is stable and not affected by the addition of natural materials and the same results for OLE.

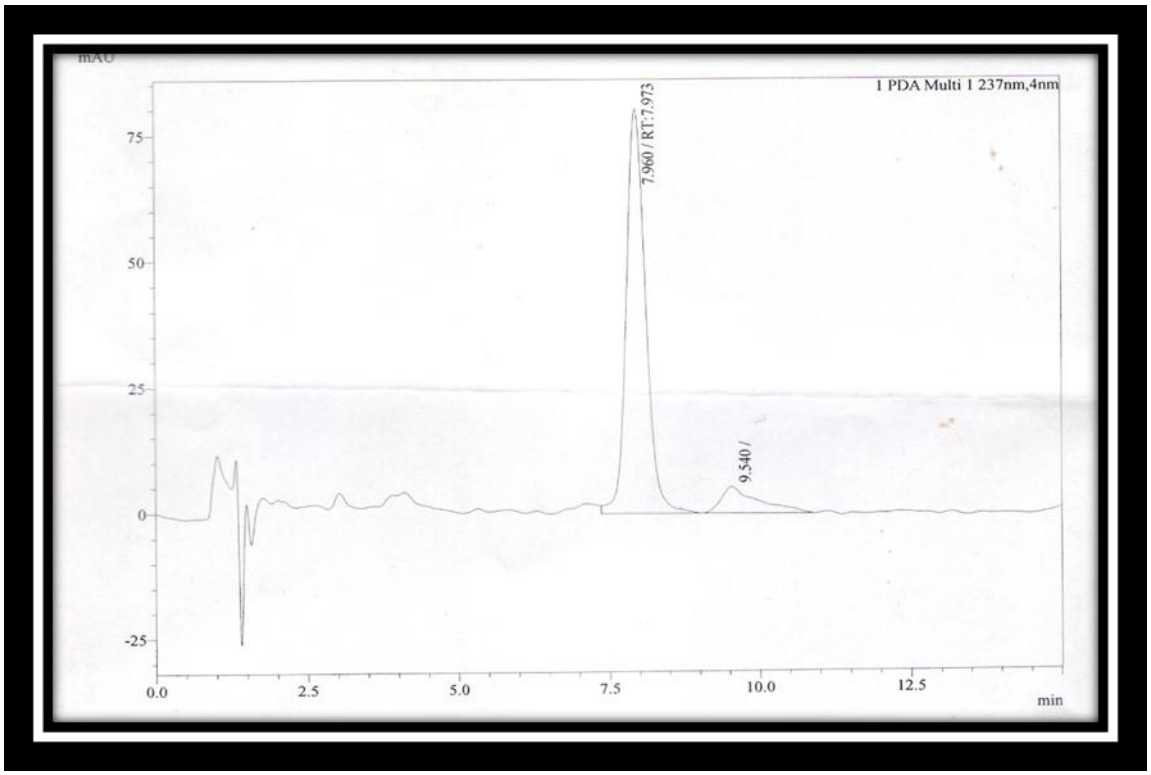


Figure (4.1): Chromatogram of Oleuropein standard

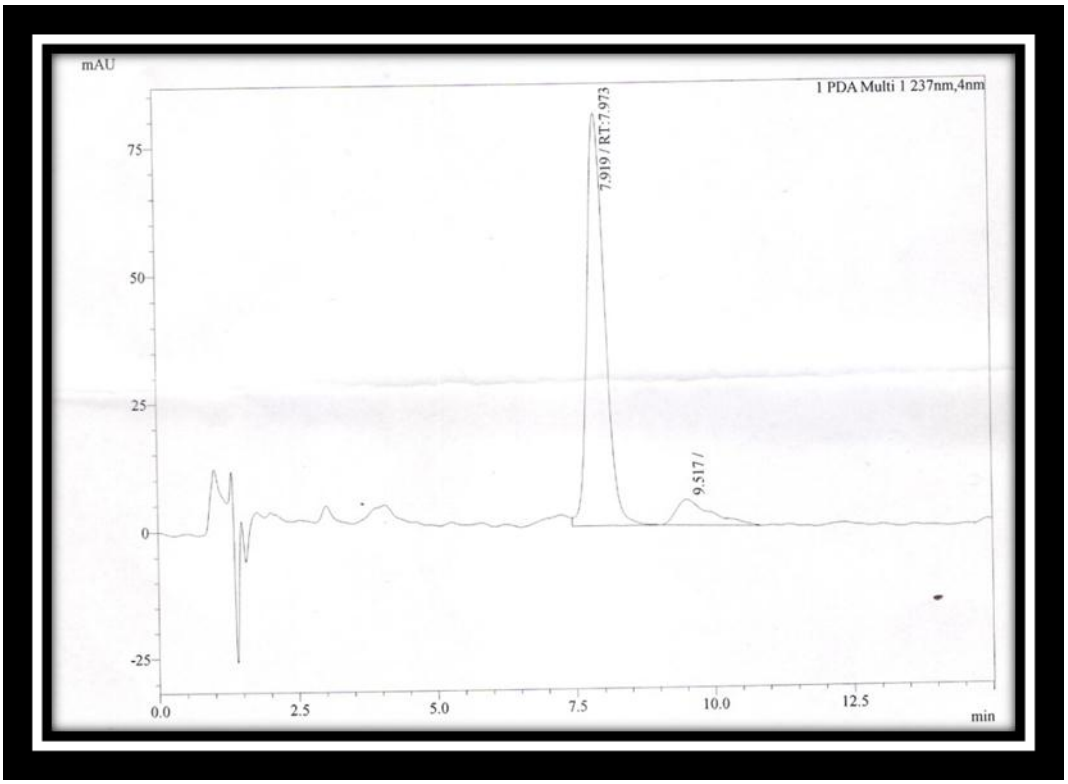


Figure (4.2): Chromatogram of Oleuropein in Dexamethasone Sodium Phosphate Oral Syrup, stored at accelerated Condition (40±2 °C /75%±5% RH) for 3 months.

