Deanship of Graduate Studies

Al-Quds University

Preparation of semi-solid dosage forms containing Psidium guava leaves extract as antimicrobial preservative

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M.Sc. Thesis

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Dedication

To the owner of the first and last credit, to the guide to the right path, God Almighty.

To you, my mother, who taught me to give without waiting in return, who planted in my heart the highest meanings of the virtues. To that great edifice that taught me noble manners, to my father who is the owner of great credit, to my honorable professors who taught me that the teachers' encouragement to their students is a strong motivation to progress.

To my brothers who are supporting me in my life. To all brothers who have proven that brothers are not bound to blood tie only.

To everyone who supported me and gave me a push forward.

Declaration

I Certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed: .....M. Atieh.....

Mohammad Daud Abdallah Atieh

Date: 21 / 08 / 2021

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Abstract

As it is known, guava leaves extract has several medicinal characteristics, such as antimicrobial, antioxidant, anti-cancer and anti-inflammatory. Several studies have been proposed to use guava leaves extract in several preparations. However, applying guava leaves extract as a preservative in a pharmaceutical product has not been studied in previously. In the present work, we try to use a natural preservative instead of chemical (methyl and propyl paraben) since many studies were warning from their effect and that it must be used at limited concentration. Guava leaves were extracted with ethanol 95.5 % as extraction solvent. The percentage yield was 20% which was acceptable. This extract was tested by HPLC; several phenolic compounds were detected. To determine the ability to use this extraction powder as a preservative, antimicrobial effectiveness test (preservative efficacy test) was conducted for extracted powder in purified water at different concentrations (0.5, 2.5, 5, 7.5, 9.5, 10) % w/w, against three bacteria, one gram-positive (S. aureus), two gram-negative (P. aeruginosa, E. coli) and two fungi: one yeast (Candida albicans) and one mold (Aspergillus (Niger) brasiliensis). The results showed that the only concentration that passed the test was 10%; this concentration was used in semi-solid pharmaceutical products. For this purpose, six different preparations (Ketoconazole shampoo, Clotrimazole Cream, Permethrin Cream, Gentamycin Cream, Ibuprofen gel, Indomethacin emulgel) were used. Chemical preservative was replaced by natural one (guava leaves extract powder) to serve as a preservative. Only three out of all six preparations (Ketoconazole shampoo, Clotrimazole Cream, Ibuprofen gel,) passed preservative efficacy test. This passed preparations were fully checked-up by chemical (drug content,) physical (odor, physical appearance) and biological test (Direct transfer test, Total count test) for three months at accelerated condition. The results confirmed that pharmaceutical preparations were stable and effective.

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List of Abbreviations

HAc	Acetic acid
ACN	Acetonitrile
ATCC	American Type Culture Collection
A. brasiliensis	Aspergillus Brasiliensis
BHIA	Brain Heart Infusion Agar
BP	British Pharmacopoeia
SCDA	Casein Digest Agar
CFU	Colony-Forming Unit
DMSO	Dimethyl sulfoxide
D. water	Distlled Water
E. Coli	Escherichia Coli
HPLC	High performance liquid chromatography
h.	Hour
LF	Laminar Flow
MIC	Minimum Inhibitory Concentration
PET	Poly Ethylene Terephthalate
PP	Polypropylene
PTFE	Polytetrafluoroethylene
P. aeruginosa	Pseudomonas aeruginosa
P. guava	psiduim guava
SABDA	Sabouraud Dextrose Agar
SABD, SABDA	Sabouraud Dextrose Broth, Agar
SCD, SCDA	Soybean-Casein Digest Broth, Agar
SCDM /SCDA	Soybean-Casein Digest Medium /Agar
TS	Sterile Saline
TYMC	Total Aerobic Yeasts and Molds
USP	United States Pharmacopeia

Chapter One

Introduction:

1. Introduction:

1.1 Guava plant

Guava is an evergreen tree or shrub 1.83 to 7.62 m high, with wide-spreading branches and squares usually found in many tropical areas. The plant flushes in the winter season, so it can be found in the market mostly in that season.

Table 1.1: Composition of nutrients present in the leaves of Psidium guava (Thomas et al., 2017).

