BIRZEIT UNIVERSITY

Faculty of Graduated Studies

Master Program Industrial Pharmaceutical Technology

MSc. Thesis

Development and Formulation of Novel Fusidic acid Cream Containing Metal Ions and Natural Products

تحضير و تطوير حامض الفيوسيدك بالأساليب الحديثة على شكل كريم موضعي يحتوي على أيونات معدنية و منتجات طبيعية

This Thesis is submitted in partial fulfillment of the requirements for the degree of Master in Industrial Pharmaceutical Technology from the Faculty of Graduate Studies at Birzeit University, Palestine.

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April, 2019

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By

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Dedication

I would like to dedicate this thesis to my parents because no one has ever been loving and unconditionally supporting than they have been. They have always been a sourse of encouragement and inspiration.

To wonderful sisters and my fiancee for your encouragement, support, love, care, and sacrifices.

Acknowledgements

In the name of Allah, the most Gracious and the most Merciful. Alhamdulillah, all praises to Allah for his blessing in completing this thesis.

It is a great pleasure to acknowledge those people who have supported me during my research.

I am heartily thankful to my supervisor Doctor Hani Shtaya for his guidance and continuous support. His motivation, advice and the interesting discussions have helped me in my research and writing of this thesis.

I will always be grateful to you for your kindness, your faith in my abilities and for putting your trust in me.

I am eternally grateful for Doctor Mohammed Farraj. I have learn so much from his huge knowledge and advice that were very insightful. Ireally appreciate your hand work and effective efforts that you have to help me in each step and also in writing of this thesis and being so patient.

I would like to express my deepest and sincere gratitude to all members of examination committee.

I am very thankful and gratitude to all the staff and physicans in microbiology laboratory at biet-jala govermental hospital for their help in facilitating the collection of specimens and recording the data.

Special thanks also go to all the wonderfull members in the administration department, Quality control department in Beit-Jala Pharmaceutical Co, Ltd Bethlehem – Palestine for their support and valuble help in molecular work and financial support. With great respect to the conusltant microbiologist Hussam Abu-Rmias in this company.

Lastly, I express my warm thanks to everyone who supported me and aided in complection of this project.

Birzeit, April 2019
Yousef Sahoury

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List of abbreviations and symbols

^{0}C	Dgrees (Clsius)
A site	Aminoacyl site
aa-Trna	Aminoacyl Trna
AS	Active substance as alone
ASPC	Active substance as part of cream
ATCC	American Type Culture Collection
CAMHB	cation-adjusted Mueller-Hinton broth
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institue
Cps	Centipoise
DD	Disc-diffusion
DPPH	Diphenyl-1-picrylhydrazy
EC	Entire cream
EDTA	Ethylene diaminetetra acetic acid
EF-G	Elongation factor G
Eos	Thyme oil
E-Test	Epsilometer test
FD	Fusidic acid
FDA	Food and Drug Administration
FLM	Fluid Lactose Medium
FRSA	Fusidic acid resistance *S. aureus*
FusA	Chromosomal gene encoding EF-G
FusB	Fusidic acid resistance gene
FusC	Fusidic acid resistance gene
FusD	Fusidic acid resistance gene
FusE	Mutation in gene encoding ribosomal protein
FusF	Fusidic acid resistant gene
G	Gram
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
H	Hour
HPLC	High performance liquid chromatography
KCL	Potassium Chloride
LF	Laminar Flow
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
Mg	Milligram
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
Min	Minutes
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Stapylococcus aureus*
NaCl	Sodium chloride
NO	Nitric oxide
Nuc	Thermostable nuclease gene
O.D	Optical density
OH	Hydroxide

OLE	Olive leaves extract
RH	Relative humidity
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SABD, SABDA	Sabouraud Dextrose Broth, Agar
SCDB, SCDA	Soybean-Casein Digest Broth, Agar
TABC	Total aerobic bacterial count
TS	Sterile saline
TYMC	Total aerobic yeasts and molds
UK	United Kingdom
USA	United States of American
WHO	World Health Organization
ZOI	Zone of inhibition

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Development and Formulation of Novel Fusidic acid Cream Containing Metal Ions and Natural Products

By
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ABSTRACT

Fusidic acid has manily been used to treat staphloccocal infections. It has been noticed a significant trend towards increased fusidic acid resistance among staphloccocal aureus when it is used as monotherapy in topical prepations with long duration of use.

The need for new antimicrobial agents has increased due to the expansion of resistance to this agent. Copper sulfate and zinc sulfate are recongnized in pharmaceutical industry for having antiviral and antibacterial properties.

This study aims to formulate various topical creams with antibacterial and antiviral activity. Oleuropine and thyme oil are natural products which have antimicrobial activity and used as preservatives in pharmaceutical cream to replace chemical preservative (paraben). Fusidic acid was used as the model drug target. Three cream formulations (F1, F2, F3) were prepared, F2 formula was successfully integrated with medications.

F2 was prepared with O/W emulsion cream base. Its physicochemical properties involving physical appearance, spreadability, homogeneity and color were evaluated. In vitro antibacterial evaluation against *staphloccocal aureus, Escherichia coli*, FRSA (*fusidic acid resistance staphloccocal aureus*), drug content, viscosity and PH was performed. Similar formulations were used, antibacterial activity for F2 was carried out and compared with marketed product. The stability survey of the formula (F2) on antibacterial activity,

physicochemical characteristics and viscosity was determined at 25^0C and 40^0C in plastic jars for over 3 months period. It was found that F2 formula was adequate in spreadability, homogeneity, PH, appearance and viscosity with pseudoplastic behavior. These values obtained showed that our formula displayed better results than the trade product. The desirable synergistic antibacterial effect for zinc and copper sulfate was observed in the optimal concentration of 1.5% against tested microorganisms.

The synergistic concenterations of 0.4% Oleuropine and 0.1% thyme oil showed very promising results as antimicrobial preservatives in pharmaceutical products. The formula (F2) showed considerable zones of inhibition (ZOI) when compared with trade products. Interestingly, considerable numbers (9.5%) of FRSA isolates possessed high resistance pattern with MIC $\geq$ 121 μg/ml, wherase whereas the majority of tested FRSA isolates (90.5%) were still at low resistance pattern with MIC $\leq$ 8 μg/ml. The physicochemical properties for the formula (F2) has proved to be stable at 4^0C and 25^0C through a 3 months period in plastic jars.

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اعداد
يوسف محمود ساحوري

المشرف
الدكتور هاني اشتيه

ملخص

يستخدم الفوسيدك كعلاج للالتهابات العنقودية بصورة رئيسية. لقد لوحظ أن هناك ميل لحدوث المقاومة ضد هذا الدواء من قبل المكورات العنقودية الذهبية بشكل كبير و خاصة عند استخدامه كعلاج موضعي وحيد لفترة طويلة من الزمن.

لقد ارتفعت متطلبات المضادات الحيوية الجديدة بسبب توسع مقاومة هذه المضادات. كبريتات النحاس و كبريتات الزنك لها خصائص مضادة للفيروسات و مضادة للبكتيريا معترف فيها في الصناعة الدوائية.

تهدف هذه الدراسة الى تشكيل عدة صياغات مختلفة من الشكل الصيدلاني الكريم ذات نشاط ضد البكتيريا, وضد الفطريات وضد الفيروسات.

المنتجات الطبيعية (Oleuropine, Thyme oil) لها نشاط مضاد للبكتيريا يستخدم كمواد حافظة في الشكل الصيدلاني الكريم لتحل محل المواد الحافظة الكيميائية (الباربين). الفيوسيدك استعمل كنموذج للدواء الكائن وتم تحضير ثلاث من الصياغات الموضعية الكريم الجديدة, وكانت النتيجة ان التركيبة (F2) ثابتة مع الأدوية. تم اعداد التركيبة (F2) بقاعدة كريم مستحلب O/W , و تقييم خصائصه الفيزيائية الكيميائية التي تشمل المظهر المادي, القابلية, الانتشار, التجانس, اللون, و تحليل مسح مضاد للجراثيم في المختبر ضد *S. aureus*, *E. coli*, FRSA (مقاومة البكتيريا العنقودية للفوسيدك), محتوى العقاقير, اللزوجة ودرجة الحموضة. تم تقييم التركيبة المصنعة حيث تم تنفيذ النشاط المضاد للبكتيريا للتركيبة (F2) و مقارنته بالمنتج التجاري, و تحديد مسح الاستقرار للصيغة (F2) على النشاط المضاد للبكتيريا, الخصائص الفيزيائية و اللزوجة عند 25 و 40 درجة مئوية في علب بلاستيكية لأكثر من 3 أشهر و الحصول على نتيجة جيدة للتركيبة (F2) حيث اظهرت نتائج الانتشار, التجانس, مقدار الحموضة, المظهر, اللزوجة مع السلوك شبه البلاستيكي انها ضمن الحدود المنصوص عليها. تشير هذه القيم الى أن الصيغة تظهر قيما أفضل من المنتج التجاري. و قد لوحظ التأثير المضاد للبكتيريا التآزري المرغوب فيه لكبريتات الزنك و كبريتات النحاس في التركيز الذي كان أفضل من 1.5% في الكائنات المجهرية المختبرة.

(0.4% Oleuropine and 0.1% Thyme oil) تراكيز تآزرية كنتائج واعدة جدا كمواد حافظة مضادة للميكروبات في المنتجات الصيدلانية. أظهرت الصيغة (F2) حساسية أعلى (Larger ZOI) بالمقارنة مع المنتجات التجارية. و من المثير للاهتمام, أن عددا كبيرا 9.5% من العزلات FRSA لديه نمط مقاومة مرتفعة (MIC≥ 121 µg/ml) بينما كانت غالبية العينات FRSA المختبرة (90.5%) لا تزال عند مستوى مقاومة منخفضة (MIC ≤ 8 µg/ml).

أثبتت الخصائص الفيزيائية الكيميائية للتركيبة (F2) أنها مستقرة عند 40 مئوي و 25 مئوي خلال فترة 3 أشهر في علب بلاستيكية.

Chapter 1

Introduction

1.1 Cream Formulation

Creams are the most common class of emulsions used in dermatological treatment [1]. Creams have been described as emulsions of a semi-solid intensity [2, 3] or emulsions of an elvated obvious viscosity [2, 3] with an ideal creamy white aspect [4] produced for topical utilization [2, 3]. More recently, however, others classified and defined creams as semi-solid dosage forms consisting of more than 20% water and volatile component about 50% hydrocarbons, wax or polyethylene glycol ingredients as a vehicle for exterior skin application [5].

The same authors (Buhse) also described creams as semi-solid mode with a plastic flow behavior to other semi-solid formulations such as ointment bases, when rated using thermogravimetric analysis (TGA), creams exhibit two or more transitive. The transitional states showed a system consisting of at least two phases. Cream formulations can be categorized as" semi-solid systems which clarify as opaque, are viscous and had a non-greasy to moderate greasy texture and tend to evaporate or absorb when rubbed onto the skin" [5]. The cream to be stable third phase, an emulsion stabilizing mode or an emulsifier, was required [1]. Inversion to liquid emulsions, as though, creams were composed to contain more emulsifiers than those desired to form an intensified monomolecular surfactant film at the liquid emulsion droplet interface [2]. It was reported that excess emulsifiers in cream formulations interact with other ingredients of these formulations at the droplet interface or in the volume phase in order to make complex products, it was reported that multiphase

textures were essential for the the preparation of long-term stable creams [2]. Pharmaceutical creams may contain one or more APIs either melted or dispersed in an O/W or W/O system [6]. Oil-in-water creams usually function as "fading creams" as if the formulation vanished without leaving any effect of their presence on the skin [2].

The physical instability of creams had been found through different physicochemical destabilizing mechanisms relying on time and temperature and these mechanisms are schematically summarized in Figure (1.1), [1, 7, 8].

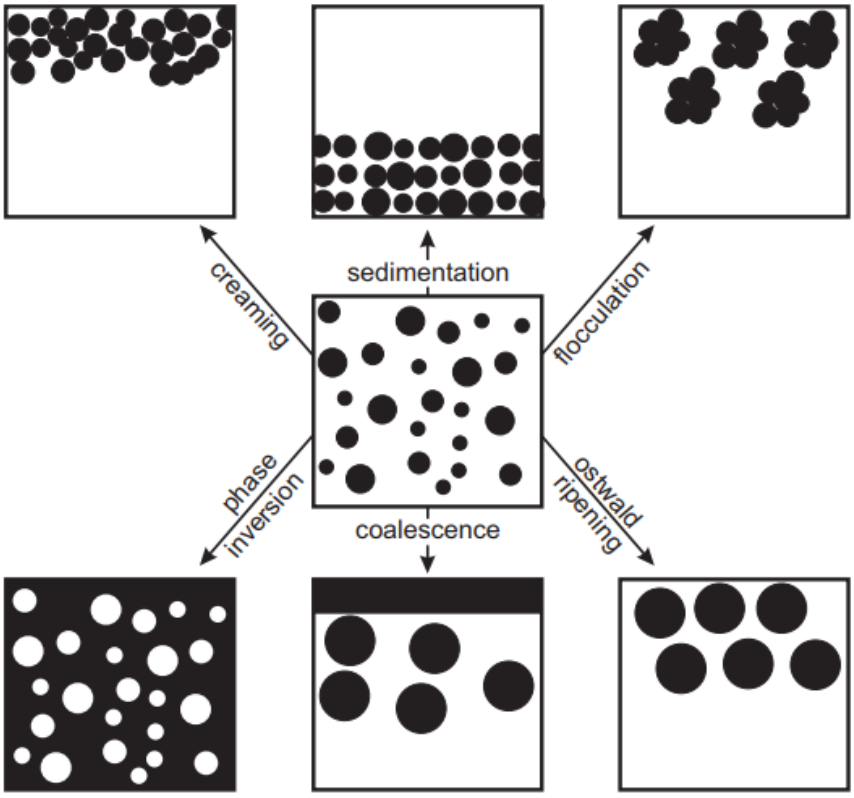

Figure 1.1: Schematic representation of mechanisms showing instability in creams [7].

1.2 Resistance of S.aureus to fusidic acid

Staphylococcus aureus (S.aureus) was one of the most significant microorganisms responsible for healthcare-associated as well as community acquired infections [9]**.**

S.aureus infections had been treated with antibiotics excessively that lead to the evolution of multi-drug resistant strains [10, 11]. Usually, three strains harbor staphylococcal chromosome cassette which encodes one or multiple antibiotic resistance genes [12].

Methicillin resistant *S. aureus* (MRSA) strains were not only resistant to methicillin or oxacillin, but they were also resistant to commonly used antibiotics because they carry several resistant genes [13, 14]. The infections caused by MRSA were very difficult to treat and had been associated with high morbidity and mortality in both hospitals and community worldwide [10].

Fusidic acid is an effective antibiotic for the treatment of many infections caused by MRSA and MSSA [15]. Fusidic acid was a narrow spectrum antibiotic, its action is bacteriostatic and it had been used clinically since the 1960s [16]. Fusidic acid prevents bacterial protein synthesis by binding to Elongation Factor G (EF- G) on the ribosomes and therefore prevents the elongation of nascent polypeptides [17]. Fusidic acid resistance in *S. aureus* usually results by either alteration or protection of the drug target, each of these mechanisms had different underlying molecular mechanisms.

In recent years, reports from various geographical areas worldwide revealed that the prevalence of resistance of *S. aureus* strains to fusidic acid remained low.

In Palestine, there is a lack of studies regarding the prevalence of fusidic acid resistance among clinical isolates of S.aureus (FRSA), simultaneously there was a reported increase in

the use of this antimicrobial agent in Palestine particularly in cases of recurrent topical infections caused by *S. aureus.*

Chapter 2

Literature review

2.1 Fusidic acid

In 1962, fusidic acid was isolated from the fungus *Gusidium coccineum* that was found in stool cultures from monkey [16, 18]. Fusidic acid structure as showen in Figure (2.1) was defined and described as chemically associated steroid compound [19].

Fusidic acid was introduced into clinical use as sodium salt; sodium fusidate, because fusidic acid as such is very soluble in water [20].

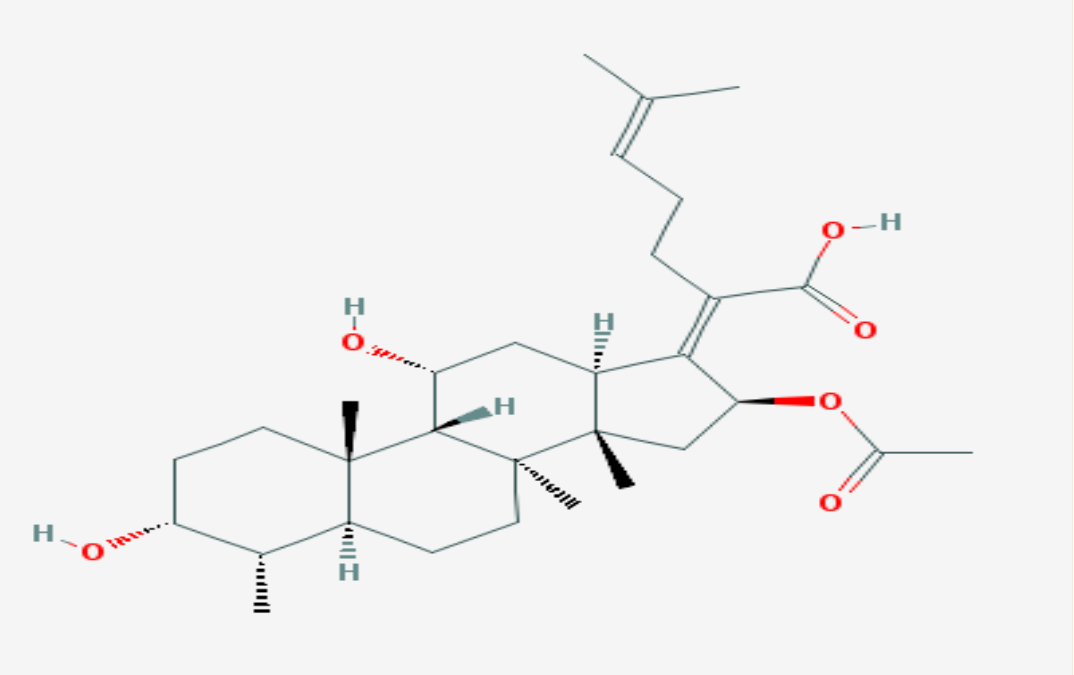

Figure 2.1: Structure of fusidic acid [21].

2.1.1 Clinical uses of fusidic acid

Fusidic acid has been utilized for many decades in different countries but has not been approved yet in USA. Currently, it is available for the treatment of acute bacterial skin and skin structure infection [22, 23]. It is available as topical agent, since it has good penetration into different body sites such as soft tissues and large abscesses [20, 24].

Fusidic acid is considered as the drug of choice for the treatment of skin and soft tissue infections (SSTIs), such as impetigo, folliculitis, erythrasma, furunculosis, abscesses and infected traumatic wounds. The topical application of fusidic acid with either betamethasone or hydrocortisone are extremely useful in the treatment of atopic infections [25].

2.1.2 Fusidic acid activity

Fusidic acid has an activity in vitro against various pathogens; it is active against gram positive bacteria such as Staphylococci, Corynebacteria and Clostridia species, while its activity against streptococci and enterococci is limited [26]. It is active on some gram negative bacteria such as *Moraxella catarrhalis* and *Neisseria meningitides*, but not on the enterobacteriaceae family or *Pseudomonas aeruginosa*. In addition, fusidic acid is active against mycobacteria but not active against yeasts or fungi [27, 28].

2.1.3 Mode of action of fusidic acid

Fusidic acid has a unique mechanism of action in the inhibition of bacterial protein b synthesis[29], as illustrated in Figure (2.2).