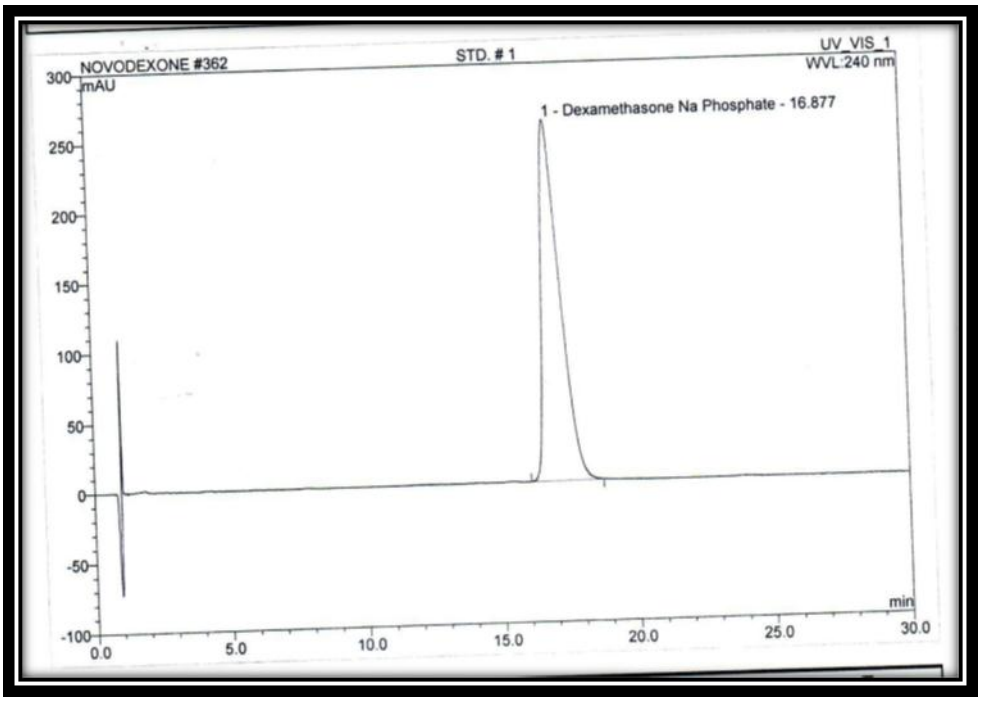


Figure (4.3): Chromatogram of (Dexamethasone sodium phosphate in formulate syrup, stured at accelerated Condition (40±2 °C /75%±5% RH) for 3 months.

4.5.1.2- Effect on pH of Dexamethasone sodium phosphate oral syrup:

As it is shown in table 1,pH of Dexamethasone sodium phosphate oral syrup was not affected by the addition of oleuropein where very minor decrease in pH was observed, still it is in the acceptable range of pH for this product. (table 4.13)

Table (4.13): Effect on pH of Dexamethasone sodium phosphate syrup:

pH (Dexamethasone sodium phosphate syrup)	Range pH (4.5-6)
0 Month	5.6
1 Month	5.6
2 Month	5.4
3 Month	5.3

4.5.1.3. Antimicrobial test of Dexamethasone sodium phosphate syrup:

4.5.1.3.1Microbial limit test – Direct transfer (broth media):

Microbial test for Dexamethasone sodium phosphate syrup with oleuropein 0.6% w/v concentration was conducted for 0,1,2, and 3 months of storage, and compared to the positive control (Dexamethasone sodium phosphate with methyl and propylparaben) and negative control (Dexamethasone sodium phosphate without any preservative).

Results showed that there is no bacterial growth using the three media shown in table (4.10), which is exactly similar to the result of Dexamethasone sodium phosphate control sample (with methyl and propylparaben preservative). This result showed the effectiveness of oleuropein as antimicrobial agent. Negative control of Dexamethasone sodium phosphate syrup without preservative showed bacterial growth (see table 4.14)

Table (4.14): Microbial limit test – Direct transfer (broth media) test for Dexamethasone sodium phosphate syrup with oleuropein 0.6% concentration:

Medium	Results of sample with oleuropein at 0.6%	Results positive control (using methyl and propyl paraben)	Results Negative control No preservative
Tryptic soy Broth	Clear	Clear	Turbid
Fluid lactose medium	Clear	Clear	Turbid
Sabouraud dextrose broth	Clear	Clear	Turbid

4.5.1.3.2 Microbial limit test –Total count:

The total microbial count of Dexamethasone sodium phosphate syrup samples was conducted and results (table 4.14) showed that samples with oleuropein is compatible to the positive control (Dexamethasone sodium phosphate syrup with methyl and propylparaben preservative) for example the number of bacterial on tryptic soy agar were found to be <10 CFU for each type of bacteria which is in accordance with the acceptable limit (<200 CFU).

The same results were obtained for yeast and mold, where results showed that Dexamethasone sodium phosphate syrup samples with oleuropein are close to that with convention chemical preservative methyl and propylparaben (Table 4.15).