S.N	Nutrients	Concentration(mg/100g)
1	Amino acid	8
2	Phosphorus	360
3	Protein	16.8
4	Carbohydrate	7
5	Vitamin B	14.80
6	Vitamin C	103.0
7	Magnesium	440.0
8	Iron	13.50
9	Starch	6.3
10	Potassium	1602

The main constituents of guava leaves are phenolic compounds, isoflavonoids, Gallic acid, catechin, epicathechin, rutin, naringenin, kaempferol (Sandra et al. ,2012)

1.2 Phenolic compounds

Polyphenols have antimicrobial activity against a broad spectrum of bacteria. Among the polyphenols, flavanols, and phenolic acids possess the highest antibacterial activity, thanks to the ability to (a) inhibit bacterial virulence factors such as enzymes and toxins, (b) interact with cytoplasmic membrane (c) suppress biofilm formation and (d) exert a synergistic effect with antibiotics (Quideau et al.,2011; D'Archivio et al., 2007; and Pereira et al., 2009)

1.2.1 Gallic acid

Gallic acid is a trihydroxybenzoic acid with the formula $C_6H_2(OH)_3CO_2H$. It is classified as a phenolic acid.

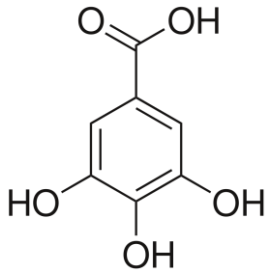


Figure 1.1: Chemical structure of Gallic acid

Gallic acid is a polyphenol natural compound found in many medicinal plant species, and it was shown to have antibacterial properties. Gallic acid was found to have a wide spectrum of antifungal activity, with minimum inhibitory concentrations for bacterial strains between 43.75 μg/mL and 83.33 μg/mL. Gallic acid is also active against three Candida strains, with minimum inhibitory concentrations between 12.5 μg/mL and 100.0 μg/mL. The most sensitive Candida species is Candida albicans (Minimum inhibitory concentrations = 12.5 μg/mL). (Jian et al., 2017)

1.2.2 Catechin

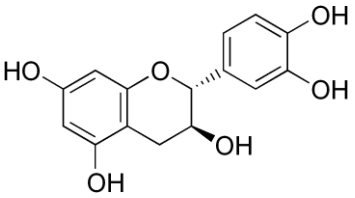


Figure 1.2: Chemical structure of Catechin

Catechin is a flavonoid (flavan-3-ol) found, for instance, in tea, some fruits like guava, and in vegetables. It has been proved that catechin has radical scavenging metal-chelating and anti-proliferative effects. Also, it was proved that catechin can inhibit HIV-1 reverse transcriptase in vitro. Many experiments showed that catechin also possessed antibacterial activity. (flavan-3-ol) possessed the ability to bind to artificial lipid bilayers. It was shown that catechin epicatechin gallate and epigallocatechin gallate could sensitize MRSA strains to β-lactam antibiotics, inclusive methicillin. It was also notified that epicatechin gallate and epigallocatechin gallate acted as a norA gene suppressor and decreased β-lactam MICs to the antibiotic breakpoint, thus improved the antimicrobial activity of those antibiotics (Miklasi et al., 2016)

1.2.3 Chlorogenic acid

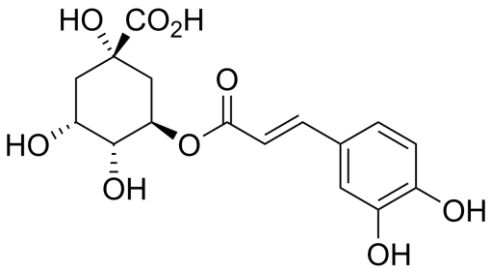


Figure 1.3: Chemical structure of Chlorogenic acid

Chlorogenic acid is a natural chemical ester that has a specific antimicrobial activity and corresponding reduction in log survival ratio. Chlorogenic acid has bactericidal effects due to the action of bioactive phenolic compounds that exert physiological changes on the microbial cell membrane, eventually resulting in cell death (Kabir et al., 2014)

Understanding the action mode of chlorogenic acid against the pathogen microorganism showed that chlorogenic acid significantly increased the outer and plasma membrane permeability, resulting in the loss of the barrier function, even inducing slight leakage of nucleotide. Chlorogenic acid bound to the outer membrane, disrupted the membrane, exhausted the intracellular potential, and released cytoplasm macromolecules, which led to cell death. It could be concluded that chlorogenic acid killed pathogenic bacteria strains by provoking irreversible permeability changes in cell membrane, causing cells to lose the ability to maintain membrane potential and cytoplasm macromolecules including nucleotide. (Lou, et al., 2011)

1.2.4 Caffeic acid

Caffeic acid (3,4-dihydroxy-cinnamic acid) is an organic compound that can be found naturally in many medicinal plants.