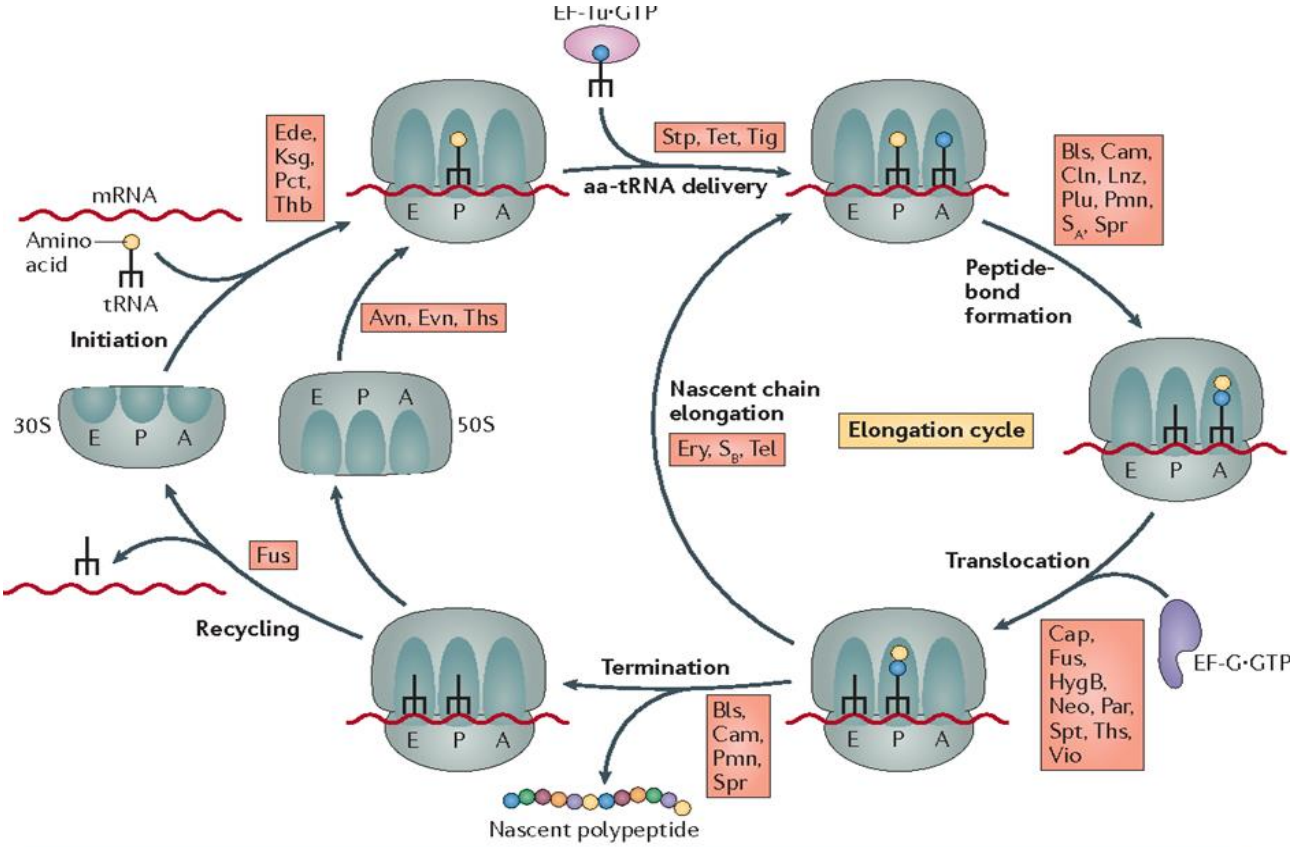

Figure 2.2: Fusidic acid target sites during bacterial protein synthesis. [30].

In prokaryotes, proteins are biosynthesized on the ribosomes in a process known as translation which is divided into four stages: initiation, elongation cycle, termination and ribosome recycling.

In the initiation stage, an initiator complex is formed in the presence of initiator factors. This complex consists of two ribosomal subunits, messenger ribonucleic acid (mRNA) and initiator transfer RNA (tRNA), which carries the first amino acid of polypeptide chain and binds to the ribosome at peptidyl (P) site.

In the elongation cycle, the formation of poly peptide chain takes place. This cycle starts when an aminoacyl tRNA (aa-tRNA) binds to the ribosome at aminoacyl (A) site by elongation factor Tu (EF-Tu). The first amino acid transfers from tRNA at P site and binds to

a new amino acid on aa-tRNA at A site in a process called peptide bond formation. Now the uncharged tRNA in the P site and the peptide-tRNA in the site move to exit (E) site and P site respectively, leaving the A site free to bind to another newly aa-tRN in order to continue the elongation. The movement of tRNAs toward E site is synchronized with the movement of mRNA, and this synchronization movement is known as translocation which is catalyzed by EF-G coupled with guanosine triphosphate (GTP). GTP is hydrolyzed into guanosine diphosphate (GDP), and then EF-G-GDP complex is released following the translocation step in order to restart the elongation cycle. Elongation stage extends until the ribosome receives a stop codon on the mRNA and the poly peptide chain is released from the ribosome in the termination phase.

In ribosome recycling stage, which is the final stage, ribosomal subunits separate and the mRNA is released. This stage is catalyzed by ribosome recycling factor (RRF) and also by EF-G combined with GTP hydrolyses [31, 32].

EF-G is the major target of fusidic acid. Fusidic acid locks the EF-G-GDP complex on the ribosome during peptide chain elongation step and ribosome recycling step and thereby inhibits protein synthesis in susceptible bacteria [33].

2.1.4 Fusidic acid resistant mechanisms

Several mechanisms of microbial resistance to fusidic acid have been determined. The best described are alterations in drug target. Alteration in drug permeability has been described, and appears to be plasmid-borne. Binding by chloramphenicol acetyltransferase type I and efflux have been also described but the predominant mechanism was unclear [24].

The mechanisms of resistance of fusidic acid among *S. aureus* and other Staphylococci have been identified and extensively studied and can be categorized into two major classes:

the first class is due to alteration of drug target site, while the second one is due to protection of drug target site and both are genetically controlled [34, 35].

The first class, fuse class resistance, is due to mutation in *fusA* chromosomal gene, which encodes for EF-G. Mutations in this gene cause amino acid exchanges within the EF-G and thereby the affinity of fusidic acid to the EF-G ribosome complex is reduced [36]. Furthermore, these mutations mostly appear in structural domain III of EF-G and less frequent in domains I and V, and they are associated with high level of resistance [37, 38].

A subset of fusA class is known as fusA-SCV class in which mutations in fusA mostly appear in structural domain V of EF-G and some in domains I and III. The mutants of this class display SCV phenotype among *S. aureus* are auxotrophic for hemin, and their resistance to aminoglycosides is increased [39].

FusE class, the fusidic acid resistant mutants carry mutation in *rplF* gene; which encodes for ribosomal protein L6 that is located at the interaction site with EF-G, these mutants also display SCV phenotype that are auxotrophic for either hemin or menadione [40].

Second class, protection of drug target site by acquiring fusidic acid resistant genes that encode fusB family proteins, these genes include *fusB, fusC* and *fusD* gene and usually confer low level of fusidic acid resistance [41, 34, 42]. Furthermore, these genes can be either chromosome or plasmid mediated [28]. The produced proteins hinder the binding of fusidic acid to EF-G or facilitate the separation of these two compounds [43].

Many studies suggested that the alteration in drug target site was the primary mechanism of resistant to fusidic acid [24]. However, nearly 10 years after fusidic acid was introduced, the horizontal acquisition of genes has been postulated to be the major mechanism encoding fusidic acid resistance among clinical *S. aureus* isolates [44].

FusB gene has been identified in both fusidic acid-resistant isolates of *S. aureus* and *S. epidermidis* and found to be carried by different genetic elements [45].

First it was identified on plasmid, PUB101, which encodes for fusidic acid resistance and also for B-lactamase and cadmium resistance [46], and can be incorporated into the chromosome as found in *S.aureus* strains in an epidemic of fusidic acid-resistant impetigo clone [34]. The FusC and FusD genes were discovered in 2007 [34]. They have been identified as encoding proteins that were similar to FusB, approximately with 45% of amino acids [35]. Both genes were located in the chromosome of clinical isolates of different Staphylococcus species [47]. *FusC* gene has been found in *S. aureus, S. intermedius and S. epedermidis,* while fusD gene has been found in *S. saprophyticus* and it is responsible for intrinsic resistance to fusidic acid [34, 48].

2.2 Synergistic activity of metal ions

Copper Sulfate pentahydrate ($CuSO_4.\ 5H_2O$) [49] was used as an agent to provide antibacterial and antiviral activities in topical formulations. Figure (2.3) illustrates the structure of $CuSO_4$. Zinc sulfate heptahydrate USP ($ZnSO_4.\ 7H_2O$) [49] was another synergistic agent that was utilized as antibacterial and antiviral agents in the topical formulations. The chemical structure for $ZnSO_4.\ 7H_2O$ is shown in Figure (2.4).

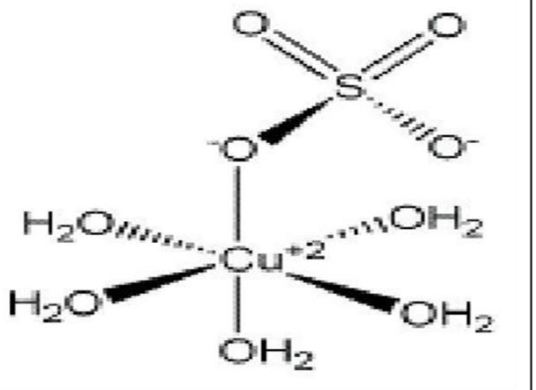

Figure (2.3): Chemical structure of Copper Sulfate Pentahydrate

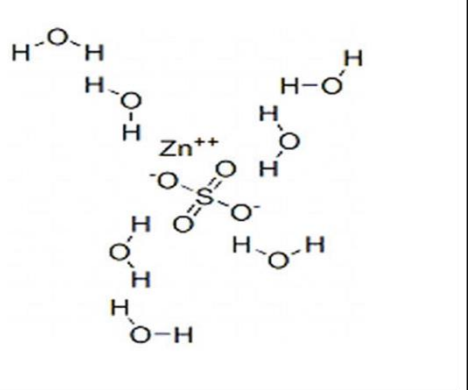

Figure (2.4): Chemical structure of Zinc Sulfate Heptahydrate

2.2.1 Copper sulfate

Copper sulfate was registered as pesticide & antimicrobial agent by USA environmental protection agency since 1956 (U.S. Government Printing Office: Washington, DC. 2009). There is evidence indicating the ability of copper inhibition of some pathogens including MRSA, Influenza virus and Pseudomonas [50, 51, 52]. Other studies showed that it has an activity against potential pathogens such as *E. Coli* [53], and used as aquatic herbicide to control the algae known as "blue stone" [54]. Copper nanoparticles were prepared form copper sulfate that has antimicrobial properties with chitosan stabilizer to give this effect [55]. Copper has been reported to have some antimicrobial mechanisms by binding to protein molecules and denaturation of DNA, as well as producing free radicals which damage the cell integrity, and inhibit some enzymes that are necessary for the functions of bacteria [56, 57].

2.2.2 Zinc sulfate

Zinc alone had been demonstrated to have effects on dermatological infections and inflammatory diseases via its effects on the activity of macrophages, neutrophils and cytokines associated inflammation [58]. Zinc has been shown to be necessary in cellular construction, metabolism in living organisms, DNA and protein synthesis and wound healing (National Institutes of Health, Office of Dietary Supplements 2013). Zinc sulfate containing products were registered for their anti-pesticide activity since 1973 in the United States (United States Environmental Protection Agency 1992). Zinc ions have excellent antibacterial activity against both gram-negative and gram positive bacteria, which are considered to be pathogens in the intestinal tract causing diarrhea [59]. The antiviral activity and mechanism of action of zinc sulfate were investigated in skin tissues and in intercellular blisters [60]. Zinc salt has selective inhibition of DNA polymerase in HSV infected cells,

thus inhibiting DNA replication of HSV [61] as well as binding to the surface glycol-protein [62]. Zinc Sulfate can be used in ophthalmic solutions to inhibit ocular infection [63].

2.2.3 Synergistic effect of zinc and copper sulfate

The presence of synergistic effects between zinc and copper against *E. coli* and *S. aureus* has been evaluated. The results showed significant activity at different combination ratios, the 3% combination of zinc and copper was found to give the best activity against the tested organisms [64].

2.3 Medicinal plants (Herbs)

Reviewing literature, it is evident that inclusive research has been accomplished to address different manifestations evaluating the effectiveness of natural plant extracts to determine their antimicrobial activity and prospect side effects in pharmaceutical products. With this in mind, in an attempt to simplify the topic and delimit the subject to some extent, this section will focus primarily on the work that has been carried out concerning the approximation of antimicrobial activity systems.

The main goal of this section is to review the theoretical and practical aspects concerning olive leaves extract (OLE) antimicrobial activities and compare their effectiveness. This includes examining OLE and thyme oil as preservatives for pharmaceuticals. Indeed, studying the antimicrobial activity of some curative plant extracts has become a significant research concern and a major challenge.

2.3.1 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) to avoid the significant side effects of synthetic drugs [65]. The pharmacological therapy of disease has

started long since the utilization of herbs [66]. The term “herbs” has a meaning herba in Latin where it means a curative plant. An herb is a plant or plant portion applied for its smell, flavor or therapeutic features. An herb is gained from seed, berries, buds, leaves, bark and roots of plants growing at most in the equatorial, the semitropical and the temperate zone. Essential oils of herbs have been utilized widely for many years in nutriment outputs, perfume products, dental and oral products due to their various medicinal features [67].

Medicinal plants term concern the used of plants, plant portions, solvent extracts, essential oils, gums, resins, secretions or other types of advanced products made from plant portions utilized as curative to supply proactive support of different physiological systems; or, in a more traditional medical concept, to treat, heal, or inhibit a disease in animals or humans [68]. Around 75% of the world populations, mostly in the developing countries, based on non-traditional medicine in their essential healthcare as announced by the World Health Organization [69]. The uses of medical herbs and phytonutrients or nutraceuticals continues to extend quickly across the world with many nations currently utilized these products for treatment of different health challenges in various national healthcare regulations -WHO, 2004.

Several reports and research had shown that plants had the ability to act against oxidation, anticoagulants, anti-sugar and inflammation, and also had a function in the control of blood pressure (antidiabetic, anti-hypertensive, anti-obesity, anticancer, hepatoprotective, gastroprotective and cardioprotective activity) [70]. Many studies and research suggested activity against herb microbes by inhibiting the outgrowth of a large diversity of bacteria [71], fungi [72, 73] and viruses [74]. Olive tree has been mentioned and blessed by God in the holy Quran. The beneficial effects of the olive components (Olea europaea L.) had been confirmed and proven in many studies and research [73]. The olive

tree contains a high percentage of the biophenols such as oleuropein, verascascide, ligstrosides, tyrosol or hydroxyl tyrosol [75].

We will discuss some studies about the activity against microbes of olive leaves and thyme oil. The activity of OLE was studied against gram positive and gram negative bacteria. The minimum inhibitory concentration was determined in the laboratory by a broth microdilution assay. Water was used as a solvent and the concentrations used were 20-320 mg/ml. The result of this study showed that Oleuropein was more toxic to gram positive bacteria than gram negative bacteria. However, the conclusion of the study was that Oleuropein was found to be effective against mycoplasmas [76]. There was a consistency with a study contacted by Aliabadi [77] in a study against microbes where the bacteria used were mainly "*Staphylococcus aureus* PTCC 1431, *Salmonella typhimurium* PTCC 1639, *Escherichia coli* PTCC 1399, *Klebsiella pneumonia* PTCC 1053 and *Bacillus cereus* PTCC 1274", as a result of the research Olive leaf aqueous extract displayed good antimicrobial efficiencies and highest inhibitory activity of 11.5 μg/ml against salmonella typhi. The results were also consistent with another reseach conducted by Gumgumjee [78]. In this study three types of gram negative bacteria and three gram of positive bacteria were tested by agar assay method and antimicrobial activity was determined by measuring the zone of inhibiton, it had shown a moderate effectiveness against all bacteria.

The effectiveness of the OLE may not be due to one main active constituent but may be due to the combined action of different compounds originally present in the plant. It was also consistent with the study where five gram-positive and five gram negative bacteria were evaluated and the zone of inhibiton (ZOI) and MIC were determined by the concentration (50 mg/ml) and the result of this study presented the highest antibacterial activity against *B. cereus* and the lowest antibacterial against *S. typhi* [79]. It was also agreed with the study conducted by Salma [80] two types of Gram positive bacteria (*S.aureus & B. cereus*) were

evaluated using the zone of inhibiton (ZOI) and MIC, where methanol was used as a solvent, and the result of this study showed that OLE was effective against the two gram positive strains tested.

In 2003, a study conducted by Markin [81] the method of *In vitro* scanning electron microscopic observation for *Candida albicans* was used. The result of this study had shown that deramatophytes were inhibited by 1.25 % (w/v) OLE after three days, *Candida albicans* was killed after 24 h in the presence of 15% (w/v) OLE. The conclusion of this study was that "Olive leaves extract" shows antibacterial and antifungal properties. In addition, a study which took place in 2014, [82] 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 was active against the tested pathogens specifically gram-negative anaerobic bacteria.

OLE was also tested against bacteria and fungi by determiming their MIC and MFC (minimum fungicidal concentration). The result of this study showed a different spectrum of antimicrobial activity and MFC values were the same as the MIC ones. The conclusion of this study was that OLE can be useful in the topical treatment of cutaneous fungal infections [83].

The effectiveness of OLE against fungus was studied using the disc diffusion method where other solvents were used (water, acetone, methanol, ethyl acetate), depending on the solvent used, it showed a different spectrum of antifungal effects [84].

In another study where the effectiveness of OLE against microbes was evaluated by determining the MIC for of microbes tested [85]. In this study, water was used as a solvent and concentration of 0.05-5.0 mg/ml, gram positive "(*Bacillus cereus*, *Bacillus subtilis* and Staphylococcus aureus)", gram negative bacteria "(*Pseudomonas aeruginosa*, *Escherichia*

coli and *Klebsiella pneumoniae*)", 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 antimicrobial effect.

The effectiveness of OLE against the intestinal bacteria using agar dilution and broth microdilution techniques; MIC, MBC and MFC was determined, by using of the extract of olive leaves (4.4 mg/ml) and microorganisms "(*H. pylori, C. jejuni, Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis, E.coli, Shigellasonnei* and *Yersinia sp*.)". The result of this study was that OLE may have a function in regulation of the composition of gastric flora by selectively decreasing level of *H.pylori* and *C. jejuni*; while the result of this study showed that olive leaf extract has not broad spectrum in action aspect perceivable -not clear activity only against- "(*H.pylori, S.aureus, C.jejuni* and *Methicillin-resistant Staphylococcus aureus*)" [86].

A study was performed to investigate the antimicrobial and antifungal activity of OLE, using bacteria and fungus by the agar disk diffusion method and measurement of diameters of inhibition zone. The microorganisms used were "*Staphylococcus aureus* ATCC 25923, *Enterococcus feacalis* ATCC 29212, *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumonia ATCC 700603, *Salmonella typhemurium* ATCC 13311) and the fungus *Cladosporium herbarum* MNHN 3369". The result of this study showed good inhibitory effects against *E.coli* and Bacillus cereus as compared to other bacteria and fungi. The conclusion of this study indicated that olive leaves were a potent source of antibacterial and antifungal activity [87].

The activity of OLE was evaluated against six bacterial pathogens: *Escherichia coli, klebsiella pneumoniae, staphylococcus aureus, enterococcus faecalis, pseudomonas aeruginosa* and *salmonella enterica* [88]. Water and methanol were used as solvents. The

result of this study showed that OLE exhibited the highest inhibitory potential against *Salmonella enterica* and *Escherichia coli* with a zone of inhibiton of 13.0 mm and 10.5 mm respectively.

In a review article which had discussed the ability of olive leaves extract as antimicrobial [89] containing 26 related scientific articles, including 21 evaluations of the effectiveness of OLE against bacteria and 3 studies of its effectiveness against viruses and 7 studies of the effectiveness against fungus, all reported significant antimicrobial activity (Figure 2.6) of OLE. Most of the articles demonstrated that different solvents and concentration had presented a significant effectiveness against microorganisms. Figures (2.5, 2.6).