Table (4.15): Microbial limit test –Total count test for Dexamethasone sodium phosphate syrup with oleuropein 0.6% w/v concentration

Medium	Limits on agar CFU #\ml	Results Sample with oleuropein at 0.6% CFU/ml	Results positive control CFU/ml	Results Negative control CFU/ml
tryptic soy agar	Bacterial <200(10^2))	<10 CFU	<10 CFU	<180 CFU
sabouraud dextrose agar	yeasts and molds <20 (10^1)	<10 CFU	<10 CFU	<10 CFU

4.5.1.3.3 Microbial limit test – membrane filtration:

The number of bacteria on tryptic soy agar was <10 CFU for each type of bacteria, and this number is associated with the standard limits (ATCC) which must be <200 CFU. While yeast and mold colony number on sabouraud dextrose agar was <10 CFU, this number is within the standard limits (which should be <20 CFU units), in comparison with the product containing natural preservatives sample results, the results of positive control were very close. The bacteria, yeasts and mold number in negative control are high compared to the positive test preparation and with the product containing natural preservatives. (see table 4.16)

Table (4.16): Microbial limit test – membrane filtration test for Dexamethasone sodium phosphate syrup with oleuropein 0.6% w/v concentration:

Medium	Limits on agar CFU/ml	Results Sample with oleuropein 0.6% CFU/ml	Results positive test CFU/ml	Results Negative control test CFU/ml
tryptic soy agar	<200(10^2)) Bacteria	<10	< 10	<120
Sabouraud dextrose agar	yeasts and molds <20 (10^1)	<10	< 10	<20

4.5.2 Sodium Chloride nasal spray with oleuropein and thyme oil

In the second formulation, Sodium Chloride nasal spray containing oleuropein (0.4% w/v concentration) and thyme oil in the concentration (0.1% v/v). The product is formulated with natural preservative instead of the chemical preservative (benzalkonium chloride) in sodium chloride The natural materials were also prepared and tested for their effectiveness against microbes through the tests mentioned previously and tests were repeated for the same stored specimen each month(for three months), another formula was prepared to without preservatives (negative control). The results of the product containing natural preservatives were compared with the preparations (positive control).

By testing the percentage (%) of oleuropein by (HPLC) in (Sodium Chloride Nasal Spray), results showed that oleuropein is stable during three months of storage at Accelerated Conditions while the % of oleuropein was 96% of day zero decreased to 95%, 94%, 92% at 1,2,3 months of storage respectively These results showed that oleuropein is stable in this formula. Chromatogram of Oleuropein standard and sample is shown infigure 4.4, 4.5).

The percentage of the active ingredient (Sodium chloride(NaCl)) for three months by titration method was as follows: 97% of zero days decreased to 96%, 95%, 95% in 1,2,3 months of storage respectively Where the results indicate that the active substance is stable and not affected by the addition of natural materials.

Figure (4.4): Chromatogram of Oleuropein standard

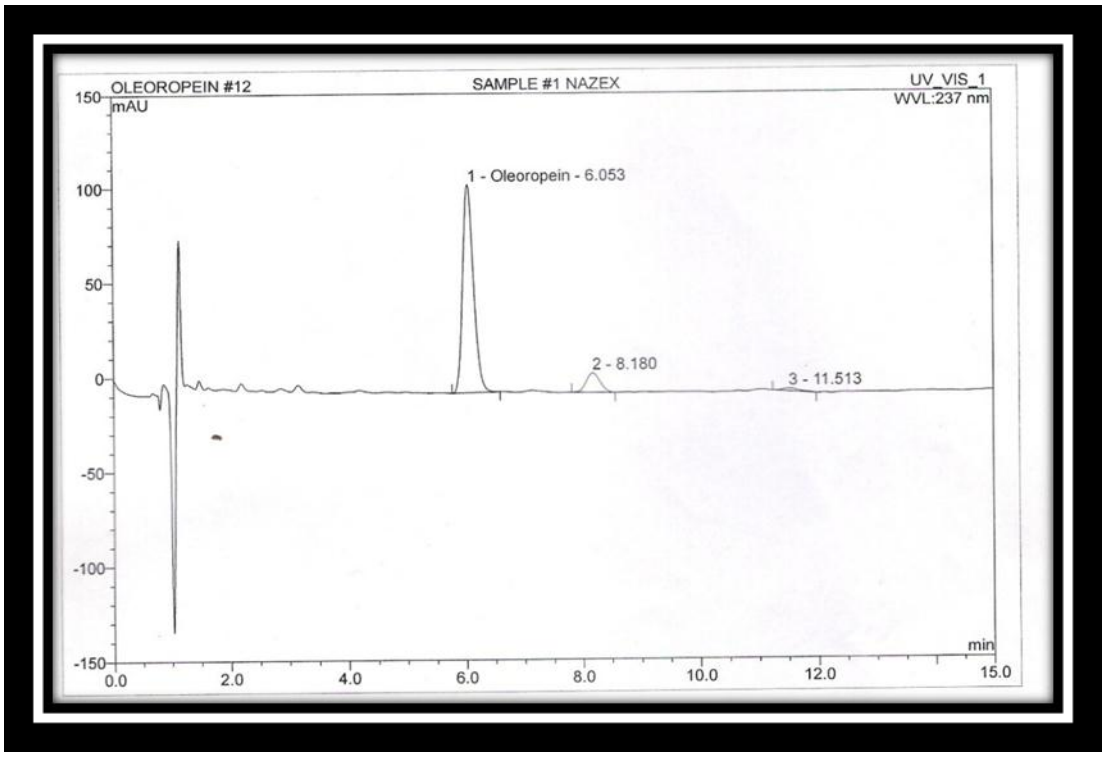


Figure (4.5): Chromatogram of Oleuropein in Sodium Chloride nasal spray, stored at accelerated Condition (40±2 °C /75%±5% RH) for 3 months.

4.5.2.2 Effect on pH of Sodium Chloride nasal spray:

As it is shown in table 10, pH of Sodium Chloride nasal spray was not effected by addition of oleuropein where very minor decrease in pH was observed ,but still in the acceptable range of pH for this product.