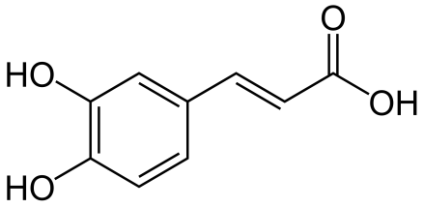


Figure 1.4: Chemical structure of Caffeic acid

Caffeic acid is a type of polyphenol that has many health benefits, including anti-bacterial, anti-inflammatory, anticancer, and antiviral abilities. (Tabassum et al., 2021)

1.3 Flavonoids

Flavonoids are a class of polyphenolic secondary metabolites found in plants with two phenyl rings - and a heterocyclic ring

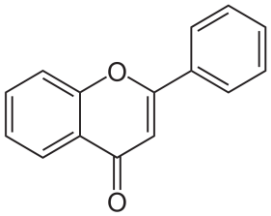


Figure 1.5: Chemical structure of flavonoids

Gauav leaves extract contains impotrant flavonoide such as quercetin and glycosides. Many of the Flavonoids have strong activity against gram-positive bacteria and have no activity or small activity against gram-negative bacteria. It was assumed that the protoplasmic membrane of the bacterial cell is the effective target of isoflavones and related compounds, and thus they showed antibacterial activity by interfering with the combination of metabolites and nutrients into bacterial cells. these compounds showed a wide domine of antibacterial activity on gram-positive bacteria, including Staphylococci, Streptococci, Actinomyces, and Lactobacillus species. Anyhow, they could also inhibited gram-negative bacteria, such as P aeruginosa, E Coli, and Klebsiella pneumonia even at slightly high concentrations, indicating that the outer membrane of gram-negative bacteria intervened with the accessibility of the compounds to the protoplasmic membrane. The second potential is that the nucleic acids in MRSA cells could beinfluenced. In addition to complete inhibition of metabolite integration of bacterial cells, the observed strong bactericidal action of these compounds beared some similarity to that induced by 4-quinolone antimicrobial agents, which prevented DNA synthesis (Tanaka et al., 2004)

1.4 Antimicrobial Activity of guava leaves extract

Bacteria are unicellular microorganisms. They were first identified by Dutchman Anton Van Leeuwenhoek, around the 1670s, after the creation of the microscope in the 16th century (Patrick, 2005). According to the most current classification status of living beings, these microorganisms are gathered in the Bacteria and Archaea domains. Among these, the most common ones belong to the Bacteria domain (University of Sao Paulo, 2015). They are among the smallest organisms in present, ranging from 0.5 to 2.0 μm in diameter, with different morphology, but generally have three basic shapes: spheres (cocci), rods (bacilli), and spirals (spirillum). In addition, they were found in groups of cells, forming arrangements that originate when dividing cells do not separate themselves from one another.

Guava leaves essential oils showed strong antimicrobial properties against P. aeruginosa, E. coli, Streptococcus faecalis, S. aureus, and Bacillus subtilis (Soliman, F., 2016). Several studies also pointed to their antioxidant and anti-proliferative activities. Qualitative analysis of aqueous and organic extracts of guava leaves tested the existence of phenolic acids, flavonoids, terpenoids, glycosides, and saponins, in which their existence was positively correlated with antimicrobial activity. Chromatographic analysis of fermented guava leaves extract confirmed the existence of gallic acid, chlorogenic acid, and rutin. These compounds have the potential to inhibiting ergosterol (fungal cell membrane component), and glucosamine (fungal cell growth indicator). Similarly, water-soluble tannins present in guava leaves extract could act as bacteriostatic agents, they proved to have an inhibitory effect on antibiotic-resistant clinical isolates of Staphylococcus aureus (Das, M., 2019). Hirudkar, in his study recognized quercetin as a main flavonoids of guava leaves with the maximum pharmacological activity (Hirudkar, J., 2020). Furthermore,

activity against bacterial and fungal pathogens was traced to triterpenoids like betulinic acid and lupeol (Ghosh, 2010). A decoction of guava leaves at various concentrations (1%, 5%, and 10%) showed an inhibitory effect on bacterial colonization and binding of bacterial enterotoxins on epithelial cells, thus altered the inflammatory response.