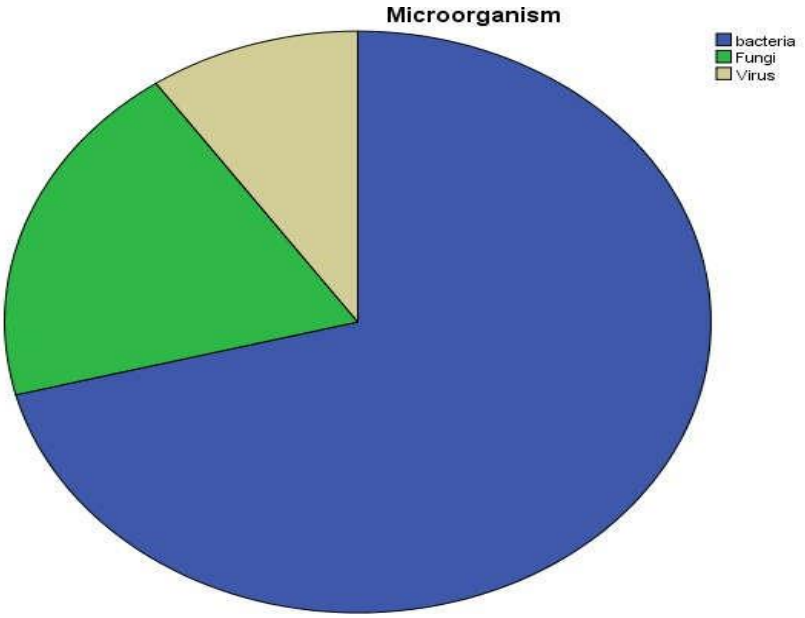

Figure (2.5): Types of microorganisms used in antimicrobial assessment of OLE

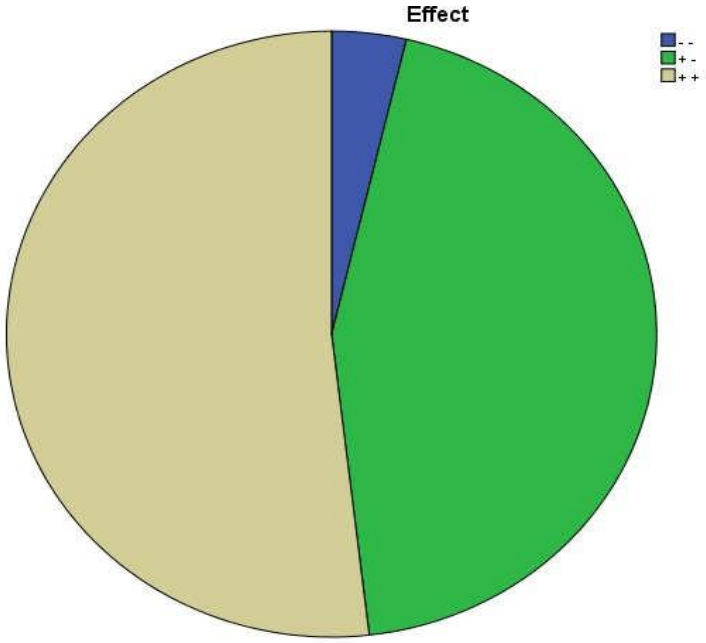

Figure (2.6): Type of antimicrobial effect of OLE. ++: studies which were effective against all tested microorganisms. +-: studies which were effective against some strains of tested microorganism.--: studies which had no effect on tested microorganism [89].

A study conducted by Mariha [90], where the activity of thyme oil against microbes was evaluated. The MICs were determined by broth microdilution method, the realization of the antibacterial effects of thyme oil was accomplished on *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Salmonella Enteritidis, Salmonella Thyphimurium* and *MRSA*". The results of this study showed that thyme oil has antibacterial activity against all tested microorganisms.

The effects of the essential oils from thyme and oregano against microbes were evaluated in a study conducted by Bülent [91]. The methods used were disc diffusion (DD) and minimum inhibitory concentration (MIC). A total of 43 microorganisms were tested including 14 fungal species, 3 types of yeasts and 26 bacteria. The results of this study showed a zone of inhibition and MIC values of 8 to 72 mm and 7.8 to 500 μg/mL, respectively. The maximal zones of inhibition of the yeast and fungi species sensitive to the essential oils were 8-74 mm and MIC values were 7.8-500 μg/mL.

The various susceptibility results of the tested microorganisms depending on the essential oils of oregano and thyme may be further investigated as a possible source of a natural antimicrobial for food industry after evaluting the toxic and side effects on humans.

Chapter 3

Objectives and significance of the thesis research

3.1 Objectives

The objectives of this study are:

1. To determine the antimicrobial activity of pure Oleuropein and thyme oil (pharmacopeial grade) on clinical bacterial and fungal isolates and compare it with routinely used preservatives.

The antimicrobial effectiveness test or efficacy test were 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).

2. To investigate the optimum concentration of Oleuropein, and thyme oil in the cream as natural preservative/antioxidant.

3. To incorporate Oleuropein, thyme oil, zinc sulfate and copper sulfate into topical formulation (containing fusidic acid as active ingredient).

4. To develop a formulation for treatment of bacterial, fungus and viral in one medical preparation.

5. To evaluate the antimicrobial effectiveness of the topical formulation mixture and compare it with pharmaceutical cream containing chemical preservatives as (positive

control product).

5. To study the stability of the topical preparation.

3.2 Significance of the research thesis

Although synthetic preservatives and antioxidants may provide high antimicrobial and antioxidant effectiveness, they are usually associated with adverse reactions and are considered to have potential undesirable effects due to chronic consumption. Currently, there is an increasing tendency for high quality, with fewer chemicals, and long-term pharmaceutical products. Thus, there is an urgent need to develop moderate pharmaceutical products for self-preservation and /or use of protected natural materials from microbiological growth during storage and used as antioxidant. So, the antimicrobial activities of thyme oil and Oleuropine have been investigated extensively. Therefore, copper sulfate and zinc sulfate are proposed to be used as anti-bacterial and anti-viral agents and expected to give synergistic effect when combined with fusidic acid. However, there is no research found that evaluated the formulations which incorporate these ingredients combined with fusidic acid. The favorable outcome of this combination may contribute to reduce the emergence of MRSA and FRSA strains, multi drug resistance, reduce toxicity of chemical preservatives and exhibit more antimicrobial activity and better effects against polymicrobial infections. Furthermore, to develop a formulation for treatment of bacteria, fungi and viruses in one medical preparation.

In this study, the characterizations of three cream formulations were evaluated, and these formulas were tested for their antimicrobial activity as compared with marketed products against *E. coli* and *S.aureus*. Furthermore. The MICs of the formulation were determinated by agar dilution assay against reference microbes and FRSA isolates. In addition, stability studies were conducted on the topical products for a three months period at room tempreture and at accelerated conditions.

Chapter four

Materials and methods

4. Materials and methods

4.1 Materials and reagents

Materials and reagents used in this study were illustrated in Tables (4.1, 4.2, 4.3). All materials used in the formulation of pharmaceutical preparations were pharmaceutical grade.

Table (4.1) : Materials of pharmaceutical preparations

No.	Materials of pharmaceutical preparations		
1	Fusidic acid	11	Cremophor RH40 (polyoxyl40 hydrogented castor oil)
2	Ethanol 96%	12	Sodium dihydrogen phosphate
3	Titreplex	13	Dibasic sodium phosphate
4	DMF	14	Cetosteroyl alcohol
5	Sucrose	15	Macrogol A6
6	Thyme oil	16	Macrogol A25
7	Glycerin	19	Parafine oil
8	Distilled water	20	Propylene glycol
9	Sodium chloride (NaCl)	21	$ZnSO_4$. $7H_2O$
10	Olueropine	22	$CuSO_4$. $5H_2O$

Table (4.2): Materials and reagents for analytical tests

No.	Materials and reagents for analytical tests		
1	80% Ethanol	14	Fluid Lactose Medium
2	Acetic acid conc.(HAC)	15	Poly sorbate 80
3	Acetonitrile	16	Phosporic acid
4	Casein Digest- soy Lecithin Polysorbate	17	Xylenole orange
5	Deionized water	18	Glacial acetic acid
6	Distilled water	19	Sterile saline
7	Ethylacetate	20	Eosin Y
8	Hexamine	21	Phosphate buffer stock solution
9	Oleuropein 40%	22	Broth media
10	Olive leaves	23	Letheen Broth, Letheen Agar
11	Ptassium dihydrogen phosphate	24	0.005 EDTA
12	Soybean-Casein Digest Broth	25	HDPE bottles, LDPE Caps, Amber Glass Bottles type III
13	Soybean-Casein Digest Medium	26	Silver nitrate

Table (4.3): Equipments and Tools

No.	Equipment and Tools
1	BrookFlied IV Viscometer # (QCA-036)
2	ERWEKA AR 401
3	Filters: Whatman No.1 filtera
4	HPLC Apparatus
5	Inactivator (Neutralizer)
6	Laminar Flow Cabinet
7	METTER TOLEDO and Precisa 2200C
8	Mixer tank
9	pH meter 211R Hanna
10	Plates
11	Rotoray evaporate
12	Sieve mesh # 120 mm
13	UV- visible Spectrophotometer
14	Steam distillation
15	Sterile petri dishes (diameter of 9 cm)
16	Thermometer
17	UNIMAX 1010 shaker
18	Vortex

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

4.2 Microbiological test methods

4.2.1 Antimicrobial effectiveness testing

This procedure was designed to evaluate the antimicrobial effectiveness of antimicrobial preservatives used in topical formulations [92].

4.2.1.1 Media:

For the cultivation of the test organisms, agar medium that optimally support their growth and the growth of the stock cultures used in this project. Soybean Casein Digest Agar/Broth and Sabouraud's Dextrose Agar/Broth were used. In addition, a suitable inactivator (neutralizer) for the specific antimicrobial properties in the product to the broth was added.

4.2.1.2 Growth promotion of the media

The selected media was used to cultivate and grow the appropriate microorganisms. It was preferable that tested microorganisms be chosen for growth promotion testing (Section 4.4.1.5). Solid agar media was used for growth promotion testing. The pour plate method was used to determine the number of colony forming units (CFU) which must be ≥ 70% of the microorganism inoculum's calculated value [92].

4.2.1.3 Test organisms:

The following standard reference organisms were selected**:**

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)

4.2.1.4 Preparation of inoculums

1. Preparatory to the test:

Inoculums were prepared by taking few colonies from the surface of an 18 to 24 hour cultures. The Culture conditions for the inoculum preparations are described in Table (4.4).

Table (4.4): 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 Sufficient sterile saline TS was added to obtain a microbial count of about $1x10^8$ CFU per mL.

2.2 The number of cells were 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)

2.3 Several dilutions were made ($10^{-3} – 10^{-6}$) and cultivated by the pour plate method.

2.4 Bacteria was inoculated for 24 hours at 35ºC± 2, and *C. albicans* at 23º C ± 2 for 2-3 days and the CFUs was 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.

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.

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

4.4 To determine the number of CFU per mL in each suspension, it was used the condition of media and microbial recovery incubation times listed in Table (4.4) to confirm the initial CFU per mL estimate.

4.2.1.5 Procedure

For purposes of testing, products had been divided into two categories and our dosage form belongs to:

a) **Category 2 products**: Fusidic acid cream

Test Procedure for topical Products:

1. The tested reagent was conducted in 5 original containers (if sufficient volume of product were available in each container and the product container could be entered aseptically).
2. Each container was inoculated with one of the prepared and standardized inoculum, and mixed:
    1. The initial concentration of applicable microorganisms in each test preparation was estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.
    2. The volume of the suspension inoculum which was used was between 0.5% and 1% of the volume of the product.
    3. The concentration of test microorganisms that was added to the product should reach a final concentration in the test preparation after inoculation between $1x10^5$ and $1x10^6$ CFU per mL of the product.
    4. The inoculated containers were incubated at 22.5 ± 2.5^oC by UNIMAX 1010 shaker and of 32.5 ± 2.5^oC by UNIMAX 1010 shaker.
    5. A sample was obtained from each container at the appropriate intervals.
    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 and the product was diluted to 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.

8. The calculated concentrations of CFU per mL present at the start of the test was used, 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 was 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).

4.2.1.6 Interpretation

"Category 2 products":

Table (4.5): The acceptance critiria for semisolid products [92]

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

4.3 Preparation of fusidic acid cream

For this study, a topical cream of fusidic acid 2% with different concentrations of olueropine, thyme oil, zinc sulfate and copper sulfate were prepared. The ingredients used are showen in Table (4.6). The concentrations of oleuropine and thyme oil were determind based on the results of the synergestic anti- microbial

effect from oleuropine and thyme oil by test preservative mentioned earlier. Finally, the formulations were tested according USP and evaluated for their stability, antimicrobial and anti fungal effect and the best formulation was selected.

Table (4.6): Illustration of the general formula for Fusidic acid cream

No.	Component	Description	Function
1	Fucidic Acid 2%	BP/USP	Active ingredient
2	Zinc Sulfate	BP/USP	Antibacterial ,antiviral,
3	Copper Sulfate	BP/USP	Antibacterial, antifungal
4	Oleuropine	In house	Antioxidant and preservative
5	Thyme oil	BP/USP	Antioxidant and preservative
6	Cetosteroyl alcohol	BP/USP	Emollient
7	Macrogol A6	BP/USP	Emulsifying agent
8	Macrogol A25	BP/USP	Emulsifying agent
9	Parafine oil	BP/USP	Vehicle
10	Propylene glycol	BP/USP	Solvent
11	Purified water	BP/USP	Vehicle

4.3.1 Formulation of cream topical

The O/W cream was prepared by preparing the oil phase and the aqueous phase seperatly at specific tempretures. Then the oil phase was slowly added to the aqueous phase by agitation and the mixture was cooled until the temperature reaches 40 °C by the following procdure:

1. Heating oil phase: added cetostearyl alcohol, Macrogol A6, A25 in a clean beaker 1, then heating to 70 °C.

2. Added Oleuropein, thyme oil to beaker 2 for 2 minutes with stirring.
3. Heating aqueous phase: transfer purified water then added propylene glycol in beaker 2, then heating for 70 °C, dissolved paraffin oil in beaker 3 for 60 °C.
4. Adding co-solvent and API: added Fusidic acid, zinc sulfate, copper sulfate, with paraffin oil until dissolved with good stirring for 2 minutes.
5. Add fusidic acid mixture to beaker 1 with stirring for 5 minutes.
6. Transfer aqueous medium from beaker 2 to beaker 1 with continuous stirring.
7. Cool the mixture until 40°C.

4.4 Chemical and physical analytical test Methods

4.4.1 Determination of Oleuropein

Phosphate buffer- pH 3.0 was prepared by mixing equal volumes of 0.01M Phosphoric acid and 0.01 M monobasic sodium phosphate. Mobile phase was prepared by filtering and degassing mixture of acetonitrile and pH 3.0 Phosphate buffer (20:80), containing 1% acetic acid. Diluent was prepared with a mixture of acetonitrile and water (200:800). Test solution was transferred about 70mg of oleuropein, accurately weighed to a 100 ml-volumetric flask, dissolved in a dilute with diluent to volume, and well mixed [93]. Chromatographic system- the liquid chromatograph was equipped with 280-nm detector and with silica-based C18 bonded phase column C18, 5 µm (5 µm,150* 4.6 mm inner diameter). The flow rate was about 1.0 ml /min; injection volume was (20.0µl).

4.4.2 Determination of Fusidic acid

Phosphate buffer- pH 3.5 was prepared by mixing equal volumes of 0.01M Phosphoric acid and 0.01 M monobasic sodium phosphate. Mobile phase- was prepared by filtering and degassing mixture of methanol, 1.0g potassium dihydrogen phosphate/500 ml purified water, pH 3.5 Phosphoric acid and acetonitrile (20:45:35). Diluent- a mixture of ethanol and purified water (70:30) were prepared. Test solution- 40 mg of fusidic acid was accurately weighed and transferred, to a 200 ml-volumetric flask and was dissolved the with Diluent. Chromatographic system- the liquid chromatograph wich was used was equipped with 235-nm detector and Lichrosphere RP-select B, 5µm (125* 4 mm). The flow rate was about 1.5 ml /min; injection volume was (20µl) [94].

4.4.3 Determination of zinc sulfate heptahydrate and copper sulfate pentaydrate

Determination of the assay for ($ZnSO_4$. $7H_2O$) and ($CuSO_4$. $5H_2O$) was made by complexmetric titration method; accurately sample was weighted and transfered to 250 ml E.M flask, purified water and 1ml of concentrated acetic acid was added, a few milligrams of xylenole orange and sufficient amount of hexamine was added and then the titration was taken place with 0.1M EDTA to achieve the green end point [95].

4.5 Total aerobic microbial count for topical finished products

The purpose of these tests was to test the qualitative and quantitative estimation of total viable aerobic count of microorganisms in finished topical dosage forms [92].

4.5.1 Plate method for bacterial count

a) Specimen was transfered accurately to a sterile bottles.

b) SCDM or (phosphate buffer of pH 7.2) was added in bottles, then the bottles was closed and mixed by swirling the bottles gently.

c) The SCDM bottle (phosphate buffer bottle) was opened, and with a sterile pipette a sample was drawn and transfered to a petri dish containing already solidified SCDA then the petri dish was covered.

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

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

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

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

h) Observation of the results 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 was registered (taking into account the initial dilution 1:10).
- **If no growth**: result was expressed $\leq$10 CFU/ml for a negative result.

4.5.2 Plate method for total aerobic yeast and molds Count

a) The same procedure as for bacterial count (section **4.5.1**) but sabouraud dextrose broth/agar (Sab.D / SDA) was used instead of SCDM/ SCDA.

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

4.5.3 Acceptance criteria for nonsterile finished products

Table (4.7): Acceptance criteria for nonsterile finished products [92]

Route of Administration	TABC (CFU/g or ml)	TYMC (CFU/g or ml)	Absence of*
Rectal	**<200 (10^3)****	**<200 (10^2)**	
Vaginal	**<200 (10^2)**	**<20 (10^1)**	*P. aeruginosa, S. aureus and C.albicans*
Cutaneous	**<200 (10^2)**	**<20 (10^1)**	*S. aureus and P. aeruginosa*

**The microorganisms listed are only examples of those microorgansims found to be objectionable in the respective product classes*

** USP/ BP acceptance criteria interpretation:

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

4.6 MIC for topical cream and substances (Minimum inhibitory concentration of active substance as part of cream (ASPC), Entire cream (EC) and active substance alone (AS))

MICs of formulation and substances was determined by agar dilution assay, by following procedure [96]:

1. Weighted different samples from formula (test sample).
2. Prepared 20 ml tryptic soy agar media and autoclaved agar then cooled to temperature of 45-50 ^oC.
3. Added the sample in volumetric glass and media then did further well mixing.
4. Poured the agar in standard petri dish (92*16 mm).

3. Prepared bacteria colonies and yeast "(*Candida albicans* (ATCC # 10231), *Aspergillus niger* (ATCC # 16404), *Escherichia coli* (ATCC # 8739), *Pseudomonas aeruginosa* (ATCC # 9027), *Staphylococcus aureus* (ATCC # 6538)" to $1*10^5$ CUF.
5. Take 20 µl from each four colonies then spread them by glass rods.
6. Incubated at 37 ^oC for 3 days bacteria, 48 hours for yeast and 7 days for molds.

4.6.1 Determination of fusidic acid MICs for FRSA (fusidic acid resistance S. aureus) strain

Refer to the same procedure as for MIC for entire cream EC (section 4.6) but it FRSA samples was used instead of bacteria and yeast colonies, a sterile cork borer was used to make a wells by punching the holes on the inoculated MH agar plates, each well was being 5 mm in diameter and the cut out of the agar was removed using a sterile needle, then a certain amount of formulation was weighed and the procdure was completed.

4.7 Determination viscosity

The viscosity of finished product (fusidic acid cream) was measured in triplicate using Brookfield IV viscometer # (QCA-036): Spindle # 4, 30 rpm, 50 ml beaker at tempreture 22^oC. The reading were taken after 3 minutes [95].

4.8 Determination of pH

The samples from the the prepared formulas were determined for pH using pH meter (pH meter 211R Hanna). Accurately weighed 5 g of the sample was dispersed in 30 ml water then heated with stirring to obtain a homogenous mixture, allowed cooling and measured the pH at 20-22 ^oC. Then let stand with

the electrode dipped in for 15 minutes and document the reading after stabilization. All measurements were carried out in triplicate [95].

4.9 Organoleptic Characters

All formulations were tested by visual observations for their texture, color and phase separation. After two minutes of applications on the skin, the feel was performed [95].