Table (4.17): Effect on pH on Sodium Chloride nasal spray

pH (Sodium Chloride nasal spray)	Range PH (5-7.5)
0 Month	6.6
1 Month	6.6
2 Month	6.5
3 Month	6.5

4.4.2.3 Microbial limit test –

4.4.2.3.1 Direct transfer (broth media):

Microbial test for Sodium Chloride nasal spray with oleuropein 0.4% w/v and thyme oil 0.1% v/v concentration was conducted at 0,1,2, and 3 months of storage, and compared to the positive control (Sodium Chloride nasal spray with benzalkonium) and negative control (Sodium Chloride nasal spray without any preservative)

Results showed that there is no bacterial growth using the three media shown in table (4.14) which is exactly similar to the result of Sodium Chloride nasal spray control sample (with benzalkonium preservative). This result showed that oleuropein and thyme oil are effective as antimicrobial agent. Negative control of Sodium Chloride nasal spray without preservative showed bacterial growth (Table 4.18)

Table (4.18): Microbial limit test – Direct transfer (broth media):

Medium	**Sample with oleuropein (0.4%) and thyme oil (0.1)**	**Results positive test (using benzalkonium chloride)**	**Results Negative control (No preservative)**
Tryptic soy Broth	Clear	Clear	Turbid
Fluid lactose medium	Clear	Clear	Turbid
Sabouraud dextrose broth	Clear	Clear	Turbid

4.5.2.3.2- Microbial limit test –Total count:

The total microbial count of Sodium Chloride nasal spray samples was conducted and results (table 1.3.2) showed that samples with oleuropein and thyme oil is compatible to the positive control (Sodium Chloride nasal spray with benzalkonium preservative) for example the number of bacteria on tryptic soy agar were found to be <10 CFU for each type of bacteria which is in accordance with the acceptable limit (<200 CFU). The same

results were obtained for yeast and mold, where results showed that Sodium Chloride nasal spray samples with oleuropein are very close to that with convention chemical preservative benzalkonium (table 4.19)

Table (4.19): Microbial limit test –Total count:

Medium	**Limits on agar CFU/ml**	**Sample with oleuropein (0.4%) and thyme oil (0.1) CFU/ml**	**Results positive test CFU/ml**	**Results Negative control test CFU/ml**
tryptic soy agar	<200(10^2)) Bacteria	<10	<10	<120
sabouraud dextrose agar	yeasts and molds <20 (10^1)	<10	<10	<18

4.5.2.3.3 - Microbial limit test – membrane filtration:

The number of bacteria in the product containing natural preservatives on tryptic soy agar was <10 CFU for each type of bacteria, and this number is associated with the standard limits (ATCC) which must be <200 CFU.

While for yeast and mold on Sabouraud dextrose agar the number of colonies in the same product were<10 CFU/ml for each type, this number is acceptable compared to the standard limits (which should be <20CFU/ml).

In positive control the number of colonies grown on agar plates for the bacteria, yeast and mold are high in comparison with the standard limits and the product containing natural preservatives. (See table 4.20)

Table (4.20): Microbial limit test – membrane filtration:

Medium	Limits on agar CFU/ml	Results Sample with oleuropein (0.4%) and thyme oil (0.1) CFU/ml	Results Positive control CFU/ml	Results of negative control CFU/ml
tryptic soy agar	<200(10^2)) Bacteria	<10	<10	<220
Sabouraud dextrose agar	yeasts and molds <20 (10^1)	<10	<10	<20

4.5.3 Vitamin D3 Oral Drops with oleuropein and thyme oil

In the third formula, oleuropein was used in the concentration (0.4% w/v), and the thyme oil used at a concentration of (0.2% v/v) in Vitamin D3 Oral Drops.

It was formulated as a natural preservative and also as an antioxidant instead of a chemical (Butylhyroxytoluene). The formulation and follow-up of the product were carried out by analyzing and testing oleuropein as a natural preservative and also test and analyzing the ratio of active ingredient in vitamin D3 by (HPLC). Natural materials were also followed in preparation in terms of their effectiveness as antimicrobial agents through tests and had for months compared with a positive test in terms of results and also compared with negative control test.

By testing the % of (oleuropein) by (HPLC) in Vitamin D3 Oral Drops, results showed that oleuropein is stable during three months of storage at Accelerated Conditions while the % of oleuropein was 95% of day zero decreased to 93.7%, 93%, 91.8% at 1,2,3 months of storage respectively. These results showed that oleuropein is stable in this formula. Chromatogram of Oleuropein standard and sample were shown (see figure 4.6, 4.7)

The percentage of the active ingredient (vitamin D3) for three months was as follows: 98% of zero days decreased to 97%, 95%, 94% in 1, 2, 3 months of storage respectively (Figure 4.8, 4.9), where the results indicate that the active substance is stable and not affected by the addition of natural materials.