1.5 Anti-inflammatory Activity of guava leaves extract

Inflammation is defined as an organism's defense mechanism against foreign substances. Systemic and local inflammatory responses tend to eradicate the triggering stimulus, assist tissue repair and healing, and develop immune memory if there is an infection so as to in the future, the host mounts a prompter and targeted response (Medzhitov, R., 2016)

When cells and body tissues are injured by biological, chemical, or physical stimuli for instance, bacteria, trauma, toxins, or heat, the inflammatory response takes place. This response is considered as one of the most significant defense mechanisms. The aim of this mechanism is to remove the injurious stimuli and beginning of the healing process.

In spite of the fact that inflammation is a defense mechanism of a body against foreign substances, it also played an important role in diseases such as diabetes, asthma, cardiovascular diseases, and cancer. Several studies suggested that the anti-inflammatory activity of Psidium guava arose primarily through inhibition of PGE2, COX-2, NO, iNOS, ERK1/2, leukocyte cell migration, and suppression of edema.

Herbal remedies offered important guidance for the improvement of drugs, such as anti-inflammatory medications. Several molecular ways were used to evaluate the anti-inflammatory properties of Psidium guava. in vitro and in vivo. In addition, the Psidium guava also indicated a significantly reduced paw withdrawal latency along with exhibited

a membrane stabilization effect by inhibiting hypotonicity-induced lysis of erythrocyte membrane. (Souri et al.,2021).

1.6 Antioxidant Activity of guava leaves extract

Oxygen is considered to be an essential element for aerobes due to its action as a terminal electron acceptor during the respiration process, which is a motivation of energy production. Nevertheless, free radicals created during metabolic processes are the cause of various ailments in the human body, (Stefanis, L. ,1997). The existence of phenolic compounds, such as gallic acid, and several others, is responsible for the antioxidant role of guava leaves extract (Chen, Y., 2007) (Farag, R., 2020). HPLC analysis of guava leaves extracts showed the presence of seven main flavonoids: quercetin, hesperetin, kaempferol, quercitrin, rutin, catchin, and apigenin, while other bioactive compounds, such as kaempfertin, isoquinoline, and corilaginoline alkaloids, were also identified (Taha, T., 2019). Flavonoids compounds are the main compounds responsible for the antioxidant properties of guava leaves extract. Many studies showed the importance of antioxidant compounds from guava leaves extract in minimizing the harmful effects of free radicals. Essential oils extracted from guava leaves were found to function as moderate antioxidants (Lee, W., 2012).

1.7 Wound healing activity of guava leaves extract

The flavonoids and tannins found in Psidium guava extract was showed to be important for wound healing due to their antibacterial activities, antioxidant, and anti-inflammatory (Mulisa et al., 2015).

Skin acts as a natural barrier against the environment and exerts an assortment of essential protective functions. When skin is compromised, either by acute or chronic injuries, the

body starts a multi-step -dynamic process at the injured position, leading to incomplete healing of the tissue and restoring the skin's barrier action. The immediate purpose of wound rebuilding is to achieve tissue integrity and homeostasis (Eming et al., 2007). The natural process of wound healing contains four overlapping, but well-defined phases: hemostasis, inflammation, proliferation, and remodeling. Hemostasis occurs upon the injury, which constitutes platelet aggregation and thereby blood clot formation (Enoch et al., 2006). The blood clot provides a provisional extracellular matrix for cell migration (Epstein et al.,1999). The inflammatory phase includes the migration of blood cells, like phagocytic neutrophils and macrophages, to the wound position (Enoch et al., 2006).

Chapter Two

- **Literature review**
- **Problem**
- **Research objectives**
- **Hypothesis**

2.1 Literature review:

Cáceres *et al. (1993)* investigated guava leaf extracts using three solvents of different polarities (n-hexane, acetone, and ethanol) as the extracting-agent. They found that the ethanol extract was the most effective against the pathogenic caused by the tested bacteria.

According to Vieira R *et al*., (2001) the antimicrobial effect of guava leaf extracts was investigated using S. aureus, and E. coli strains. Three solvents were used as extractants, namely, distilled water, acetone, and ethanol. Acetone and ethanol were tested at four different concentrations (20%, 50%, 60%, and 80%). Guava sprout extracts at all studied concentrations showed inhibitory effects on the growth of E. coli and Staphylococcus aureus strains as revealed by the Plate count agar. These results were expected because of the presence of halos ranging from 15 to 32 mm for the E. coli and from 15 to 30 mm for the S. aureus. It should be noted that guava leaves extracts that were obtained with 60% alcohol and 60% acetone produced massive halos in both species of bacteria.