4.10 Homogeneity test

Test was hold by squeezing a small amount of the formulated cream between index finger and thumb, then the homogeneity of the formulations' was being evaluated using fingers; appearance of the coarse particles, consistency of the formulations [95].

4.11 Spreadability test

The spreadability of the creams was determined by measuring the spreading diameter of 1g of sample between two horizontal glass plates dimensions 10 cm X 20 cm after one minute. The standard weight 25 g was applied on the upper plate [97].

4.12 Stability study

The final optimal formulation developed was stored at 25^0C and 40^0C in plastic container. The physicochemical properties of the formula was evaluated after 3 months to describe the physical stability of the formula. The viscosity of the formula was determined every month for over a 3 months period. The formula was tested for antibacterial activity against "*Candida albicans* (ATCC # 10231), *Aspergillus niger* (ATCC # 16404), *Escherichia coli* (ATCC # 8739), *Pseudomonas aeruginosa* (ATCC # 9027), *Staphylococcus aureus* (ATCC # 6538)" by using the agar well diffusion assay, at time intervals of 1 and 3

months, MIC for entire cream and substances for these colonies and FRSA samples [95].

Chapter Five

Results & Discussion

5.1 Growth promotion of the media

The results of the growth promotion test showed that all the media used to grow the microorganisms (Sabouraud dextrose agar, soybean casein digest, sabouraud medium and soybean casein digest medium) were effective on microorganism used in this study and corresponded to the SOP system. These conditions were determined in the validation study of the sample based upon the conditions of media and microbial recovery incubation times listed in appendices. The results are showen in Tables (5.1, 5.2, 5.3, 5.4).

5.2 Antimicrobial effectiveness testing

The antimicrobial activity of oleuropein , thyme oil and their combination were tested at different concentrations using distilled water as solvent. The following Tables (5.1 ,5.2, 5.3, 5.4, 5.5) show the antimicrobial activities of oleuropein at different concentrations.

In this test, different microorganisms that can cause infections in human body were used. Therefore, three types of bacteria, one yeast and one mold were tested for antimicrobial activity. The bacteria strains include one gram positive *"(Staphylococcusaureus* (ATCC NO. 6538)) and two gram negative *(Pseudomonas aeruginosa* (ATCC NO. 9027)), and (*Escherichia coli* (ATCC NO. 3739), the yeast *Candida albicans* (ATCC NO. 10231) and the mold *Aspergillus niger* (ATCC NO. 16404)"

5.2.1 Oleuropein

For Oleuropein three concentrations were tested, 0.2, 0.4 and 0.6% w/v. Using 0.2% w/v oleuropein, the results are shown in Table (5.1). There was 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. The same results (as for *Pseudomonas aeruginosa*) were obtained for *Escherichia coli*. Furthermore, there was complete inhibition of these three bacterial strains after 28 days (i.e. no increase from the 14 days count to 28 days). This results obtained imply that the concentration of 0.2% w/v oleuropein can be used as antimicrobial agent against the three strains of bacteria tested. However, the oleuropein to be used as a preservative at this concentration should be also effective against yeast and mold in addition to those three types of strains bacteria tested. As shown from the results for oleuropein at 0.2% w/v, there was an increase in the count of yeast (*Candida albicans*) and mold *(Aspergillus niger*) at 14 days, which indicates that oleuropein at this concentration cannot be used as preservative since it is not effective against yeast and mold but can be used as antimicrobial agent against the three types of bacteria tested. Therefore, it became necessary to increase the concentration from 0.2% to 0.4% w/v. At this concentration, oleuropein was found to be effective against the three bacterial strains same as 0.2% concentration, but more than 10 fold reduction in the counts were obtained at 14 days and no increase in the counts after 28 days as shown in Table (5.2). Regarding the activity against yeast and mold, the results showed that it wasn't effective against yeast and

mold, as shown in Table (5.2). Increasing the concentration of oleuropein to 0.6% has resulted in complete inhibition of all three bacterial strains after 7, 14 and 28 days. In addition, there was no increase in the yeast and mold counts from initial inoculum at 14 and 28 days as shown in Table (5.3). It was apparent that the results obtained with oleuropein at the new higher concenteration of 0.6% w/v has the desirable antibacterial and antifungal effects as well as adequate preservation effects. The results obtained in our study is in agreement with previous studies conducted to determined the antibacterial properities of Oleuropine in different concenterations [98, 99] .

Table (5.1): 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	>10^3	385				>10^3	
		7	0	0	0	0	0				>10^3	1600
		14	0	0	0	0	0				>10^3	>10^3
		28	0	0	0	0	0				770	60
P. aeroginosa	9027	0	>>10^3	>10^3	>10^3	317	120			180	260	
		7	>10^3	512	230	51	0				>10^3	1200
		14	103	25	10	0	0				>10^3	400
		28	0	0	0	0	0			>10^3	375	
E.coli	8739	0	>>10^3	>10^3	>10^3	>10^3	450			449	32	
		7	>10^3	501	220	85	10				>10^3	>10^3
		14	100	29	0	0	0				>10^3	590
		28	0	0	0	0	0				>10^3	100
C. albicans	10231	0	>10^3	>10^3	>10^3	>10^3	420		920	115	25	
		7	>10^3	>10^3	>10^3	1520	450			>10^3	1450	
		14	>10^3	>10^3	>10^3	>10^3	215			>10^3	>10^3	
		28	>10^3	>10^3	>10^3	490	240				1260	
A. Niger	16404	0	>>10^3	>10^3	400	45	20	>10^3	480	60	5	
		7	>10^3	>10^3	120	30	5		90	5	1	
		14	>10^3	>10^3	306	10	4		25	4	1	
		28	>10^3	370	30	5	0		65	10	2	

Table (5.2): 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	>10^3	380			525	40	
		7	0	0	0	0	0				>10^3	>10^3
		14	0	0	0	0	0				>10^3	>10^3
		28	0	0	0	0	0				750	60
P. aeruginosa	9027	0	>>10^3	>10^3	>10^3	400	120			165	10	
		7	>10^3	310	35	10	0				>10^3	>10^3
		14	0	0	0	0	0				>10^3	430
		28	0	0	0	0	0			>10^3	360	
E.coli	8739	0	>>10^3	>10^3	>10^3	>10^3	200			432	30	
		7	>10^3	500	281	120	5				>10^3	>10^3
		14	0	0	0	0	0				>10^3	580
		28	0	0	0	0	0				892	100
C. albican	10231	0	>>10^3	>10^3	620	105	10		890	100	20	
		7	>>10^3	>10^3	>10^3	>10^3	300			>10^3	>10^3	
		14	>10^3	>10^3	>10^3	600	60			>10^3	>10^3	
		28	>10^3	>10^3	>10^3	311	120				1205	
A.niger	16404	0	>>10^3	>10^3	400	75	5	>10^3	475	65	5	
		7	>10^3	300	100	25	4		85	8	1	
		14	>10^3	>10^3	200	11	2		25	3	1	
		28	477	220	10	1	0		60	5	1	

Table (5.3): 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	419	80			565	40	
		7	0	0	0	0	0				>10^3	1670
		14	0	0	0	0	0				>10^3	825
		28	0	0	0	0	0				770	60
P. aeruginosa	9027	0	>10^3	>10^3	300	35	13			185	20	
		7	>10^3	70	15	5	0				>10^3	1200
		14	0	0	0	0	0				>10^3	450
		28	0	0	0	0	0			>10^3	365	
E.coli	8739	0	>10^3	>10^3	>10^3	565	45			435	30	
		7	>10^3	400	85	15	9				>10^3	>10^3
		14	0	0	0	0	0				>10^3	550
		28	0	0	0	0	0				>10^3	85
C. albican	10231	0	>>10^3	>10^3	633	100	18		928	115	28	
		7	>10^3	>10^3	>10^3	>10^3	308			>10^3	1380	
		14	511	417	312	155	9			>10^3	>10^3	
		28	200	280	80	5	0				1200	
A. niger	16404	0	>10^3	>10^3	265	30	5	>10^3	472	60	5	
		7	330	55	5	0	0		98	8	1	
		14	160	31	3	0	0		38	5	1	
		28	240	21	5	0	0		62	15	2	

5.2.2 Thyme oil

The same tests conducted with Oleuropine has been repeated using thyme oil at 0.1% v/v concentration. At this concentration, the results showed that it is effective against the three bacteria only, Table (5.4). The results of the present study along with those of previous studies [100, 101] conducted on thyme oil taking into consideration to use the lowest concentration of the oil in order to minimize the strong stinging effects due to its odor and it is also the highest concentration dissolved in water.

Table (5.4): Antimicrobial activity of thyme oil (0.1 %) in distilled water against three types 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	300	79			577	45	
		7	>10^3	411	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	318	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	>10^3	445	246			>10^3	1450	
		14	>10^3	>10^3	431	212	109			>10^3	>10^3	
		28	>10^3	>10^3	450	320	150				1269	
A. Niger	16404	0	>10^3	>10^3	>10^3	>10^3	623	>10^3	484	69	5	
		7	>10^3	>10^3	>10^3	732	435		90	6	1	
		14	300	255	513	420	390		28	4	1	
		28	421	240	500	450	400		66	10	2	

5.2.3 Synergistic effect between oleuropine and thyme oil

The use of a combination of thyme oil and oleuropein at concentrations of 0.2% and 0.4% w/v respectively showed inhibitory activity against the three bacterial strains as well as the yeast and mold tested as shown in Table (5.5). This implies that thyme oil/oleuropein combination can be used as natural preservative system in pharmaceutical preparations. Combination of the two oils produced adequate synergistic effect against the parthogens included in this study

at the determined concentrations. The results obtained conforms to a great extent with previous studies conducted on these two oils [102].

Table (5.5): Antimicrobial activity of oleuropine (0.4 w/v %) and thyme oil (0.1 v/v %) in distilled water against three types of bacteria , one yeast and one molds.

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	$>10^3$	$>10^3$	$>10^3$	58	15		928	115	28	
		7	$>10^3$	$>10^3$	560	245	223			$>10^3$	1450	
		14	501	400	280	109	8			$>10^3$	$>10^3$	
		28	210	250	31	0	0				1269	
A. Niger	16404	0	$>10^3$	$>10^3$	185	21	0	$>10^3$	484	69	5	
		7	$>10^3$	24	2	0	0		90	6	1	
		14	192	16	18	0	0		28	4	1	
		28	113	9	0	0	0		66	10	2	

To rule out the presence of systematic error in our results, it was necessary to validate these results. Validation of the results has been done by taking pairs of successive form in a dilution series and comparing the cfu obtained by the following formula:

$$\frac{|2L_{cfu} - H_{cfu}|}{\sqrt{2L_{cfu} + H_{cfu}}} \leq 1.96$$ ……Equation [4.1]

L *cfu*: # of colonies on the plate with lower count (greater dilution)
H *cfu*: # of colonies on plate with higher count (less dilution)

All of the estimated cfu from successive paired in the dilution series must agree within the limits (value of 1.96).

Data analysis of the antimicrobial activity of oleuropine (0.4 w/v %) and thyme oil (0.1 v/v %) against the three types of bacteria , the yeast and the molds showed , more than a 2.0 log reduction from the initial count at 14 days, and no increase in counts thereafter at 28 days for all bacteria strains as well as the yeast and mold. The results of our work agree with the acceptance critiria for semisolid products [92].

5.3 Development and evaluation of topical creams

In order to test the effectiveness of natural substances (oleuropein and thyme oil) as natural preservatives and as antioxidants in pharmaceutical preparations, fusidic acid which can be used for all ages was selected as model drug 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 preparation used was fusidic acid cream. The natural materials were introduced in accordance with the results of the antimicrobial susceptibility tests. The pharmaceutical cream was 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 proportion and consistency of natural materials as well as stability and proportion of the active materials through analysis by HPLC.

The cream base excipients were selected based on the trade product Zydex TM (Beit- Jala Pharmaceutical Co, Ltd Bethlehem - Palestine). Formulation which contains 2% w/w fusidic acid with the excipients: Cetosteroyl alcohol, Macrogol A6, Macrogol A25, Parafine oil, Propylene glycol, parbenes was evaluated. The formula developed in this study was based on the obove mentioned formula, but without using the chemical preservatives - parabens. Formulation development studies were innovated by incorporation of medicinal plants (Oleuropine and thyme oil) that possess highly degree of antibacterial effect in mention trails (Section 5.2). Also, Metal ions, copper sulfate and zinc sulfate which had been studied as antibacterial agents in previous research were used. The desired synergistic antibacterial activity for these ions was observed in the concentration that was better than 2% against *Staphyloccus aureus* and *Escherichia coli* as tested on these microorganisms [55, 59, 64]**.**

Therefore, in the present research the concentrations of 1% , 1.5% and 2% from metals ions were used in the formulations F1, F2, F3 Table (5.6). No interaction between Zydex TM formula and additives according USP references. This will be proven by further studies of physical stability.

Three different cream bases were formulated using various composition of zinc sulfate, copper sulfate, oleuropine and thyme oil. The investigated quantities of each ingredient composition in cream formulation are given in Table (5.6). All formulations were prepared in a small scale and filled in plastic containers.

Table (5.6): General formula for new fusidic acid cream with indication of function for each component

No.	Component	F1	F2	F3	Function
1	Fusidic Acid	2%	2%	2%	Active ingredient
2	Zinc Sulfate	**1%**	**1.5%**	**2%**	Antibacterial ,antiviral,
3	Copper Sulfate	**1%**	**1.5%**	**2%**	Antibacterial, antifungus
4	Oleuropine	**0.2%**	**0.4%**	**0.6%**	Antioxidant and preservative
5	Thyme oil	**0.1%**	**0.1%**	**0.1%**	Antioxidant and preservative
6	Cetosteroyl alcohol	7g	7g	7g	Emollient
7	Macrogol A6	1.5g	1.5g	1.5g	Emulsifying agent
8	Macrogol A25	1.5g	1.5g	1.5g	Emulsifying agent
9	Parafine oil	12g	12g	12g	Vehicle
10	Propylene glycol	8g	8g	8g	Solvent
11	Purified water	q.s	q.s	q.s	Vehicle

All formulations were evaluated at zero time (sample #1), 1 month (sample #2) and after three months (sample #3) to describe antibacterial activity and physical stability of these formulations at accelerated conditions (40±2°C / 75% ±5% RH). The mean ± standard deviation (n= 3) of zone of inhibition for microorganisms strains for each formulations are shown in Table (5.7). In vitro antibacterial activity was studied by measuring the diameter of the zone of inhibition on the agar plate as mentioned in sections (4.6.4 - 4.6.5) for bacteria, yeast and FRSA samples.

The results in Table (5.7) showed that formula (F1) did not appear to have a desirable antibacterial activity against *E.coli*. The acceptable reported quality control limits for *S.aureus* quality and *E.*coli are 19-27 mm and 19-26 mm respectively according to the Clinical Laboratory Standards Institute (CLSI) guidelines [103]. There were no zones of inhibition observed on the agar plates with FRSA samples, therefore, they were discontinued from further evaluation for this formula.

Formulations F2 and F3, were prepared from the same ingredients but with different compositions of oleuropine, thyme oil, zinc sulfate, copper sulfate. Both formulations showed desirable antibacterial activity. There were distinct zones of inhibition observed on the agar plates with the control and other bacterial strains tested in two formulations. The zones of inhibition had been increased as the concentration oleuropine, thyme oil, zinc sulfate, copper sulfate increased. This indicated that the antibacterial activity against *S.aureus*, *E.coli*, FRSA increases with was increasing concentrations in oleuropine, thyme oil, zinc sulfate and copper sulfate.

Two of the cream formulations (F2 and F3) were observed to have similar measurement for the diameter of the zone of inhibition on the agar plate. In this work the least concentrations of excipients that could give the best antibacterial effect as preservative was evaluated, taking into consideration to avoid using high concentrations that could give toxicity out of USP limits.

From the results in Table (5.7), the physical appearance of formula (F3) was unacceptable which was bold blue-greenish in nature, the color formed due to increasing the concentration of oleuropine and copper sulfate. On the other hand, it was found that formula (F2) exhibited better spreadability than formula (F3) as shown in Table (5.7).

Table (5.7): Evaluation of different topical formulation at 0, 1 and 3 months at accelerated conditions (40±2°C / 75% ±5% RH) (n=3)

Characteristic /sample	Zone of inhibition(mm)								
	F1			F2			F3		
	S.aureus	E.coli	FRSA	S.aureus	E.coli	FRSA	S.aureus	E.coli	FRSA
Control (Fucidin TM)	18.3	0	0	18.3	0	0	18.3	0	0
Sample test (1)	22.9±0.2	14.5±1.1	15.4±0.6	39.1±0.4	21.6±1.3	36.7±0.8	39.6±0.4	24.6±0.7	38.8±0.5
Sample test (2)	22.4±0.3	14.3±1.0	15.2±.5	36.8±0.5	21.4±1.4	34.5±0.7	38.2±0.2	23.8±0.9	37.6±0.8
Sample test (3)	21.2±0.2	12.6±1.4	14.1±.6	35.6±0.4	20.3±1.3	33.8±0.8	37.4±0.1	22.7±0.8	36.4±0.5
Appearance	Light green			Light green			Bold blue-greenish		
Spreadablity	Good spreadability			Good spreadability			Thick spreadability		

Therefore, it can be concluded that the safe and desirable antibacterial activity was observed in formulation (F2), and hence, F2 was chosen as the final formulation to prepare the topical cream for this project. Therefore, further studies are required conducted for formula (F2) only. For this purpose samples from formula (F2) were stored at room temreture (25^0C) and at accelerated conditions (40±2°C / 75% ±5% RH). These samples were evaluated at zero time, one month and after three months to evaluate its antibacterial activity, physical and chemical stability.

5.3.1 Physicochemical properties evaluations

The physicochemical properties of the cream formulation (F2) were investigated for 3 month period at 25^0C and 40^0C. The results of general appearance shown in Table (5.8). From the results, it is concluded that good appearance and homogeneity are observed for the cream formulation. The physical appearance of the cream formulation was light green in nature, which was the color obtained from the natural oleuropine and copper sulfate. The odor of the formulation was scented by the smell of thyme. On the other hand, the texture of the cream was smooth with no phase separation. The physicochemical properties for F2 formula at both conditions remained the relatively similar.

Table (5.8) : Physicochemical evaluations for F2 formula at zero time, 1 month and after 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)

Formulation	Odor	Physical appearance	Feel after application	Texture	Color	Phase separation	Homogeneity
F2 (Zero month)	Light smell of thyme	Opaque	Moisture	Smooth	Light green	No	Homogenous
F2 (One month)	Light smell of thyme	Opaque	Moisture	Smooth	Light green	No	Homogenous
F2 (Three month)	Light smell of thyme	Opaque	Moisture	Smooth	Light green	No	Homogenous

5.3.2 Spreadability test

The spreadability of the cream formulations is related to the efficacy of the topical treatment which predicate on the patient spreading the semisolid formulation in an even layer to manage a standard dose [104].

The values in Figure (5.1) illustrated the spreading diameter after one minute.Y axis represent spreadability diameter after one minute (mm), X axis represents formulation storage during three month period at 25°C to 40°C. This indicated the range of the area to which the formulation easily expands on application to skin or the affected portion by a little amount of shear [105]. No apparent change in spreadability was observed with increased storage conditions from 25°C to 40°C (n=3). The mean ± standard deviation of spreadability for each sample from F2 formula was 23.48±0.3. From the result, it was supposed that the new formula (F2) displayed better spreadability than trade products.