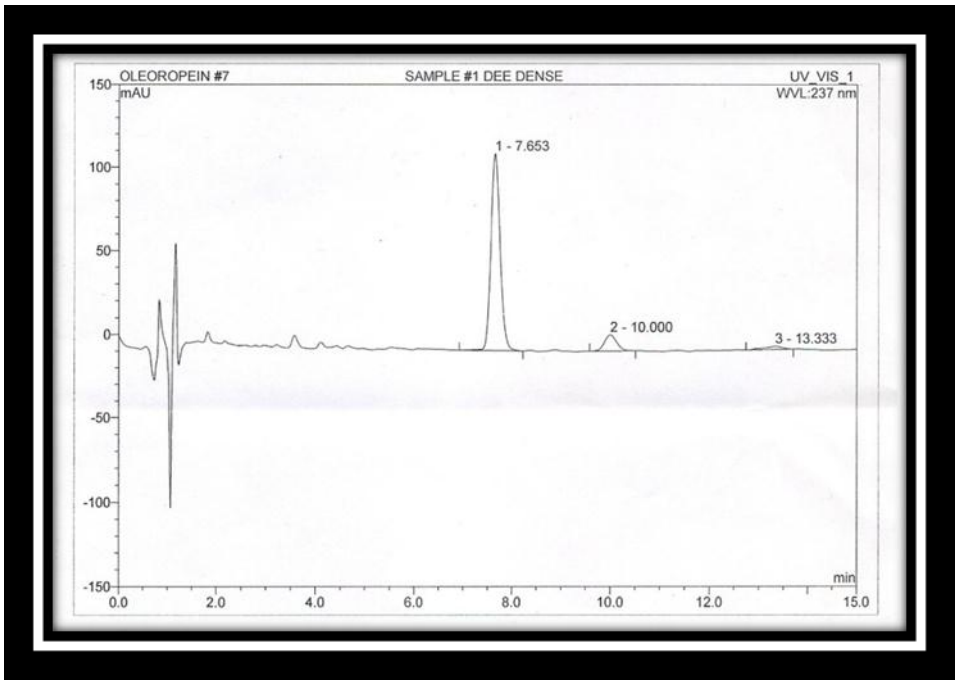


Figure (4.6): Chromatogram of Oleuropein standard

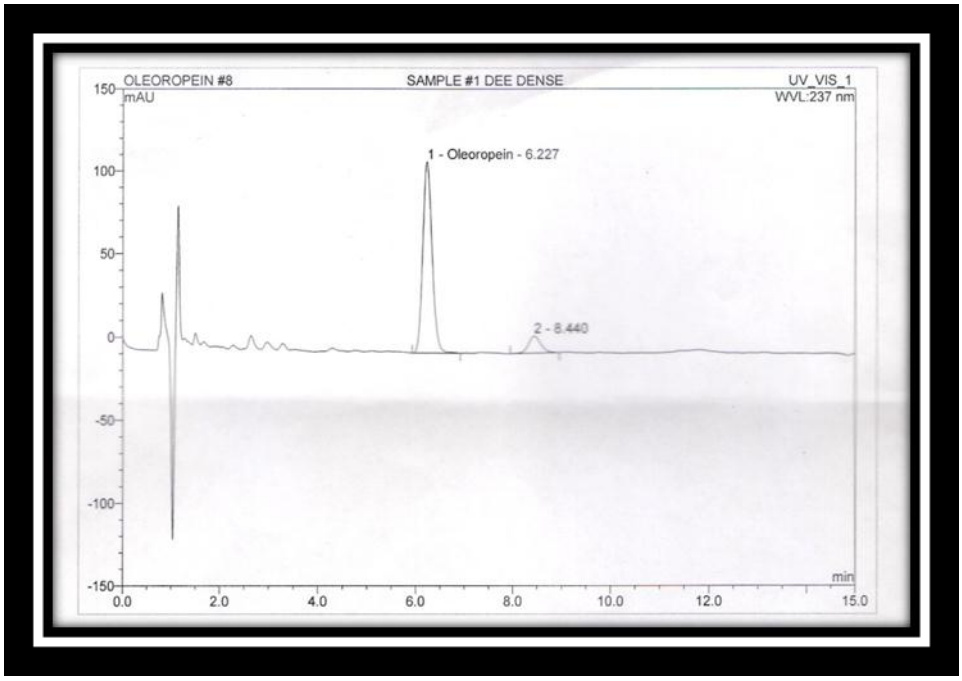


Figure (4.7): Chromatogram of Oleuropein in Vitamin D3 Oral Drops, stored at accelerated Condition (40±2 °C /75%±5% RH) for 3 months