Nwinyi *et al*., (2008) investigated the antibacterial activity of Psidium guava. Fresh leaves of P. guava were dried for 5 hours in an oven at 60^{o}C and then milled to powder. Two solvents were used for the preparation of the extracts, namely 60% (v/v) ethanol, and

distilled water. 250 g of the dried powdered leaves of P. guava was extracted with 200 ml of distilled. water for 3 h using the reflux method. The solution was filtered using Whatman No.1 filter paper and the supernatant was then concentrated to 4.0 ml using vacuum rotatory evaporator. The prepared extracts were kept at 4^{o}C in a refrigerator for at least 24 hours before the subsequent tests. With Ethanol, the extracts were prepared by weighing out 250 g of the dried powdered leaves of Psidium guava and extracted with 200 mL 60% ethanol for 5 h using a Soxhlet extractor. The extracts were concentrated by fractional distillation. The microbial effects of both aqueous and ethanolic extracts were studied at different inhibitory concentrations. The minimum inhibitory concentrations were higher for the aqueous extracts compared to the ethanolic extracts. It should be noted that higher concentrations of the both extracts were needed to inhibit Escherichia coli compared to those required to inhibit S. aureus. The minimum inhibitory concentration of the aqueous extraction of P. guava was 5.0 mg/ ml for E. coli and 2.5 mg/ ml for Staphylococcus aureus, whereas the minimum inhibitory concentrations for the Ethanolic extract were 1.25 mg/ ml for E. coli, and 0.625 mg /ml for Staphylococcus aureus. This study showed that Ethanolic extraction of the Psidium guava offers greater inhibitory effects on the organisms. This was attributable to the potency of ethanol to extract essential oils (terpenoids, e. g limonene and globulol) that are inhibitory to the organisms. Many studies were conducted to identify the essential oil present in guava leaves extract and investigated its activity against microorganism; among these studies Arian et al., (2019) in their research "Essential Oil from Psidium guajava Leaves: An Excellent Source of β-Caryophyllene", and Rogers et al., (2013) in their research "Antimicrobial Activities of Leaf Extracts of Guava (Psidium guajava L.) on Two Gram-Negative and Gram-Positive Bacteria".

Priya *et al.,* (2010) investigated the antimicrobial potential of essential oil, and solvent-

based extracts, extracted from Psidium guava leaves. They were screened against the selected Gram-positive and Gram-Negative bacterial strains as well as fungal strains. 20 grams of dried powered Leaves of Psidium guava were used for different solvent extraction such as methanol, acetone and hexane. The percentage yield of the extracts were 11.6% for acetone, 13.6% for methanol and 6.7% for hexane. These extracts and essential oil (terpenoids) were investigated against 5 Gram-positive (Bacillus cereus, Bacillus subtilis, Lactobacillus lactis, Staphylococcus aureus) 5 Gram-negative bacteria (Azotobactor species, Agrobacterium rhizogene, Enterobactor aerogenes, Glucanobactor oxydans, Pseudomonas fluorescens, and 3 fungal microbes (Candida tropicalis, Aspergillus (Niger), Aspergillus aculeatus). The methanolic extract of P. guava leaf was active against both Gram-negative bacteria and Gram-positive, indicating the broad-spectrum of its antibiotic activity. These extracts also prevented bacterial growth (for examples, P. fluorescens, Bacillus subtilis, Agrobacterium rhizogene, and Klebsiella aerogenes). All of the three solvent-based extracts of Psidium guava showed good antimicrobial activity. The acetone extract was highly active against gram-positive as well as fungal strains, whereas all of the extracts were even with respect to what? the weak antibacterial activity against Gram-negative bacteria could be attributed to the existence of an outer membrane active against gram-negative strains. The complex composition of essential oils (terpenoids) present a variety of pharmacological resources and a great chance for the development of new drugs.

Dhiman *et al*., (2011) examined the chemical composition, and the antimicrobial potential of methanolic extract of Psidium guava (using in vitro approach). The leaves were dried at room temperature for 2 weeks then milled to a coarse powder using a homogenizer for simple extraction of active compounds. The powdered material (100 g) was loaded into a soxhlet apparatus and extracted for up to 4 hours with 300 mL petroleum ether (60–80 °C)

to remove the fat content. The residue was then extracted with methanol for an additional 4 hours. The extract was filtered, and then the solvent was evaporated by a rotary vacuum evaporator to 3.0 mL. A stock solution of the extract was prepared by dissolving 0.01 g of plant extract in 10 ml DMSO; from this stock solution, the working solutions were prepared at concentrations 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/ml for the determination of MIC. For the antimicrobial assay, concentrations used ranged from 1 μg/ml to 20 μg/ml. The test against bacterial strains (Bacillus subtilis, Escherichia coli, and Staphylococcus aureus), and fungal strains (Candida albicans and Aspergillus (Niger)) showed good antibacterial activity of the extract against both gram-positive and gram-negative species. The result of this antibacterial activity test might indicate a broad spectrum of the antibacterial activity of the extract. Such findings are encouraging towards using these extracts as a source of antibiotic substances in drug developments that aims to control bacterial infections.