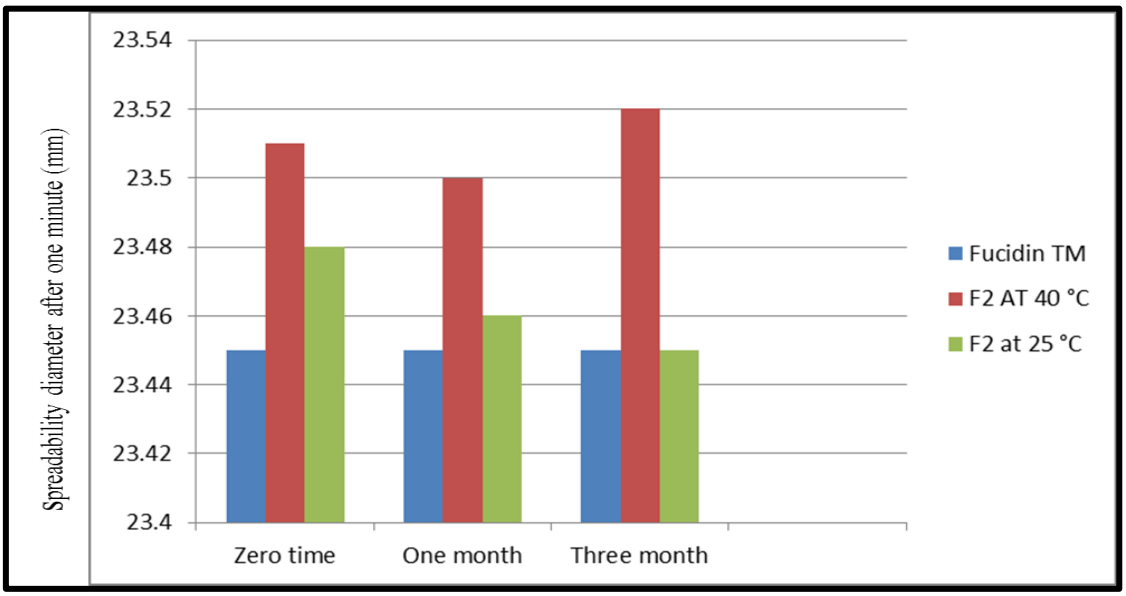

Figure (5.1): Spreadability values for F2 and fucidin TM at zero time, 1 month and after 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)

5.3.3 Fusidic acid cream with natural preservative (oleuropein)

The formulation (F2) and follow-up of the preparation were done by analyzing and testing the assay of the natural substance oleuropein as a natural preservative. Also, active substance (fusidic acid) was determined by HPLC and the natural material was followed up in the preparation in terms of its

effectiveness as antimicrobial for three months. The pharmaceutical cream was stored at 25°C and 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 percentage of oleuropein by (HPLC) in fusidic acid cream, results showed that oleuropein is stable during three months of storage at 25°C, 98.6% at zero time decreased to 97.3%, 95% at 1, 3 month of storage respectively. While the % of oleuropein at 40°C (98%, 97%, 95%) at 0, 1, 3 months of storage respectively and as abserved these results were very close to the results samples at room tempreture. That's mean that oleuropein is stable in this formula. Chromatogram of Oleuropein standard and sample is shown in Figure (5.2, 5.3) at accelerated conditions (40±2°C / 75%± 5% RH).

The percentage of the active ingredient fusidic acid for three months storage at 25°C and 40°C was similar , 102% at zero days decreased to 99%, 98% in 1, 3 months of storage respectively as shown in Figure (5.4), (5.5). These results indicate that the active substance is stable and has not been affected by the addition of natural materials and metals .

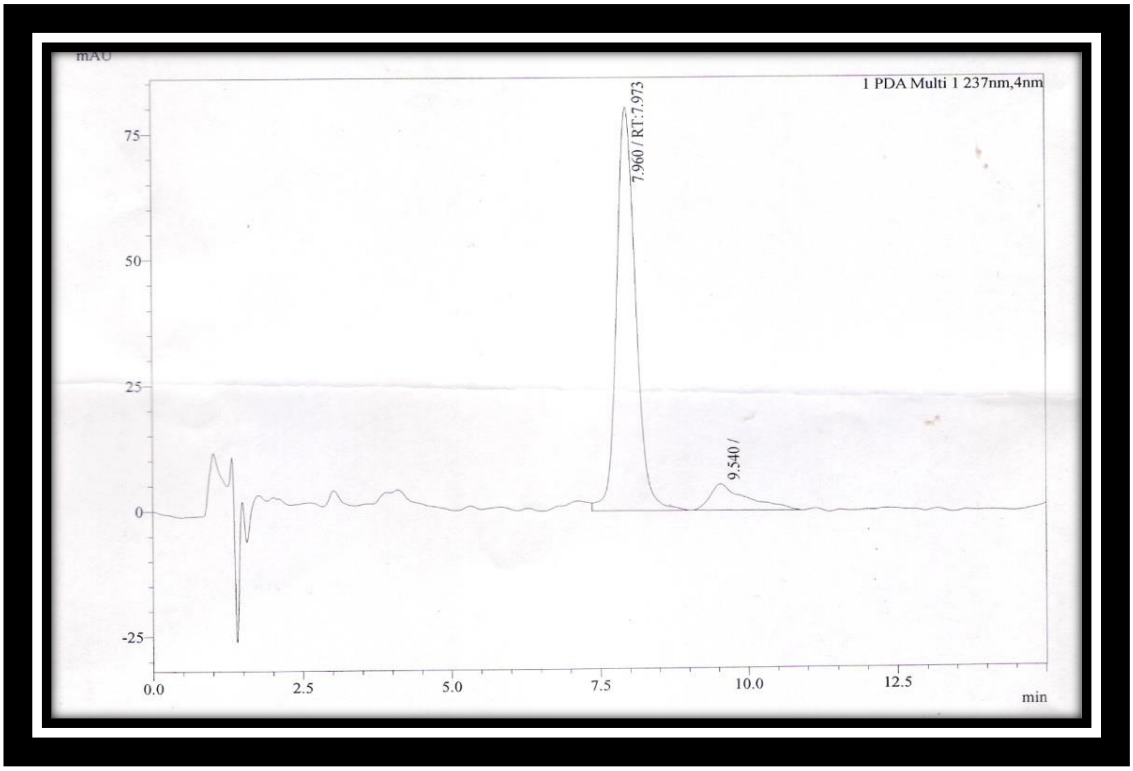

Figure (5.2): Chromatogram of Oleuropein standard

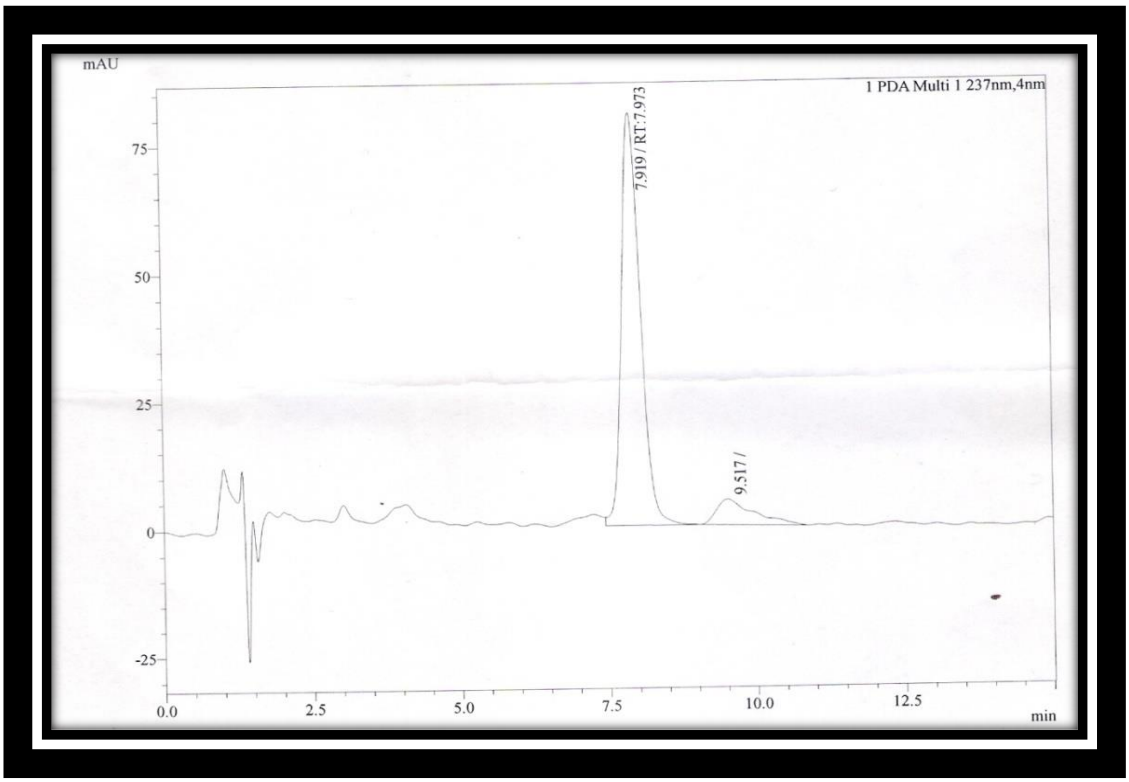

Figure (5.3): Chromatogram of Oleuropein in fusidic acid cream at third month, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

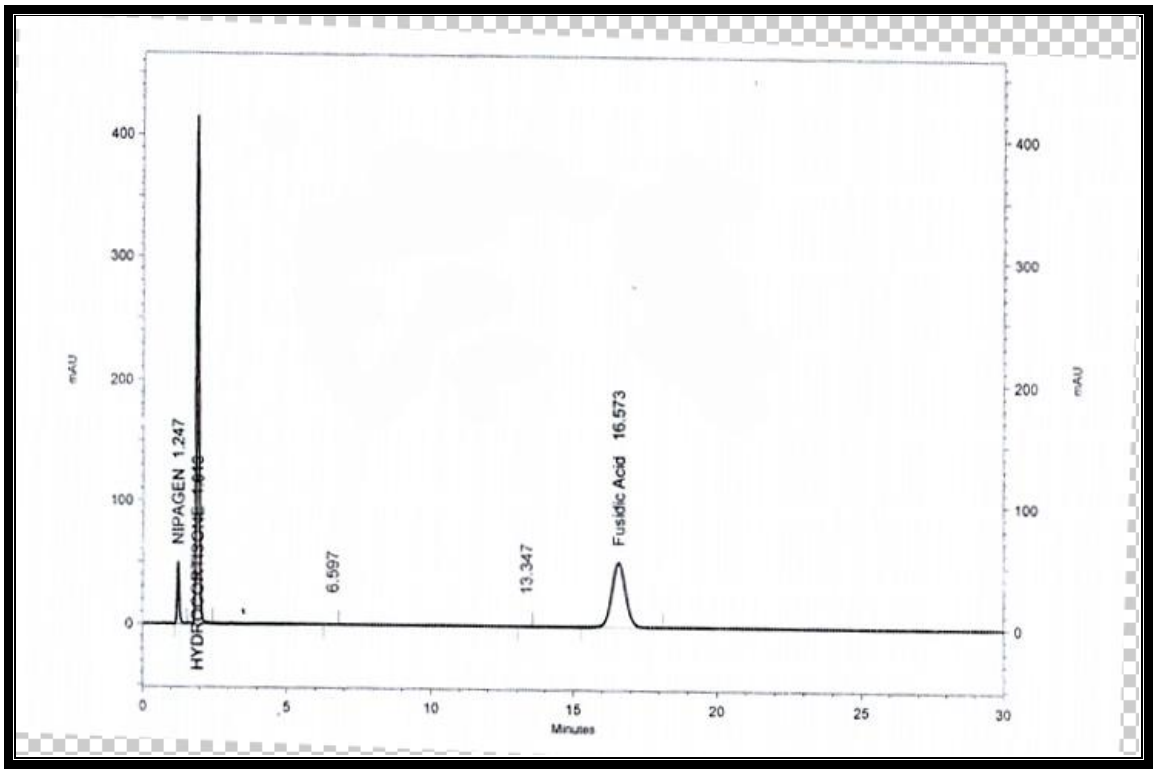

Figure (5.4): Chromatogram of fusidic acid standard

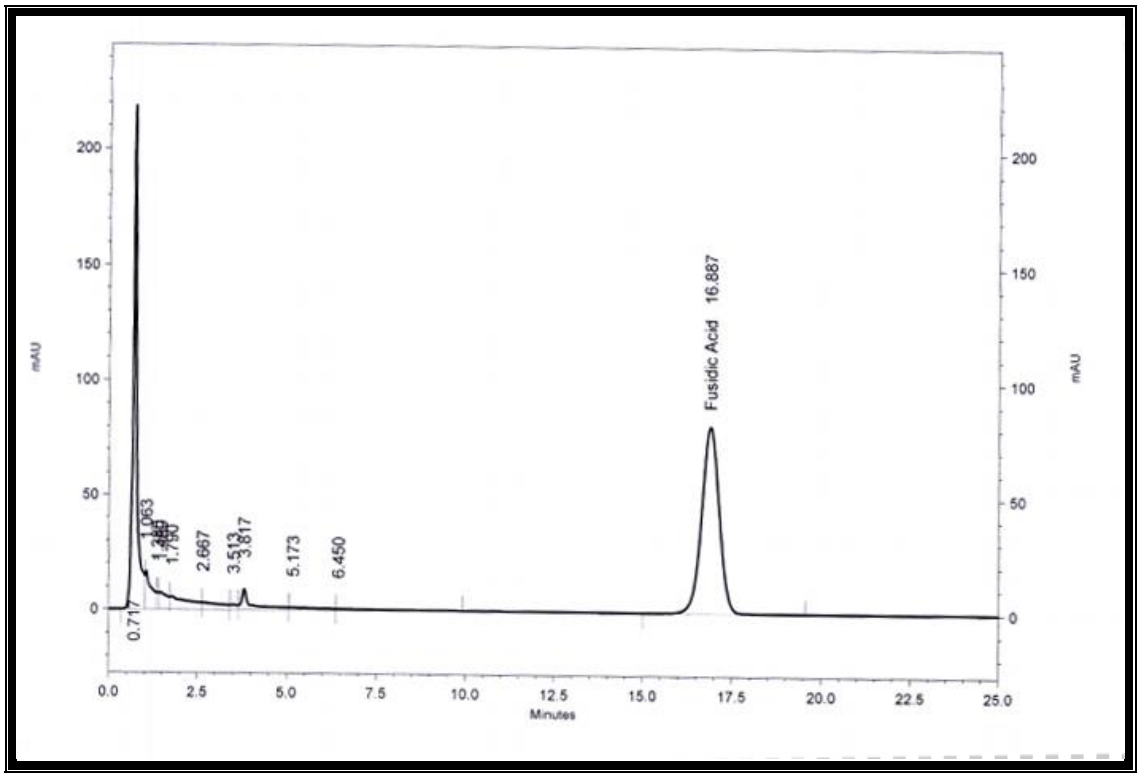

Figure (5.5): Chromatogram of fusidic acid cream at third month, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

The percent of fusidic acid and oleuropine in different conditions was calculated and presented in Table (5.9), (n=3).

Table (5.9): Percent fusidic acid and oleuropine content in the formula (F2) at zero time, 1 month and 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)(n=3)

Assay of agents at different conditions	Zero Time	One Month	Three Month
Fusidic acid at 25°C	102%±0.11	99%±0.13	98%±0.13
Fusidic acid at 40°C	101.4%±0.12	98.9%±0.10	97.8%±0.12
Oleuropine at 25°C	98.6±0.24	97.3%±0.26	95%±0.28
Oleuropine at 40°C	98%±0.37	97%±0.42	95%±0.43

5.3.4 Determination of zinc sulfate and copper sulfate

The determination of these ions content in the formula (F2) was conducted using complexmetric titration method due to unavailability of ICP-MS technique for analysis of trace elements. The percent content calculated shown in Table (5.10) after achievement of the green end point with 0.1M EDTA at accelerated conditions (40±2°C / 75%±5% RH). However, a somewhat low percent of content was obtained from the EDTA titration analysis from zero to third month which decreased between 99.8-96.2 % for zinc sulfate and 98.7-93.4% for copper sulfate at 25°C, While, it was analyzed at 40°C the data had less variability 99.3-95.4% for zinc sulfate and 98.5-92% for copper sulfate at zero time and third month. One of the causes for getting such a decreased percent of element content might be due to simple interaction with cetostearyl alcohol with metal salts but in this formula this incompatibility was relatively less. Dilution of the sample solution with ethanol in ratio 1:1 approximately can avoid high temperatures to titrate. The results in this study was better than previous research projects, that was conducted by using ICP-MS analysis. Relatively low percent of metal ions was obtained between 24% for copper ion and 21% for zinc ion [64, 95].

Table (5.10): Percent ions content in the formula (F2) at zero time at accelerated conditions (40±2°C / 75% ±5% RH)

Ions % = TS * C1 *C2 *100/C3 **TS:** Titration consumption in ml **C1:** Corresponds ions in mg/ml (0.1 mol/L * molar mass g/mol) **C2:** Titer EDTA (dimensionless unit) **C3:** Sample weight in mg
At zero time: **ZnSO4.7H2O %** = 51.8 * (0.1*287.579) *0.1 *100/150 = **99.3%**
At zero time: **CuSO4.5H2O %** = 59.2 * (0.1*249.680) * 0.1 * 100/150 = **98.5%**

Table (5.11): Percent zinc sulfate and copper sulfate content in the formula (F2) at zero time and 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH) (n=3)

Assay of agents at different conditions	Zero Time	Three Month
Zinc Sulfate at 25°C	99.8%±0.82	96.2%±0.48
Zinc Sulfate at 40°C	99.3%±0.73	95.4%±0.53
Copper Sulfate at 25°C	98.7±1.23	93.4%±1.60
Copper Sulfate at 40°C	98.5%±1.28	92%±1.53

5.3.5 Effect on pH of Fusidic acid cream

The PH of fusidic acid cream (F2) was not affected by the addition of oleuropein and other exceipients where very minor decrease in pH was observed, similar to the pH of skin in the range of 4-6 [106]. The pH of the cream formulation without the additions; oleuropine, thyme oil, zinc sulfate and copper sulfate shown in Table (5.12) was found to be 5.0, at 25°C and accelerated conditions (40±2°C / 75% ±5% RH) for three month (n=3). The pH was

decreased to 4.22 and 4.17 after three months at both conditions, when the additions were incorporated into the base.

Table (5.12): Effect on pH of fusidic acid cream at zero month and 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)(n=3)

Formulation	PH			
	Control	Drug (Zero time)	Drug (One month)	Drug (Three month)
F2 at 25°C	5.0	4.23±0.05	4.22±0.04	4.19±0.05
F2 at 40°C	5.0	4.21±0.02	4.19±0.04	4.16±0.03

5.3.6 Viscosity measurement

The viscosity of F2 formula and fucidin TM was determined at different shear stresses using a IV viscometer Brookfield. The viscosity was informed in a unit of centipoise-cps. Figure (5.7) exhibited turns per minute- X axis (rpm or shear stress) vs. the apparent viscosity for formulation- Y axis, for fucidin TM and F2 , at 25°C and 40°C storage conditions at zero time and three month at different shear stress values (n=3). The viscosities for the cream formulations at 30 rpm were found to be 15,160±1.25 cps for F2, 14,550±1.05 cps for fucidin TM. The required viscosity limits (8000-16000 cps) according for non-sterile semisolid products at 30 rpm [95]. The viscosity decreased over shear stress, it was clearly explained that the viscosity of F2 at 0 time and 3 month at 40°C was found between 8000-16000 cps. It is supposed that a non-Newtonian conduct with pseudoplastic flow was watched in both formulas. It is obvious to observe from Figure (5.7) that as the rpm or shear stress increased, the viscosity decreased in both of the formulations. In conclusion, the resistance to flow fucidin TM was low when compare to F2 by pouring the formulations out of the container.