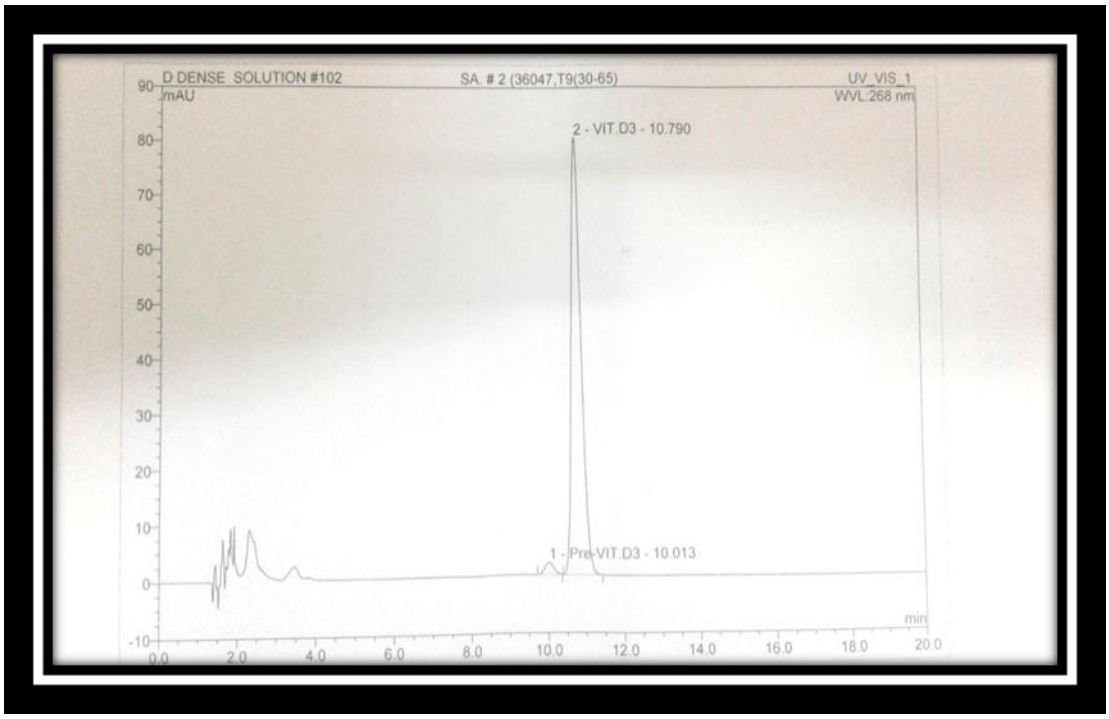


Figure (4.8): Chromatogram of vitamin D3 standard

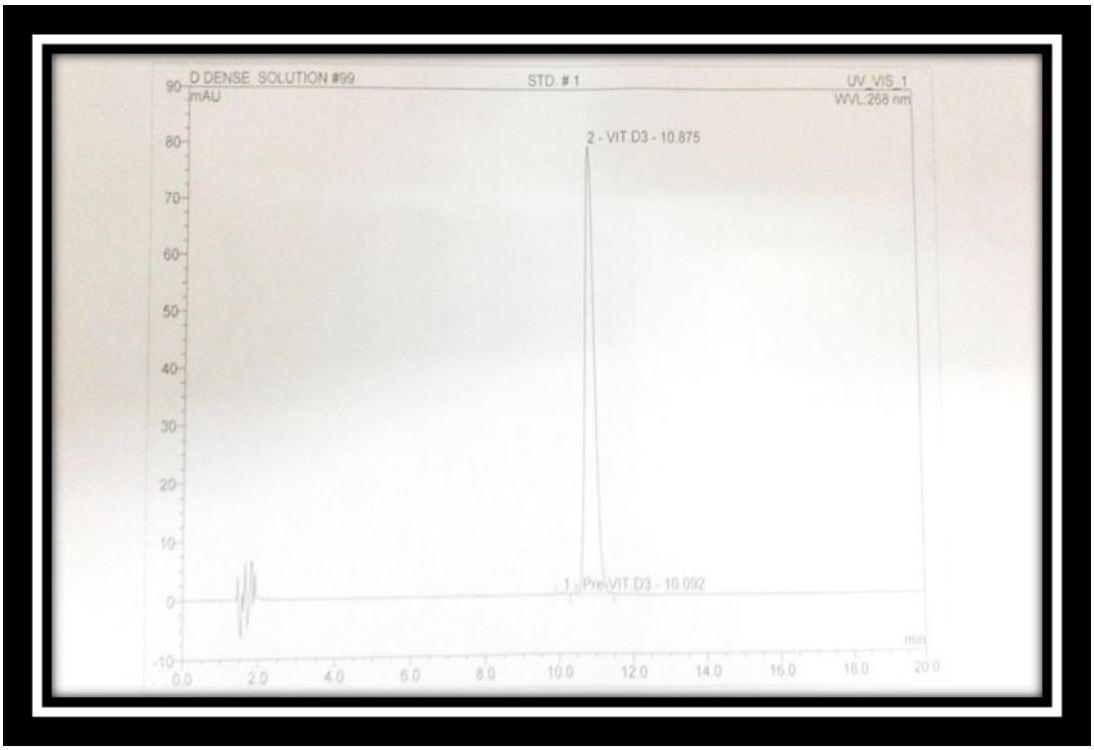


Figure (4.9): Chromatogram of vitamin D3 in Vitamin D3 Oral Drops, stored at accelerated Condition (40±2 °C /75%±5% RH) for 3 months

4.5.3.2 Effect on pH of Vitamin D3 Oral Drops

As it is shown in table, pH of Vitamin D3 Oral Drops were not affected by the addition of oleuropein where very minor decrease in pH was observed, still it is in the acceptable range of pH for this product.

Table (4.21): Effect on pH on Vitamin D3 Oral Drops

pH (Vitamin D3 Oral Drops)	Range PH (2-4)
0 Day	3.0
1 Month	3.1
2 Month	2.9
3 Month	2.9

4.5.3.3 Microbial limit test

4.5.3.3.1 Direct transfer (broth media):

Microbial test for Vitamin D3 Oral Drops with oleuropein 0.4% w/v and thyme oil 0.2% v/v concentration was conducted at 0,1,2, and 3 months of storage, and compared to the positive control (Vitamin D3 Oral Drops with glycerin).

Results showed that there is no bacterial growth using the three media shown in table(4.18) which is less than the result of Vitamin D3 Oral Drops control sample (with glycerin preservative). This refers to the use of natural substances (oleuropein or thyme oil) with the chemical (glycerin) which showed results better than those with the use of (glycerin) only (see table(4.22).

Table (4.22): Microbial limit test – Direct transfer (broth media):

Medium	**Sample with oleuropein (0.4%) and thyme oil (0.2)**	**Results positive test (using glycerin)**	**Negative control No preservative**
Tryptic soy Broth	Clear	Clear	Clear
Fluid lactose medium	Clear	Clear	Clear
Sabouraud dextrose broth	Clear	Clear	Clear

4.5.3.3.2 Microbial limit test –Total count and Membrane filtration:

The number of bacteria on tryptic soy agar was <10 CFU for each type of bacteria, and this number is associated with the standard limits (ATCC) which must be <200 CFU. While for yeast and mold on Sabouraud dextrose agar was <10 CFU for each type, this number is compared to the standard limits (which should be <20 CFU units), in comparison with the positive preparation, the results show that the product containing natural preservatives show results that are very close to those obtained from positive test preparation. (Table 4.23)

Table (4.23): Microbial limit test –Total count and Membrane filtration:

Medium	**Limits on agar CFU #\ml**	**Sample with oleuropein (0.4%) and thyme oil (0.2) CFU/ml**	**Results positive control CFU/ml**	**Results Negative control CFU/ml**
tryptic soy agar	<200(10^2)) Bacteria	<10	<10	<10
Sabouraud dextrose agar	yeasts and molds <20 (10^1)	<10	<10	<10

4.6 Stability of pharmaceutical preparations

The results showed that pharmaceutical product (Dexamethasone Sodium Phosphate Oral Syrup) When oleuropein was used at a concentration of 0.6% w/v was stable for three months with no Precipitation, appearance and color stable and pH remained within the allowable range (4.5- 6). The active ingredient (*Dexamethasone sodium phosphate*) and oleuropein were within the allowable range.

However, when the same product was used with (OLE) at a concentration of 0.4% w/v, all the results were stable except that after a month of preparation there was some Precipitation because some substances (OLE) did not completely dissolve and needed a surfactant. As shown in the table (4.24)

As well as for the pharmaceutical product (Sodium Chloride Nasal spray) where oleuropein was used at 0.4% w/v and thyme oil at 0.1% v/v concentration. The results for three months were stable. There was no precipitation, appearance and, color stable and pH within the permissible range of (7.5 - 6) and active ingredient (sodium chloride (NaCl) and (oleuropein) within the allowable range as shown in the table (4.24)

And also for the third pharmaceutical product (Vitamin D3 Oral Drops) When oleuropein was used, with a concentration of 0.4% w/v and thyme oil at a concentration of 0.2%. v/v The results for three months are stable with no Precipitation, appearance, color stable pH within the permissible range (2-4), the active ingredient (vitamin D3 crystals) and oleuropein within the allowable range. As shown in the table (4.24)

Table (4.24): Physical parameters and assay results of pharmaceutical formulations Accelerated Condition at (40± 2 °C /75%±5% RH):

Comparisons	Time	Precipitation	appearance	Color	pH	Assay of oleuropein	Assay of Active material
Dexamethasone Sodium Phosphate Oral Syrup	0 day	Negative	Uniform	Red	5.6	98%	96%
	1 Month	Negative	Uniform	Red	5.6	97%	94 %
	2 Month	Negative	Uniform	Red	5.4	96%	94%
	3 Month	Negative	Uniform	Red	5.3	95%	92%
Sodium Chloride Nasal Spray	0 Day	Negative	Uniform	Brown	6.6	96%	97%
	1 Month	negative	Uniform	Brown	6.6	95%	95%
	2 Month	negative	Uniform	brown	6.5	94%	93%
	3 Month	Negative	Uniform	brown	6.5	92%	92 %
Vitamin D3 Oral Drops	0 Day	negative	Uniform	Golden	3.0	95%	98 %
	1 Month	negative	Uniform	Golden	3.1	93.7%	97 %
	2 Month	negative	Uniform	Golden	2.9	93.%	95 %
	3 Month	Negative	Uniform	Golden	2.9	91.8%	94 %

4.7 Microbial stability of pharmaceutical preparations

Pharmaceutical preparations were tested in terms of the numbers of bacteria, yeasts, and mold. Tryptic soy agar was used as a medium for the bacteria, while Sabouraud dextrose agar was used for yeasts and mold. The result was less than < 10 CFU of bacteria, yeasts, and mold. The results were close to that of the positive test. The same result was obtained for the pharmaceuticals (Sodium Chloride Nasal Spray) and (Vitamin D3 Oral Drops). As shown in the table (4.25).

Dexamethasone Sodium Phosphate Oral Syrup was also tested using Membrane filtration, yeast, and mold. Tryptic soy agar was used, (Sabouraud dextrose agar) for three months. The result was less than 10 CFU of bacteria, yeast and mold. The results were close to that of a positive test. The same results were obtained for pharmaceuticals Sodium Chloride Nasal Spray and Vitamin D3 Oral Drops as shown in Table (4.25).

Table (4.25): Antimicrobial limit test of pharmaceutical syrup formulations at

	Month	Result	Positive	Control test

accelerated conditions (40±2 °C /75%±5% RH):

	(Novodexon)		test	
Type of test	**month**	**Result**	**Positive**	**Control test**
Direct transfer(broth media)	**Dexamethasone Sodium Phosphate Oral Syrup**			
	0	Clear	Clear	Turbid
	1	Clear	Clear	Turbid
	2	Clear	Clear	Turbid
	3	Clear	Clear	Turbid
Total count	**Dexamethasone Sodium Phosphate Oral Syrup**			
	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<100CFU
	2	<10 CFU	<10 CFU	<180CFU
	3	<10 CFU	<10 CFU	<180 CFU
Membrane Filtration	**Dexamethasone Sodium Phosphate Oral Syrup**			
	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<100 CFU
	2	<10 CFU	<10 CFU	<120CFU
	3	<10 CFU	<10 CFU	<120 CFU
Direct transfer(broth media)	**Sodium Chloride Nasal Spray**			
	0	Clear	Clear	Turbid
	1	Clear	Clear	Turbid
	2	Clear	Clear	Turbid
	3	Clear	Clear	Turbid
Total count	**Sodium Chloride Nasal Spray**			
	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<80 CFU
	2	<10 CFU	<10 CFU	<100 CFU
	3	<10 CFU	<10 CFU	< 120 CFU
Membrane Filtration	**Sodium Chloride Nasal Spray**			
	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<90 CFU
	2	<10 CFU	<10 CFU	<110 CFU
	3	<10 CFU	<10 CFU	<220 CFU
Direct transfer(broth media)	**Vitamin D3 Oral Drops**			
	0	Clear	Clear	Clear
	1	Clear	Clear	Clear
	2	Clear	Clear	Clear
	3	Clear	Clear	Clear
Total count and membrane filtration	**Vitamin D3 Oral Drops**			
	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<10 CFU
	2	<10 CFU	<10 CFU	<10 CFU
	3	<10 CFU	<10 CFU	<10 CFU