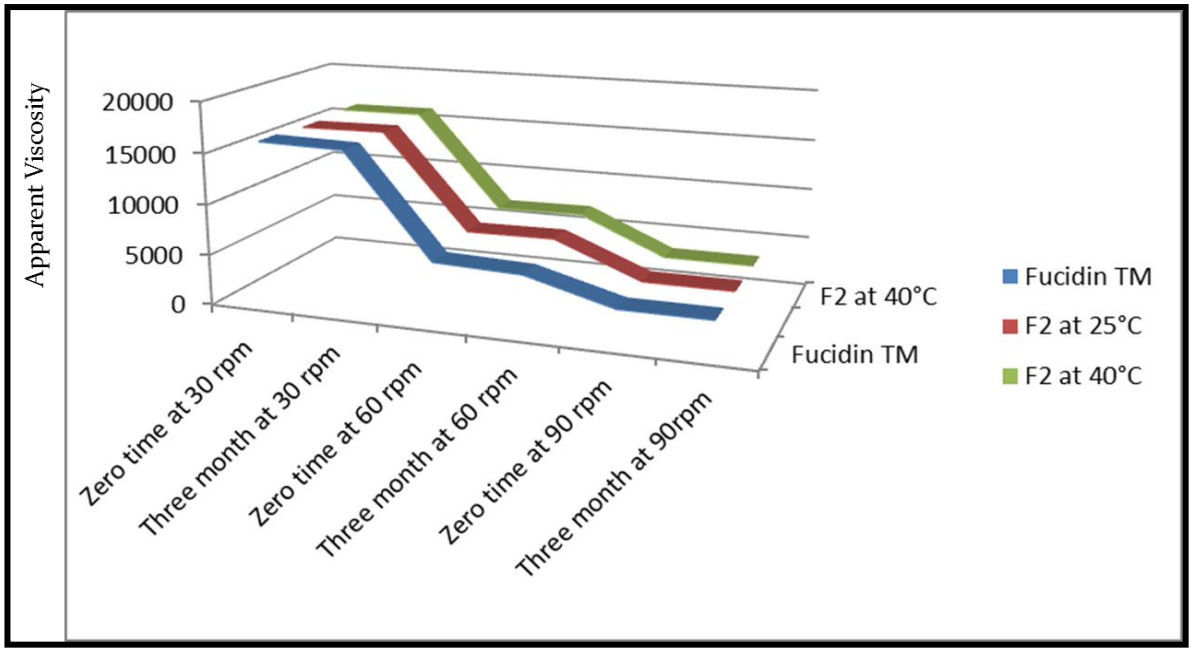

Figure (5.6): Viscosity data of F2 formula and fucidin TM at zero month and three month at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)

5.4 Antimicrobial test of fusidic acid cream

5.4.1 Direct transfer (broth media):

Microbial test for fusidic acid cream with oleuropein 0.4% w/v and thyme oil 0.1% v/v concentration was conducted at zero time and 3 months of storage at 25°C and accelerated conditions (40±2°C / 75% ±5% RH), and compared to the positive control (fusidic acid cream with parabens) and negative control (fusidic acid without any preservative).

Results showed that there was no bacterial growth using the three media shown in the Tables in (Appendix 1, 2, 3, 4) attachments which is exactly similar to the result of fusidic acid control sample (with parabens preservative) at zero time and after 3 month at 25°C and 40°C. This result showed that oleuropein and thyme oil are effective as antimicrobial agent. Negative control of fusidic acid without preservative showed bacterial growth as shown in Table (5.13).

Table (5.13): Microbial limit test – Direct transfer (broth media) at zero time and 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)

Medium	Sample with Oleuropein (0.4%) and thyme oil (0.1)	Results positive test (using parabens)	Results Negative control (No preservative)
Tryptic Soy Broth	Clear	Clear	Turbid
Fluid Lactose Medium	Clear	Clear	Turbid
Sabouraud Dextrose Broth	Clear	Clear	Turbid

5.4.2 Microbial limit test –Total count:

The total microbial count of fusidic acid cream samples was conducted and results are shown in Table (5.14) at 25°C and 40°C at zero time and 3 month, showed that samples with oleuropein and thyme oil are compatible to the positive control (fusidic acid cream with parabens 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, results showed that fusidic acid samples with oleuropein and thyme oil are almost identical to that with conventional chemical preservative parabens at zero time and 3 months at 25°C and 40°C.

Table (5.14): Microbial limit test –Total count at zero time and 3 months at 25°C and accelerated conditions (40±2°C / 75% ±5% RH)

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

5.4.3 Determination the MICs for cream formulations, their active substances and FRSA sample

Fusidic acid is primarily active against gram positive bacteria such as *Staphylococus spp*., with no activity against *E.coli, Pseudo.aerginosa* and *C.albicans*, same as the results shown with the fucidin TM (Control) reflecting growth of the microorganisms tested on agar plates except staphylococci. Interestingly, the new cream formulation which contains fusidic acid with added substances has broad antimicrobial activity in vitro, it was highly active against all microbes by inhibits growth of all pathogens as compared with fucidin TM, Figures (5.7- 5.11) after storage at accelerated conditions (40±2°C / 75% ±5% RH) at zero time only. So that, the goal has been achieved to a develop formulation for the treatment of bacteria, fungi and viruses in one medical preparation.

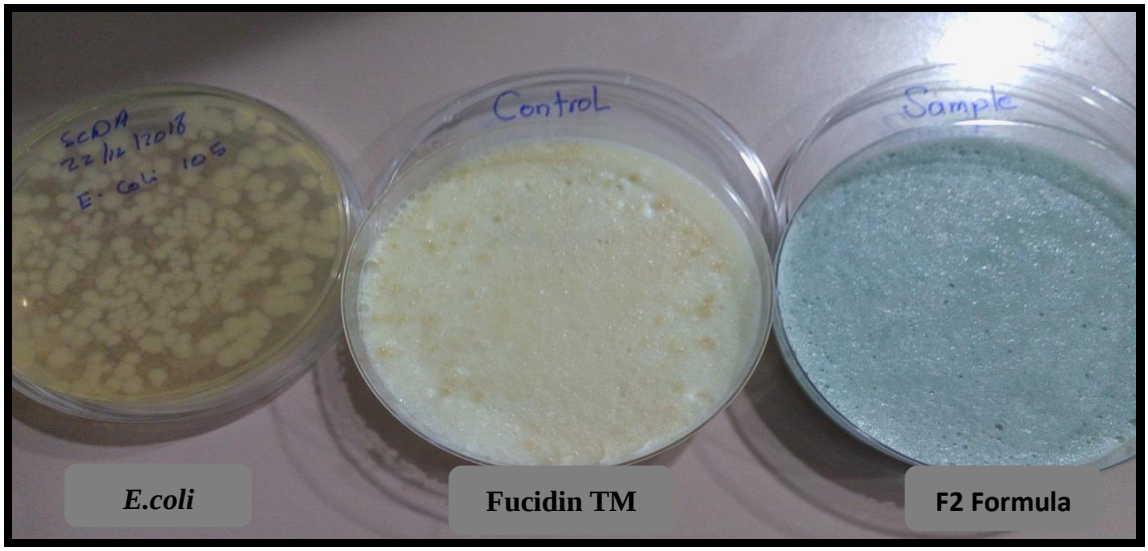

Figure (5.7): Fucidin ™ and F2 effect on *E.coli* isolate

Figure (5.8): Fucidin ™ and F2 effect on *C.albicans* isolate

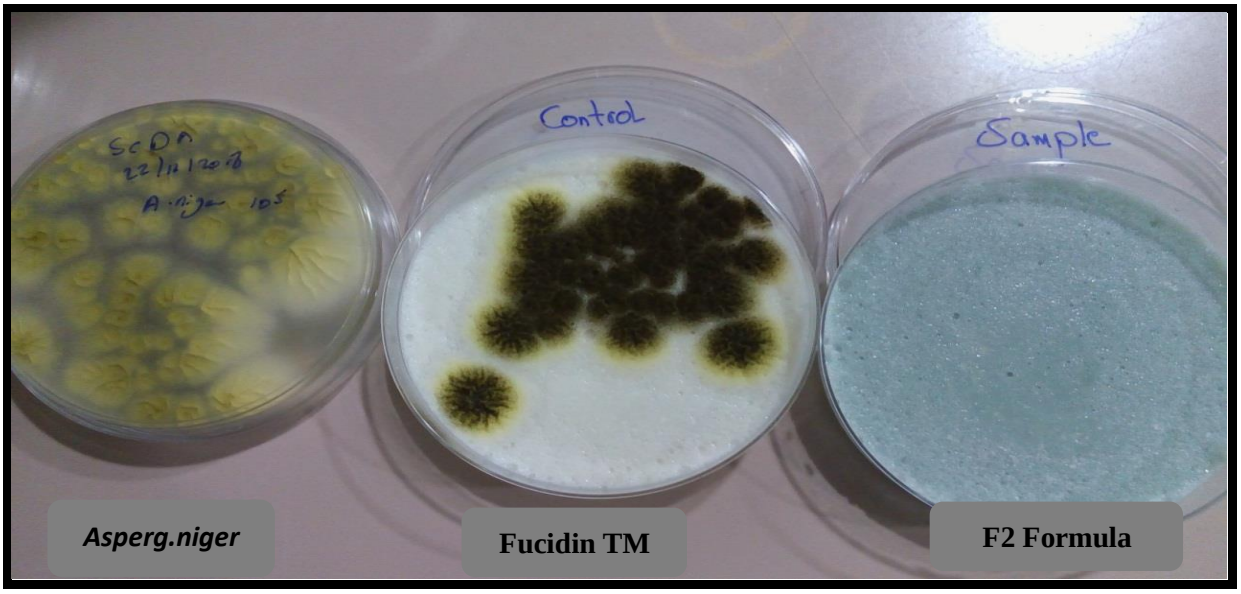

Figure (5.9): Fucidin **TM** and F2 effect on *Asperg.niger* isolate

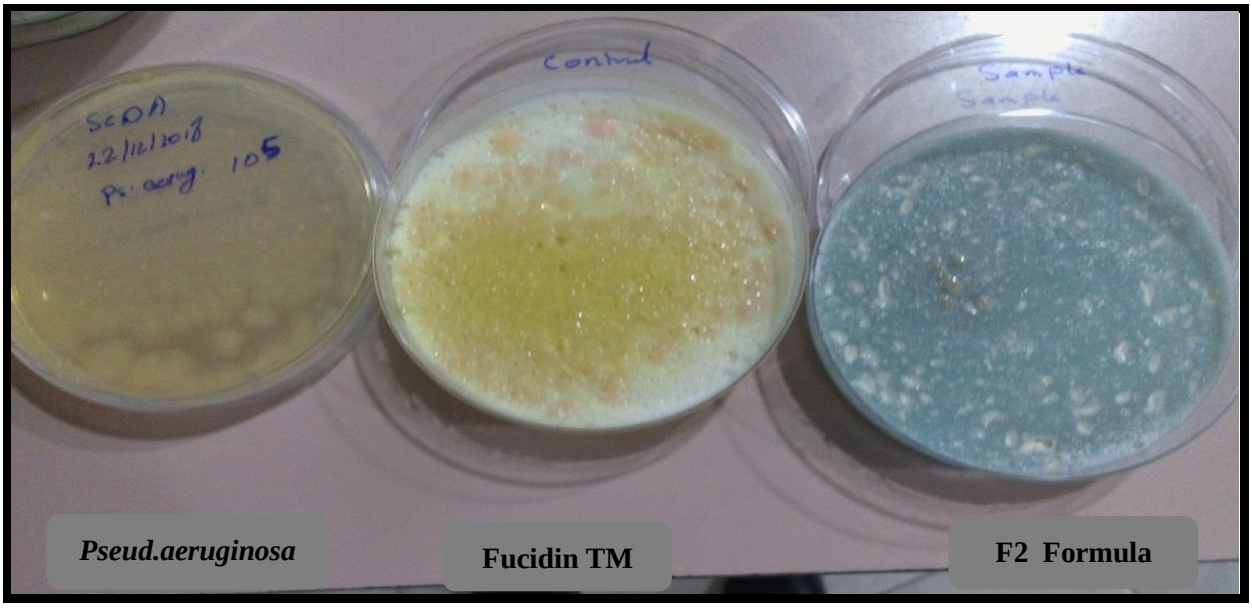

Figure (5.10): Fucidin **TM** and F2 effect on *Pseud. aeruginosa* isolate

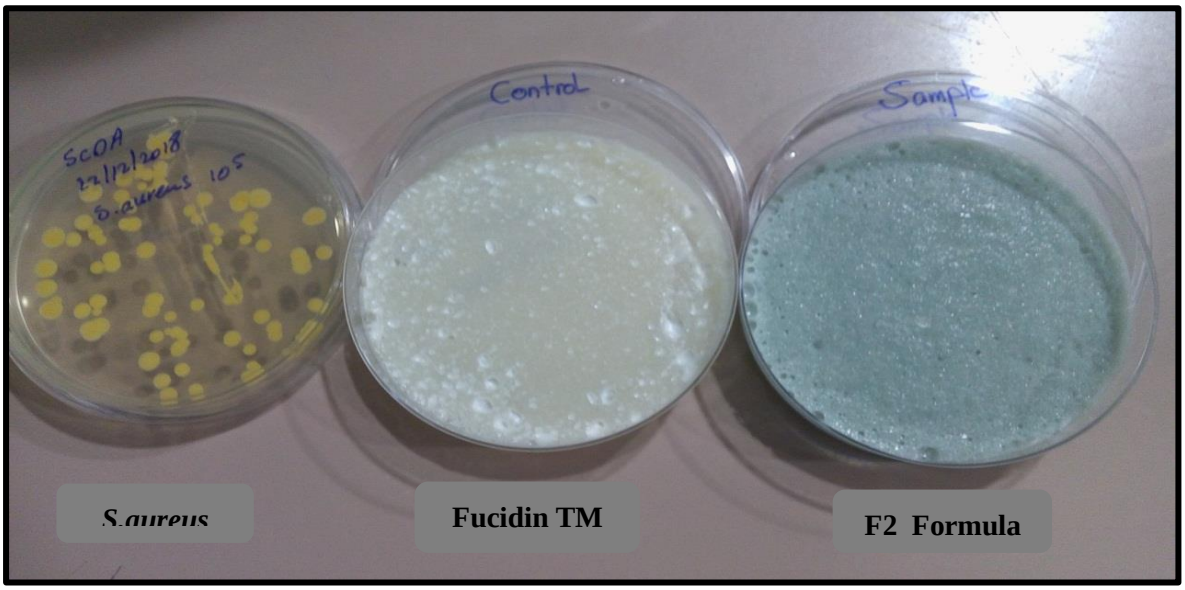

Figure (5.11): Fucidin ™ and F2 effect on *S.aureus* isolate

Susceptibility of FRSA to F2 formula was investigated by broth microdilution method. Microdilution method was performed in sterile microtiter plates. The inoculum density was set to 0.5 McFarland, diluted 10 times in sterile saline and 5 µl of this suspension was inoculated in 0.1 ml of CAMHB-Cation adjusted Mueller-Hinton Broth to reach final inoculum of 5 x 10^4 cfu/well. Active substance was diluted in DMSO and added to CAMHB from 2560 µg/ml to 1.25µg/ml by two-fold dilution in 96-well microtiter plates. After inoculation, plates were incubated at 36°C for 24 hours. MIC was determined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in broth dilution susceptibility test [96, 107]. From wells without visible growth 10 µl were subcultivated to CAMHA and incubated at 36°C for 24 h.

The MIC has been determined for cream formulation and their active substances by the agar dilution assay against FRSA strains to $1*10^5$ CUF were used for preparation of the inoculums.

The F2 formula 1% (0.5 g cream in final volume 50 ml agar) was prepared and further dilution series with agar to get concentration 0.5-0.0001%, the lowest concentration of formula that prevented bacterial growth is considered to be the minimum inhibitory concentration of that formula against all pathogens is 580 µg/ml (MIC for entire cream). The MICs for cream formulation and its substance after storage at accelerated conditions (40±2°C / 75% ±5% RH) at zero time only are presented in Table (5.14).

Table (5.15): MICs of active ingredients as part of cream(ASPC), entire cream(EC) and active ingredient alone(AS)

F2 formula and its substances	Concentration of substance	MICs against pathogens
EC	*The lowest concentration of formula that prevented bacterial growth*	580 µg/ml
Zinc sulfate(ASPC)	1.5%	870 µg/ml
Copper sulfate(ASPC)	1.5%	870 µg/ml
Oleuropine(ASPC)	0.4%	232 µg/ml
Thyme(ASPC)	0.1%	58 µg/ml
Fusidic acid(AS)	2%	1160 µg/ml

"MIC of active substance as part of cream(ASPC) is calculated as the concentration of active substance in the cream multiplied by MIC for the entire cream(EC)"

Our results showed that the strongest activity was seen against FRSA (MIC 58 µg/ml, 232 µg/ml for thyme oil and oleuropine as part of cream formulation

respectively), but there was also good effect from other excipients on the same pathogens (MIC 870 μg/ml for zinc sulfate and copper sulfate, 1160 μg/ml for fusidic acid as part of the cream or alone). In this study the additives (Oleuropine, thyme oil, copper sulfate, zinc sulfate) showed moderatly strong activity against FRSA strains with specific MIC values.

One issue with this MIC study is the difficultly of explicating and applying *in vitro* information to the *in vivo* situation. Specifically, it is strict to convert an MIC value in μg/ml obtained with the agar dilution assay into proposal on the size of cream needed to be used in well-orderd to reach analogous concentration on the the skin. Even if such a conversion was easily made it is significant to remember that the *in vitro* and *in vivo* mediums can differ significantly with regards to various factors such as temperture, pH and salt concentrations.

One purpose of this realization was to test if basic components of the formulation had additional or synergistic effects with active ingerdient of that formulation. Our data support the entity of such effects. MIC values for the active substance as part of a formulation give additive effect with active ingredient on gram negtive bacteria, yeasts, molds, virus infection and FRSA strains.

The data can be important in the handling of superficial skin infections and in the follow up of reduce the utilize of systemic antibotics. It also shed importance of the necessity for modren, topical, non-resistance reinforcement, antimicrobial therapy altrenatives for skin disease of infection.

In a vision of rising antibiotic resistance the development of new antibiotic topical preparation is a significant work for the future application and further research.

Another study was performed to test the antibacterial activity for F2 formulation against FRSA colonies. Twenty three clinical isolates of FRSA from a total of 161 *S.aureus* isolates were collected from Palestinian hospital between January and October 2018. The isolates were recovered from various sites of infection; from blood, respiratory secretions, skin, soft tissue (the highest proportion of these isolates) and urine, and obtained from inpatients and outpatients, both females and males. The range of resistance of the FRSA isolates to antimicrobial agents was from 0% for vancomycin to 100% for pencillin G . Then, the samples were subsequently proccessed from cultring and identification using different biochemical tests. For long storage of *S.aureus* postive cultures, TSB containing 15% glycerol has been used and stored at -80^{0}C.

Figure (5.12-B) shows results of antibacterial activity for F2 formula aganist FRSA colonies by measuring the zone of inhibition in millimters after storage at accelerated conditions (40±2°C / 75% ±5% RH) at zero time only, the zone of inhibition can be determined as the clear area round the sensitive well or disc with an antimicrobial agent on the agar surface. The bigger the zone of inhibition the greater and better the inhibition of specific antimicrobial agent.

There were no zones of inhibition observed on the agar plates with fucidin ™ in FRSA samples (Figure 5.12-A), but the zone of inhibition was increased in F2 plate to average (37.25 mm) from first to third month. Therefore, it can be concluded that the desirable antibacterial activity was observed in F2 and management of superfiacial skin infections (atopic dermititis, diaper rash, skin and soft tissue infection) that have fusidic acid resistant isolates of *S.aureus* causing bullous impetigo in patients, this study has proved that the additive excipients had

synergistic antimicrobial effect when they were utilized together in the trial prepared formulations.