Chapter Five: Conclusion and Future work

5.1 Conclusions

1. The efficiency of oleuropein against microbes was verified, with the optimum concentration being 0.6% w/v, which can be used in pharmaceuticals as preservatives.
2. The effectiveness of OLE against microbes was verified where the optimum concentration was 0.4% w/v, which can be used in pharmaceuticals as preservatives.
3. The effectiveness of thyme oil against microbes was verified where the concentration was 0.1% v/v, effective against bacterial only.
4. The effectiveness of a mixture between oleuropein and thyme oil against microbes was verified where the concentration was (0.4% w/v and 0.1% v/v,) which can be used in pharmaceuticals as preservatives.
5. The effectiveness of thyme oil against microbes was verified where the concentration was 0.1% v/v, which can be used antibacterial only.
6. OLE is better than oleuropein where the concentration used is less.
7. The efficacy of oleuropein and thyme oil was verified within the pharmaceutical preparations as it carried a synergistic activity between them and the result against microbes was acceptable.
8. Addition of natural substances oleuropein and thyme oil did not affect the pH of the pharmaceutical formulations.
9. The effectiveness of pharmaceutical preparations against microbes was verified by the presence of natural substances oleuropein and thyme oil and the result was acceptable.
10. Addition of natural substances did not affect the content of active ingredient in pharmaceuticals for three months.
11. Natural substances in the pharmaceutical formulations were stable and effective.

5.2 Future work:

1. Evaluation of Oleuropein and thyme oil in pharmaceutical syrup for one year

2. Evaluation of Oleuropein and thyme oil in pharmaceutical semi-solids as preservative and antioxidant.
3. Evaluation of Oleuropein and OLE in the coating of tablets as preservatives.

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SOP

استخدام مستخلصات الأوراق الطبيعية كبديل للمواد الحافظة الاصطناعية ومضادات الأكسدة في الشراب الصيدلاني

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الملخص

تعتبر المواد الحافظة المصنعة ومضادات الأكسدة (الكيميائية) ذات فعالية عالية ضد الميكروبات وضد الأكسدة، ولكن هناك رغبة متزايدة للحد من استخدام المواد الكيميائية؛ لما لها من آثار جانبية كبيرة وسمية خاصة على الأطفال عند استخدامها لفترات طويلة، وبالتالي أصبحت هناك حاجة ملحة لتطوير منتجات صيدلانية باستخدام مواد طبيعية لها فعالية ضد الميكروبات المسببة للأمراض، ولها قدرة للعمل ضد الميكروبات، وأيضاً القدرة ضد عملية الأكسدة.

الهدف من هذه الدراسة هو تقييم مواد طبيعية: زيت الزعتر ومستخلص أوراق الزيتون و(oleuropein) ضد ثلاث أنواع بكتيريا؛ اثنتين سالبة غرام، وواحدة موجبة غرام، ونوعين من الفطريات (خميرة وعفن)، حيث تم اختبار مستخلص أوراق الزيتون بثلاث تراكيز مختلفة (0.2%، 0.3%، 0.4%)، و(oleuropein) بثلاث تراكيز مختلفة (0.2%، 0.4%، 0.6%)، وزيت الزعتر بتركيز (0.1%)، وخليط من زيت الزعتر و(oleuropein) بتركيز (0.1% و0.4%) حيث كان التركيز الأمثل هو (0.6%) من (oleuropein) و(0.4%) من مستخلص أوراق الزيتون وخليط بين (oleuropein) وزيت الزعتر (0.1% و 0.4%) حيث أظهر الخليط نشاط تآزري بين (oleuropein) وزيت الزعتر التي يمكن أن تستخدم كمواد حافظة.

بناءً على هذه النتائج تم استخدام المواد الطبيعية (oleuropein) وزيت الزعتر ومستخلص أوراق الزيتون داخل ثلاثة مستحضرات صيدلانية، حيث تم استخدام مادة (oleuropein) بتركيز (0.6%)

في المستحضر الصيدلاني (Dexamethasone Sodium Phosphate Oral Syrup). وأيضاً تم استخدام مادة مستخلص أوراق الزيتون بتركيز (0.4%) في نفس المستحضر. وتم استخدام مادة (oleuropein) بتركيز (0.4%) وزيت الزعتر بتركيز (0.1%) داخل المستحضر الصيدلاني (Sodium Chloride Nasal Spray) وتم استخدام مادة (oleuropein) بتركيز (0.4%) وزيت الزعتر بتركيز (0.2%) داخل المستحضر الصيدلاني (Vitamin D3 Oral Drops).

تم اختبار ثباتية واستقرار المستحضرات الصيدلانية لمدة ثلاث شهور حيث تم اختبار فعالية المستحضرات الصيدلانية ضد الميكروبات وضد الأكسدة، وأيضاً تم اختبار خصائص الفيزيائية للمستحضرات الصيدلانية مثل الرقم الهيدروجيني واللون والترسيب والمظهر ونسبة المادة الفعالة، وأيضاً المادة الطبيعية (oleuropein).

حيث أشارت النتائج ثباتية المستحضرات الصيدلانية لمدة ثلاث شهور من الحفظ تحت الظروف المتسارعة من حيث الفعالية ضد الميكروبات، وأيضاً الخصائص الفيزيائية للمستحضرات الصيدلانية بالإضافة إلى نسبة مادة (oleuropein) والمادة الفعالة والرقم الهيدروجيني.