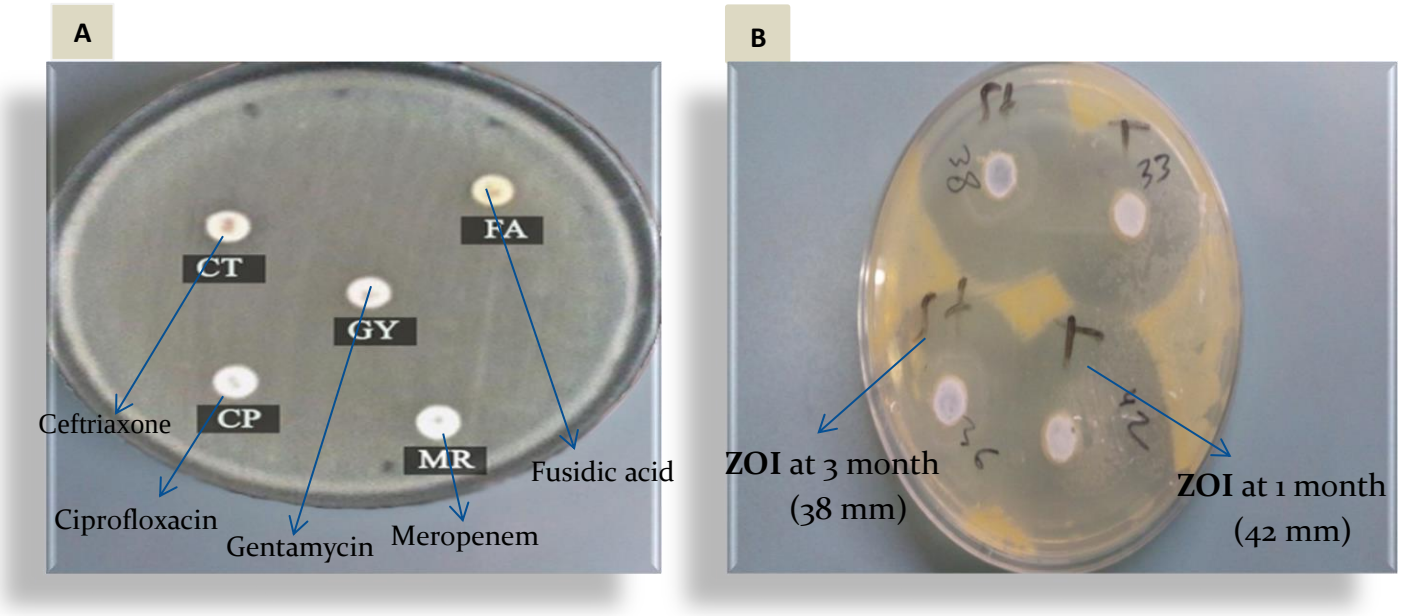

Figure (5.12): Antimicrobial susceptibility testing of S.aureus against fusidic acid and other antibiotics by disk diffusion method
(A): FRSA plate shows the resistance effect of different antimicrobial agents on *S.aureus* isolate (Breast cancer and Nodular melanoma sample case resistance to *CT*:Ceftriaxone, *FA*:Fusidic acid, *GY*:Gentamycin, *CP*:Ciprofloxacin, *MR*:Meropenem)).
(B): FRSA plate F2 formula sensitive S.aureus shows zone of inhibition> 22 mm in diameter compared to FRSA(A) without any zone ,T: at zero time , st: at third month.

5.5 Stability of pharmaceutical preparation

The results showed that pharmaceutical cream where oleuropein was used at 0.4% w/v and thyme oil at 0.1% v/v concentration were stable for three months. There was no precipitation, appearance and, color stable and pH within the required range of (4-6), active ingredient fusidic acid and oleuropein content within the allowable range as shown in the Table (5.15).

The formulation F2 was stored at 25°C and accelerated conditions (40±2°C / 75% ±5% RH) for 3 months in plastic containers (n=3). The formula was analyzed for the pH, color, physical appearance, precipitation, assay of fusidic

acid and oleuropine, phase separation, homogeneity and antibacterial activity for 3 months. The physicochemical evaluation for the F2 is given in Table (5.15). The color of the formula remained the same after month 3 at three months for both tempretures. Other characteristic's (precipitation, appearance, homogeneity) remained the same as month one for both tempreture. While, phase separation was noticed in the formula at 40°C and the greenish blue color of liquid diffused out from the cream base was observed. As the results showed, the stability survey for the formula at 40°C was finished due to the physical instability at this high temperature degree.

Table (5.16): Physical parameters and assay results of formula condition at zero time and 3 month at 25°C and accelerated condition (40±2°C /75%±5% RH) (n=3)

Comparisons	Time	Precipitation	Appearance	Color	PH	Assay of Zinc sulfate	Assay of Copper sulfate	Assay of Oleuropein	Assay of Fusidic acid
F2 at 40°C	0 Time	Negative	Uniform	Light green	4.21± 0.02	99.3%± 0.73	98.5%± 1.25	98%± 0.37	101.4%± 0.12
	1Month	Negative	Uniform	Light green	4.19± 0.04	96.8%± 0.59	98.3%± 1.21	97%± 0.42	98.9%± 0.10
	3Month	Negative	Uniform	Light green	4.16± 0.03	95.4%± 0.53	92%± 1.53	95%± 0.43	97.8%± 0.12
F2 at 25°C	0 Time	Negative	Uniform	Light green	4.23± 0.05	99.8%± 0.82	98.7 %± 1.23	98.6%± 0.42	102%± 0.11
	1Month	Negative	Uniform	Light green	4.22± 0.04	99.6%± 0.79	96.4%± 1.05	97.3%± 0.26	99%± 0.13
	3Month	Negative	Uniform	Light green	4.19± 0.05	99.3%± 0.73	93.4%± 1.60	95%± 0.28	98%± 0.13

5.6 Microbial stability of pharmaceutical preparation

Pharmaceutical preparation was tested in terms of the numbers of bacteria, yeasts, and mold, at zero time and 3 month at 25°C and 40°C . Tryptic soy agar was used as a medium for the bacteria, while sabouraud dextrose agar was used for

yeasts and mold. The results were < 10 CFU of bacteria, yeasts, and mold. The results were close to that of the positive test as shown in Table (5.15).

Table (5.17): Antimicrobial limit test of results stability study of pharmaceutical cream formulations at zero time and 3 month at 25°C and accelerated condition (40±2°C /75%±5% RH)

Microbial limit test	Period (months)	Sample	Positive test	Negative test
F2				
Direct transfer (broth media)	0	Clear	Clear	Turbid
	1	Clear	Clear	Turbid
	2	Clear	Clear	Turbid
	3	Clear	Clear	Turbid
F2				
Total count	0	<10 CFU	<10 CFU	<10 CFU
	1	<10 CFU	<10 CFU	<95 CFU
	2	<10 CFU	<10 CFU	<160 CFU
	3	<10 CFU	<10 CFU	<155 CFU

By using the agar well diffusion assay for a 3 months period at 40°C, 25°C and 4°C in plastic containers (n=3). The zones of inhibition for formula (F2) are reported in Table (5.16). The antibacterial activity did not alter overtime with plastic material containers for the formula, since the diameters zone of inhibition did not vary overtime, and there is no significant variation in the zones of inhibition between the various temperature, while the temperature did convert the physical stability of formula. Unfortunately, a lowering in the zones of inhibition were observed at both 25°C and 4°C over a 3 months period.

Table (5.18): Antibacterial activity of F2 formulation over three months period, at 4°C, 25°C and 40°C.(n=3)

	Period	Zone of inhibition (mm)		
		Temperature (°C)		
		4 °C	25 °C	40 °C
Plastic container	Zero time	24.42±0.06	25.35±0.21	24.8±1.41
	One month	23.54±0.05	24.55±0.28	-
	Two months	23.23±0.08	23.85±0.22	-
	Three months	22.8±0.07	22.38±0.24	-

Chapter Six
Conclusion

6.1 Conclusions

In this study, we developed a formula of topical cream containing natural preservative and other excipients (zinc sulfate and copper sulfate) in one medical preparation that has antifungal, antibacterial and antivirus properties. During the process of the formulation we noticed the absence of interaction between the excipients on one hand and the drug on the other. This maintained the physical stability without alteration. The results observed in this study obviously showed that the formula appear pseudo-plastic flow and that the antibacterial activity was better than the trade agents when compared with the quantity of agents that were used. Although lower percent (zinc sulfate and copper sulfate) content was analyzed using EDTA titration method, the antibacterial activity for the formula was not altered.

The efficiency of oleuropein against microbes was verified, with the optimum concentration being 0.6%, which can be used in pharmaceuticals as preservative. The efficacy of oleuropein and thyme oil were verified within the pharmaceutical preparations as it carried a synergistic activity between them and their combined effects against microbes was acceptable. Verification of the addition of these natural substances did not affect the pH for three months. The effectiveness of pharmaceutical cream against microbes was verified by the presence of natural substances, additive excipients and the result was acceptable. F2 formula reduces the emergence of FRSA strains, multi drug resistance, reduce

toxicity of chemical preservatives and better effects against polymicrobial infections. Addition of natural substances did not affect the proportion of active ingredient within pharmaceuticals for three months at room and accelerated condition (40±2°C /75%±5% RH). Natural substances were stable and effective for three months at both conditions.

6.2 Future work

1. Evaluation of Oleuropein and thyme oil in pharmaceutical cream for the year and investigate other dosage form such pediatric syrup formulations and coating in Tablets.
2. Ex-vivo permeation of the formula, human test, skin irritation studies should be performed.
3. Development a microneedle patch contains oleuropine and sliver nitrate new drug delivery method that responds to: HT29, SW620 colon cancer cells with 10-100 μM.

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Appendices

Appendix1 : Growth promotion of the Sabouraud Dextrose Agar

Medium : Sabouraud Dextrose Agar				
Test Done	Manufacturer Specification's	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 days	490	>1000	>1000
	2) As. Niger 16404/ 5 days	0	3	50

Appendix2: 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

Appendix 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

Appendix 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

Appendix 5: Preparation of 0.5 McFraland standard tube (1 to 2 x10^8 CFU/ml)

-Mix the following chemicals

-0.5 ml of 1.175% barium chloride dehydrate

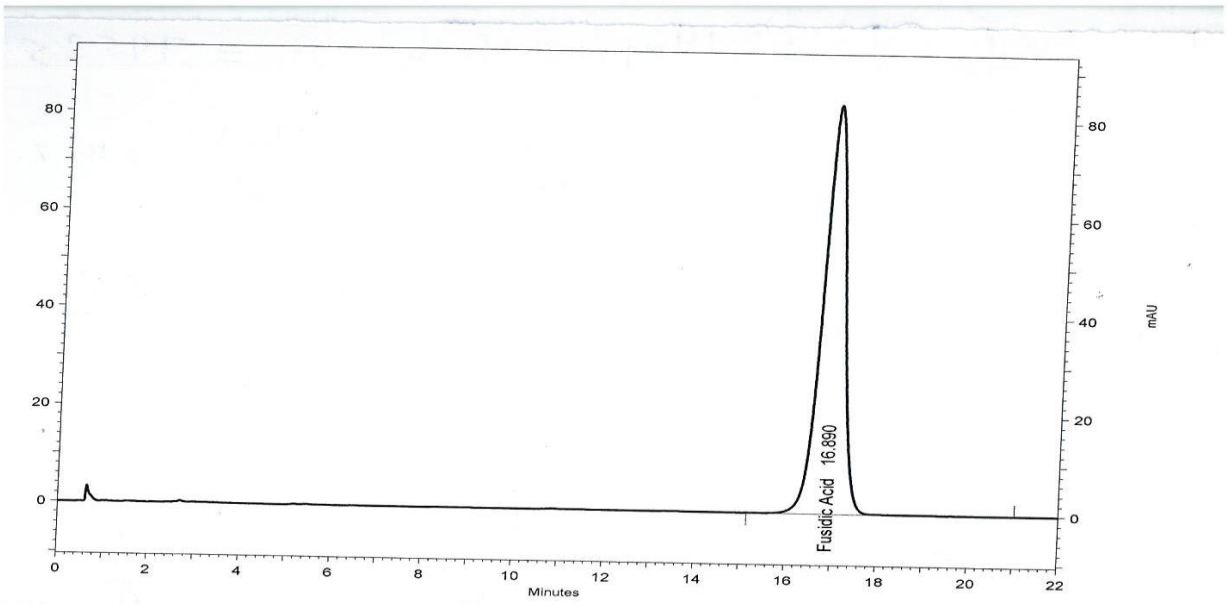
- 99.5 ml of 1% sulfuric acid

-Check the optical density (OD) at wavelength of 625 nm.

The accepted range is 0.08 to 0.10 OD

Appendix 6

Chromatograms of Fusidic acid and Oleuropein at zero time, one month and third month stored at accelerated conditions (40 ±2°C / 75% ± 5% RH)

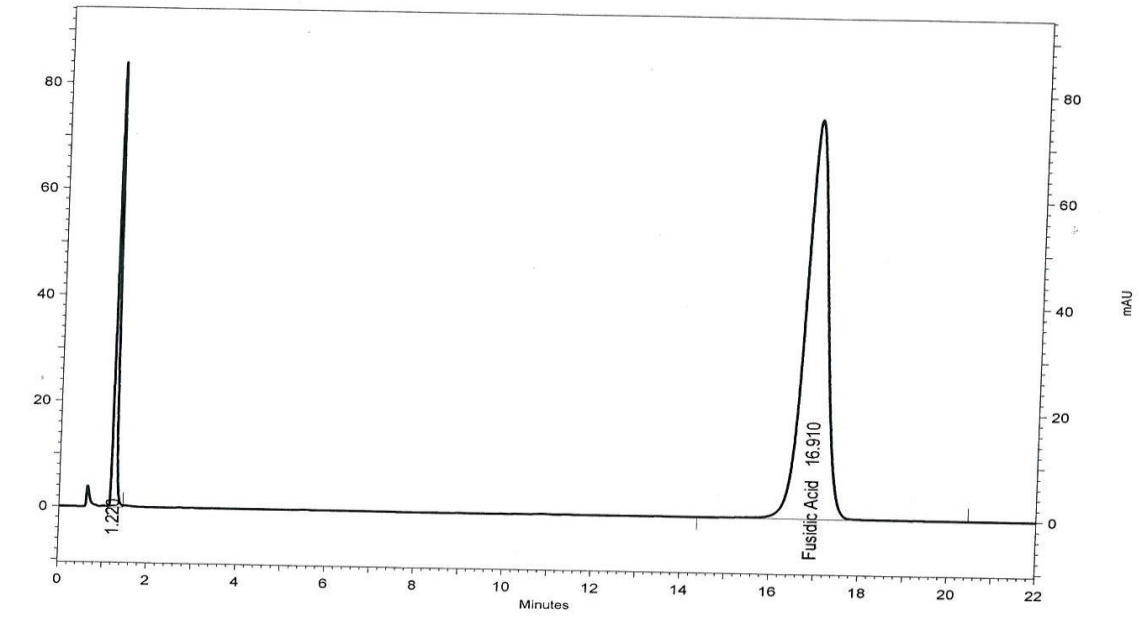


-WL1 235 nm
sults

Peak Number	Name	Retention Time	Area	Area Percent
1	Fusidic Acid	16.890	11270550	100.000
Totals			11270550	100.000

– Fusidic acid 3 month trails #1 –
(Standrd)

Appendix 6.1: Chromatogram of fusidic acid standard at third month trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

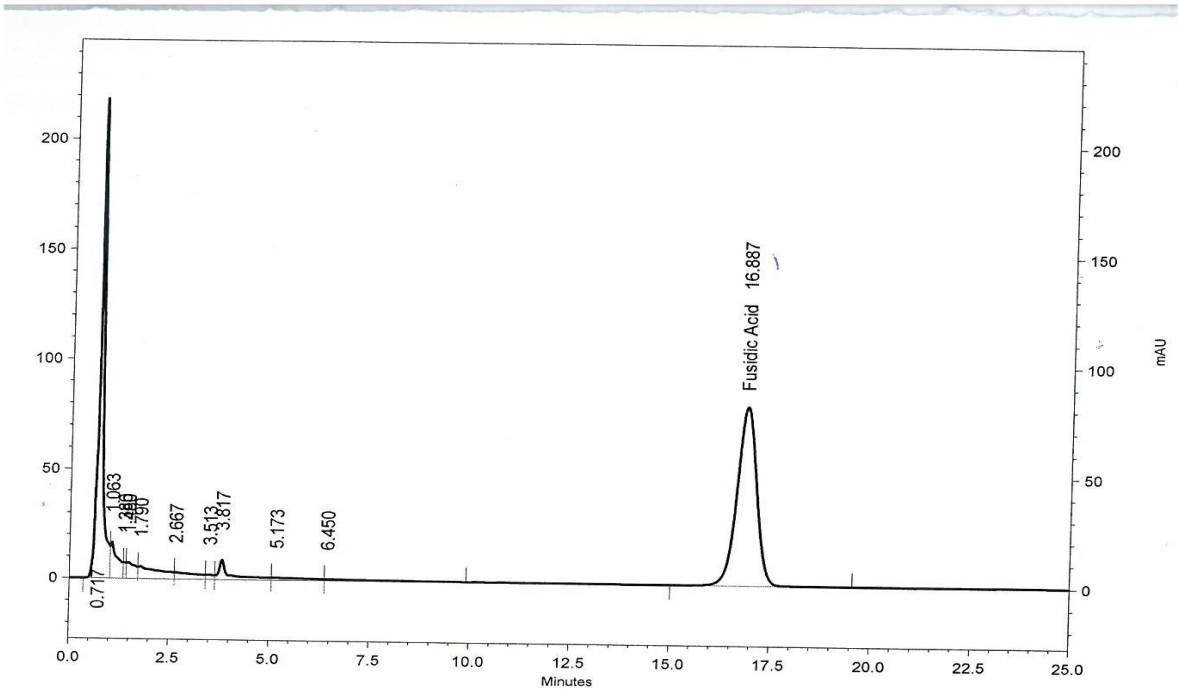


-WL1 235 nm
sults

Peak Number	Name	Retention Time	Area	Area Percent
1		1.220	1465385	12.454
2	Fusidic Acid	16.910	10301411	87.546
Totals			11766796	100.000

– Fusidic acid 3 month #1 –
(Sample)

<u>Appendix 6.2</u>: Chromatogram of fusidic acid sample at third month trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

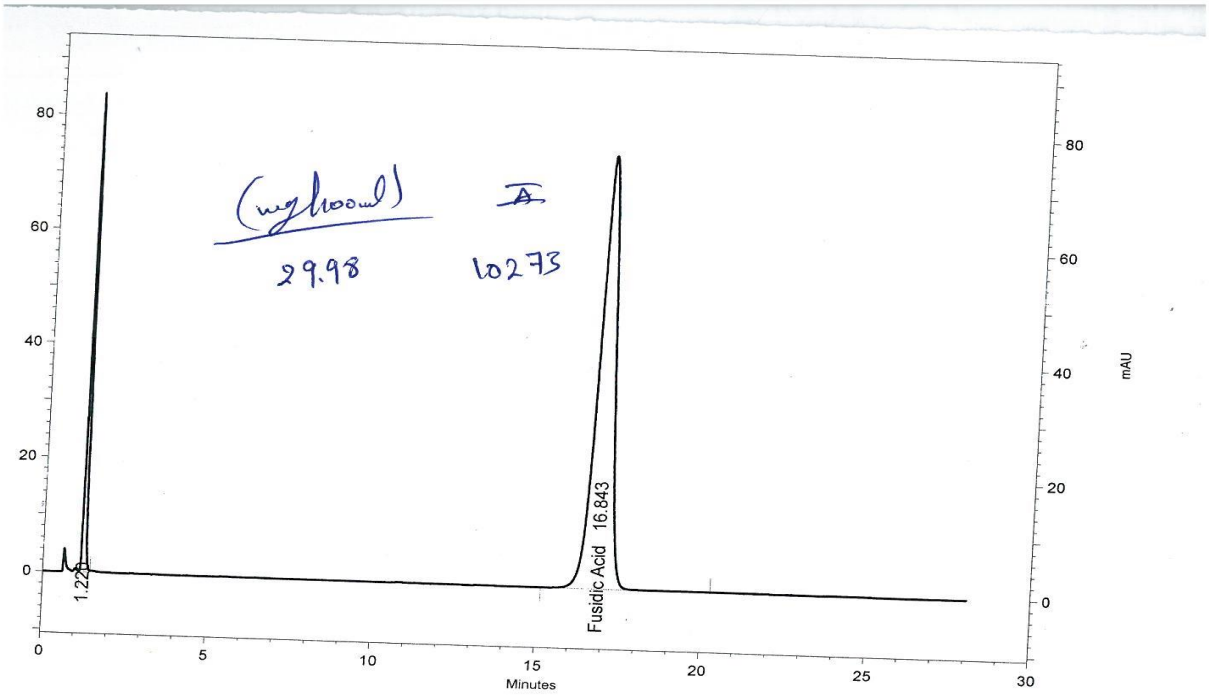


V-WL1 235 nm
esults

Peak Number	Name	Retention Time	Area	Area Percent
1		0.717	6462745	30.046
2		1.063	833096	3.873
3		1.380	119296	0.555
4		1.480	450452	2.094
5		1.790	844563	3.926
6		2.667	436676	2.030
7		3.513	106294	0.494
8		3.817	671923	3.124
9		5.173	251343	1.169
10		6.450	259468	1.206

- Fusidic acid 1 month trails #1 -
(Sample)

<u>Appendix 6.3</u>: Chromatogram of fusidic acid sample at one month trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months



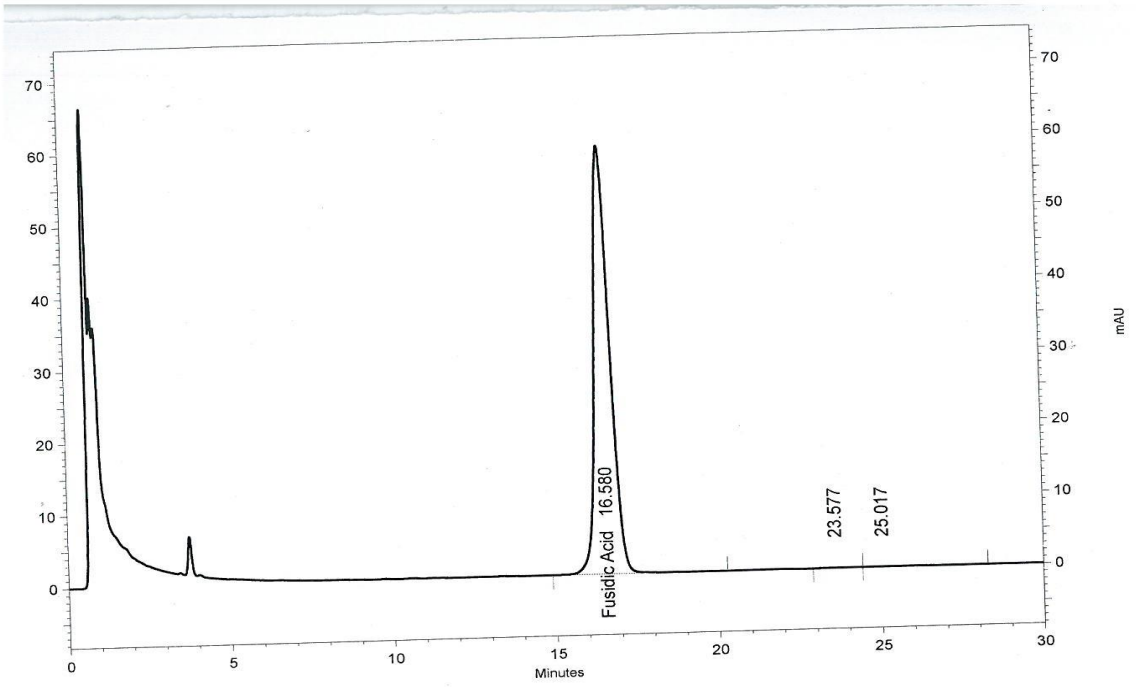
(mg/100ml) A
29.98 10273

VL1 235 nm
ılts

Peak Number	Name	Retention Time	Area	Area Percent
1		1.220	1464133	12.504
2	Fusidic Acid	16.843	10244863	87.496
Totals			11708996	100.000

– Fusidic acid 1 month trails #1 –
(Standrd)

Apendix 6.4: Chromatogram of fusidic acid standard at one month trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

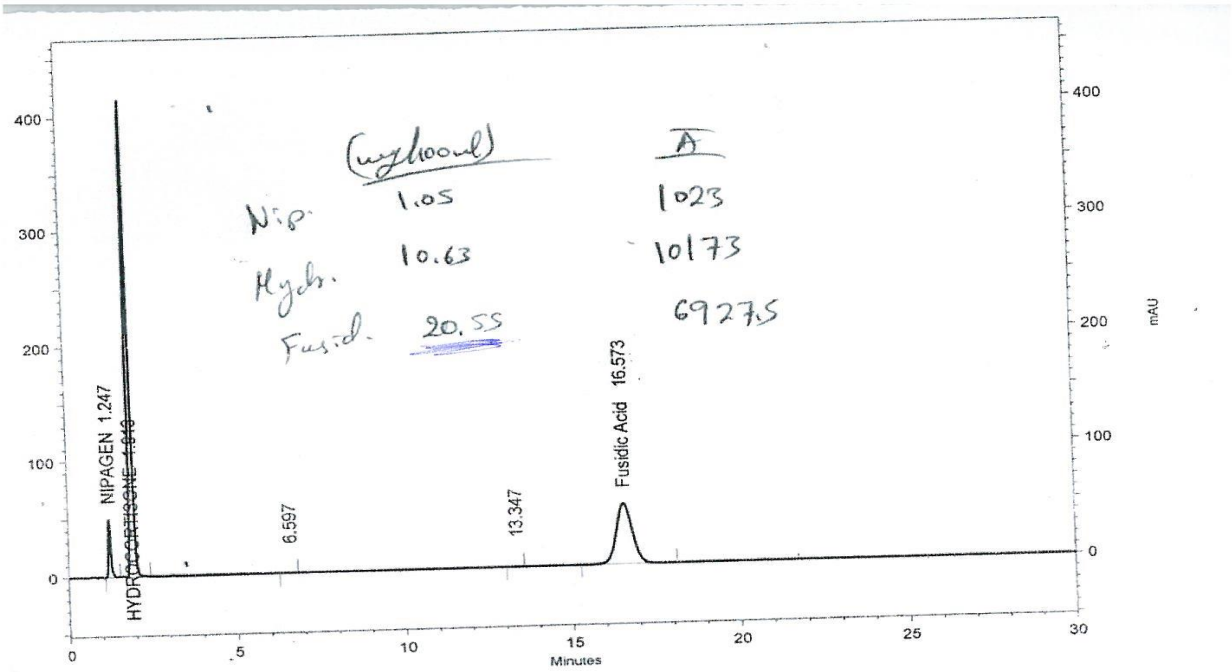


UV-WL1 235 nm
Results

Peak Number	Name	Retention Time	Area	Area Percent
1	Fusidic Acid	16.580	7959153	99.753
2		23.577	6729	0.084
3		25.017	13010	0.163
Totals			7978892	100.000

- Fusidic acid 0 month trails #3 -
(Sample)

<u>Appendix 6.5</u>: Chromatogram of fusidic acid sample at zero time trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

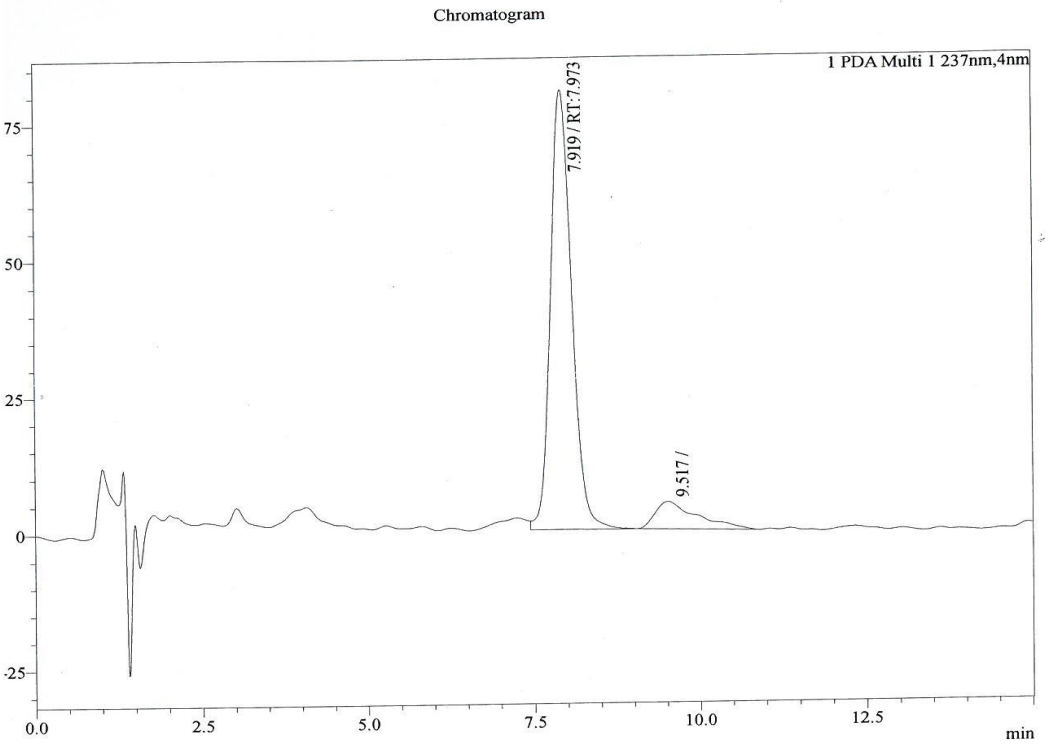


UV-WL1 235 nm
Results

Peak Number	Name	Retention Time	Area	Area Percent
1	NIPAGEN	1.247	1021276	5.645
2	HYDROCORTISONE	1.913	10156623	56.138
3		6.597	3936	0.022
4		13.347	1291	0.007
5	Fusidic Acid	16.573	6909230	38.189
Totals			18092356	100.000

– Fusidic acid 0 month trails #3 –
(standrd)

<u>Appendix 6.6</u>: Chromatogram of fusidic acid standard at zero time trails #3, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months

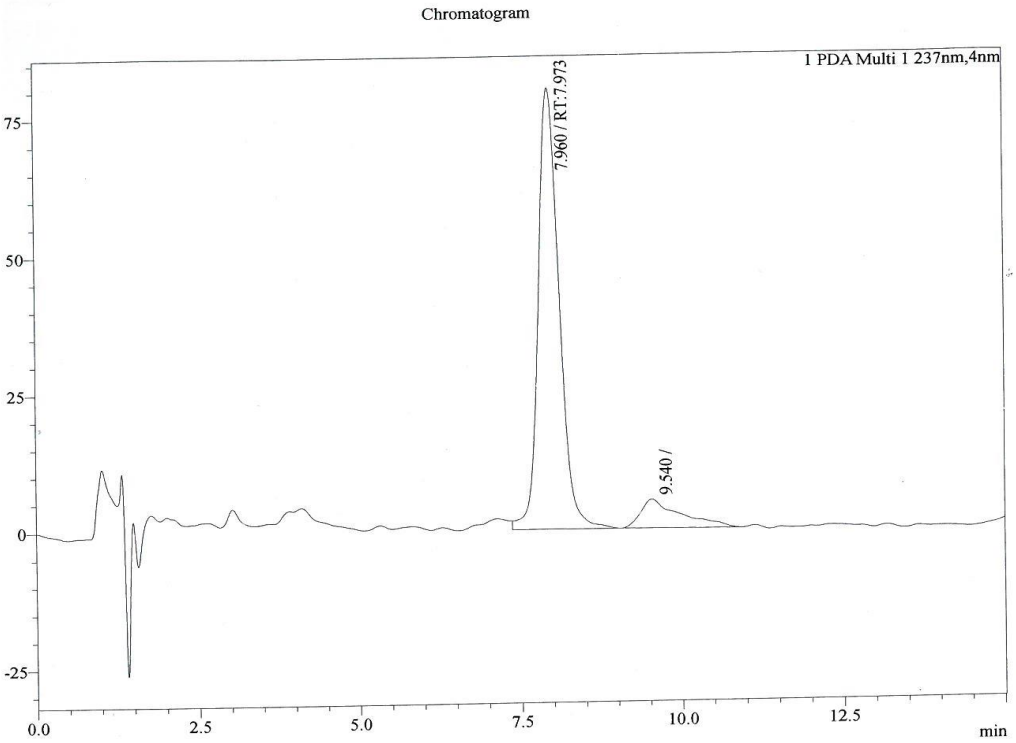


Peak Table

1 237nm						
	Ret. Time	Name	Area	Height	Area%	Peak Purity Index
1	7.919	RT:7.973	1784045	80594	89.049	Not calculated
2	9.517		219402	4988	10.951	Not calculated
tal			2003446	85582	100.000	

- oleuropine 3 month trails #2 -
(standrd)

<u>Appendix 6.7</u>: Chromatogram of oleuropine standard at three month trails #2, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months



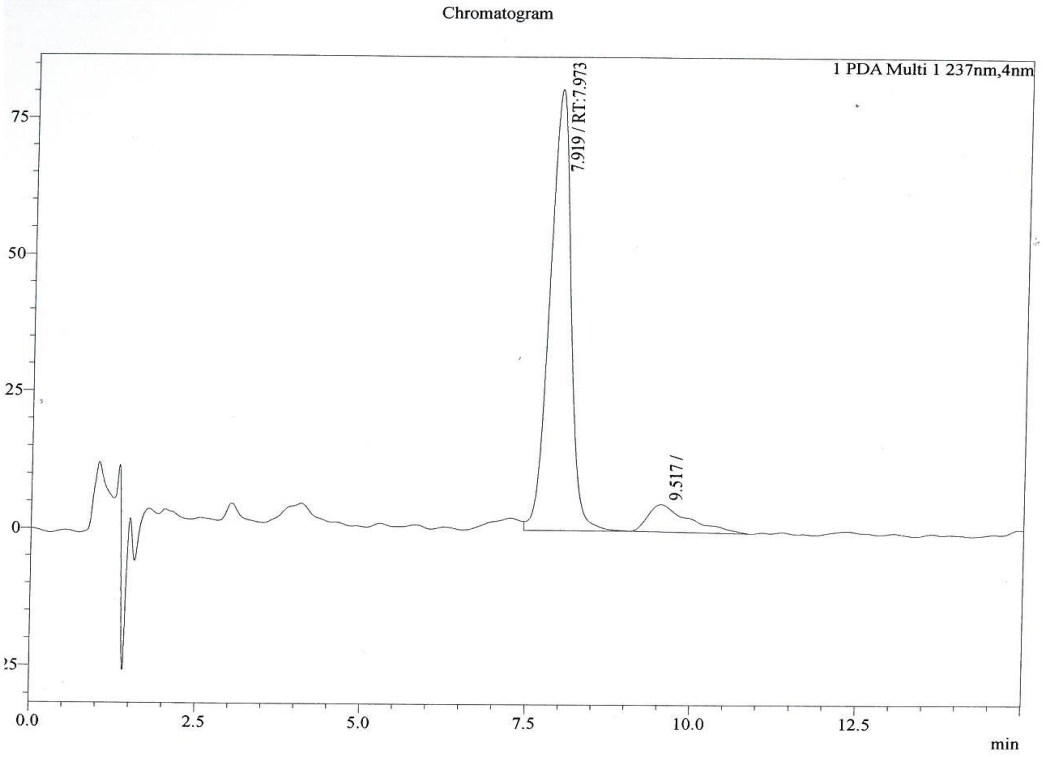
Peak Table

h1 237nm

#	Ret. Time	Name	Area	Height	Area%	Peak Purity Index
1	7.960	RT:7.973	1809260	80413	89.017	Not calculated
2	9.540		223228	5192	10.983	Not calculated
otal			2032488	85606	100.000	

- Oleuropine 3 month trails #2 -
(Sample)

<u>Appendix 6.8</u>: Chromatogram of oleuropine sample at three month trails #2, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months



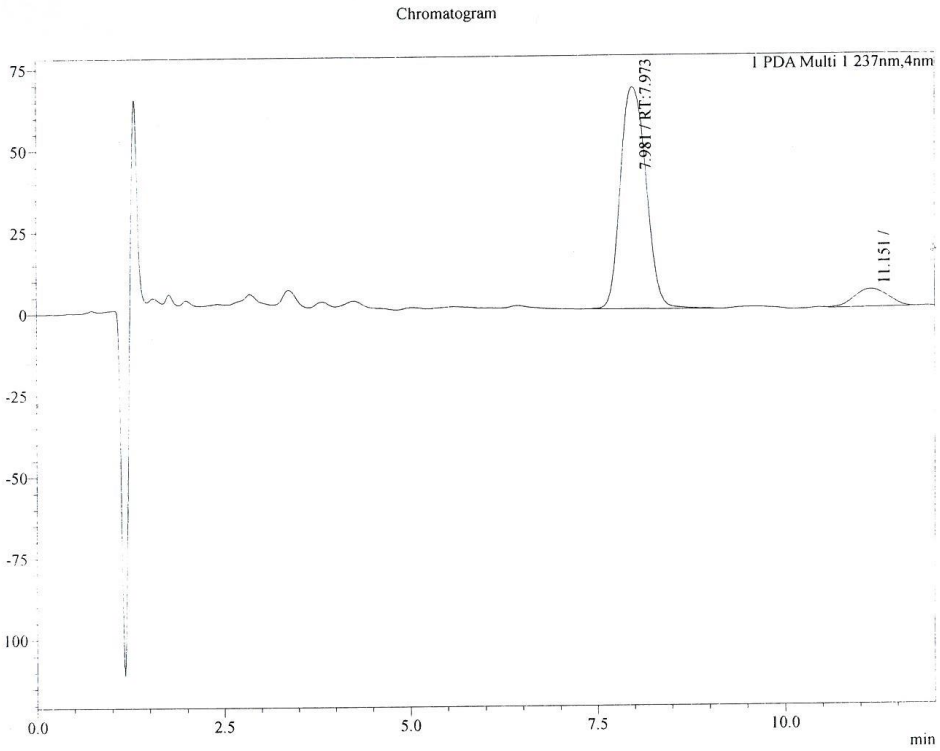
Peak Table

237nm

Ret. Time	Name	Area	Height	Area%	Peak Purity Index
7.919	RT:7.973	1784045	80594	89.049	Not calculated
9.517		219402	4988	10.951	Not calculated
		2003446	85582	100.000	

- oleuropine 1 month trails #2 -
(standrd)

<u>Appendix 6.9</u>: Chromatogram of oleuropine standard at one month trails #2, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months



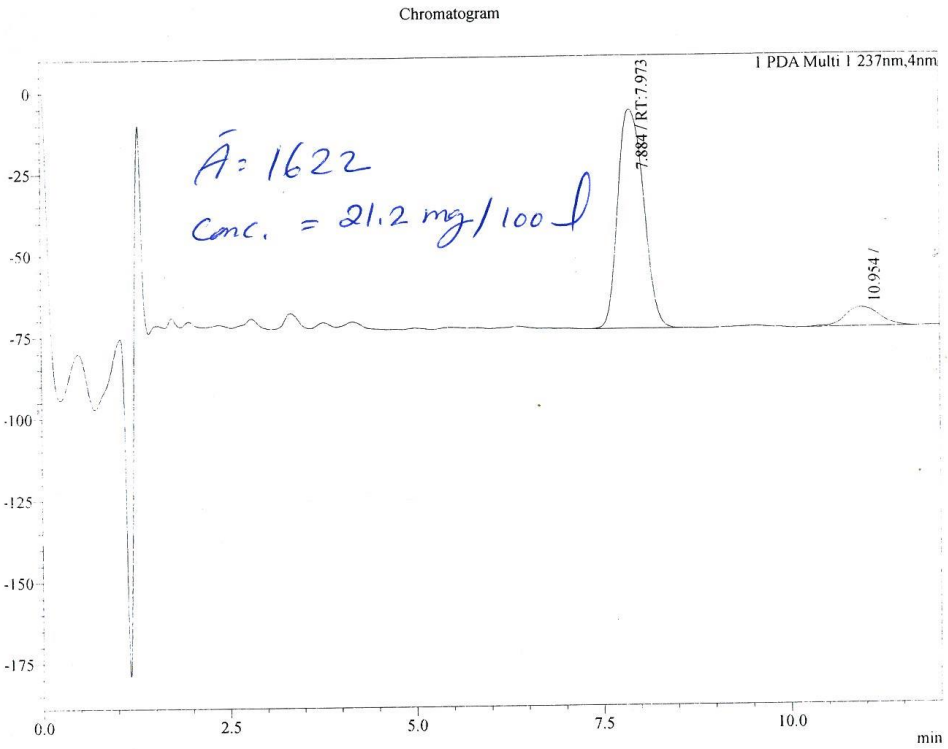
Peak Table

h1 237nm

#	Ret. Time	Name	Area	Height	Area%	Peak Purity Index
1	7.981	RT:7.973	1632725	67887	90.425	Not calculated
2	11.151		172886	5491	9.575	Not calculated
otal			1805612	73377	100.000	

- oleuropine 0 month trails # 1)
(Sample)

<u>Appendix 6.10</u>: Chromatogram of oleuropine sample at zero time trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months



Peak Table

Ch1 237nm

#	Ret. Time	Name	Area	Height	Area%	Peak Purity Index
1	7.884 RT:7.973		1610375	67090	89.552	Not calculated
2	10.954		187877	5809	10.448	Not calculated
Total			1798252	72899	100.000	

- oleuropine 0 month trails #1)
(Standrd)

<u>Appendix 6.11</u>: Chromatogram of oleuropine standard at zero time trails #1, stored at accelerated conditions (40 ±2°C / 75% ± 5% RH) for 3 months