Porwal *et al*., (2012) reported several other techniques used in the extraction of biologically active compounds from Guava Leaves. Among them are:

- Ultrasound extraction
- Supercritical fluid extraction
- Soxhlet extraction
- Steam distillation

To get good Antibacterial properties, Pelegrini (2008), Amit (2011), and Ryu (2012), suggested that extraction should be done using methanol, ethanol, ethyl acetate, and water. The antibacterial results of guava leave extracts extracted by these for different extractants are shown in Table 2.1 below.

Table 2.1: Inhibition against bacteria exhibits by methanolic extract as compared to other extracts (Porwal et al., 2012)

Extracts	Zone of inhibition (mm)
Aqueous extract	8-16nm
Ethanolic extract	14-21nm
Methanolic extract	27-30nm
Ethyl acetate	20-25nm

Mushtaq *et al.*, (2014) extracted guava leaves with different solvents. 23 important microorganisms were used in the antimicrobial tests. Acetone extract was highly active against fungal strains, while it showed moderate activity against the gram-negative bacterial.

Jayachandran *et al.*, (2018) studied Total Phenolic Compounds, Flavonoid Content, Phenolic Acids Composition, Vitamin-C and Vitamin-E present in guava leaf extracts extracted by different solvent systems including methanol/water (1: 1, v/v), ethanol/water (1: 1, v/v), absolute ethanol (95%), methanol, and water. The extracts obtained from guava leaf were tested for their total flavonoid and phenolic contents. The percentage yields of guava leaf extracts were as follows: 5.1% for methanol/water (1: 1, v/v), 5.5% for ethanol/water (1: 1, v/v), 4.6% for ethanol, 4.1% for methanol and 4.4% for water. The percentage yields of guava leaf extracts were calculated on the dry weight basis. Maximum total flavonoid content (65.12 mg/g) was found in ethanol-alone extract, while the maximum total phenolic content (174.56 mg/g) was obtained by the ethanol/water (1: 1, v/v) extracting agent.

Ekom and Tamokou (2018) reported that the extract of Psidium guava leaves showed a significant increase in the percentage of wound closure at the infected site and had an important antibacterial activity against Staphylococcus aureus. Indeed, during the proliferative phase of wound healing, the wound contraction improved the antibreccial activity by draging the edges of the wound towards the center. The influence of Psidium guava extract on wound contraction indicated that the extract has a healing action because wound contractions were considered as 88% of the healing process.

2.2 Problems:

In recent years, pharmaceutical research went through a transition that made a greater use of natural products, especially in all skin treatment products. To our knowledge, not many natural ingredients are incorporated as preservatives in a pharmaceutical product. Therefore, natural ingredients that could be extracted from, Psidium guava leaves (which contains a large amount of antimicrobial and antioxidants) might exhibit significant health benefits. Psidium guava is known for its antispasmodic activity, antioxidant properties, and anti-inflammatory functions that could help to treat antimicrobial infections. Natural compounds have interesting properties. Using natural compounds as preservatives in pharmaceutical formulations might provide solution to several problems that are normally encountered upon using conventional chemical ingredients as preservatives. Therefore, it is crucial to examine the potential applications of natural ingredients such as those present in guava leaves in pharmaceutical formulations, mainly as preservatives alternative to conventional of chemical ingredients.

2.3 Objectives:

- To extract guava leaves using ethanol (95.5 %).

- To examine the antimicrobial activity of guava leaves extract.
- To determine the minimum concentration of guava leaves extract that could be used as a preservative in the formulations of a number of pharmaceutical products.
- To study the feasibility and effectiveness of using natural products extracted from guava leaves in the formulations of medical shampoo, cream, and gel- as preservatives alternative to conventional chemical ingredients.

2.4 Questions:

- How could we use guava leave extract in pharmaceutical preparations (shampoo, gel and cream)?
- Would Psidium guava leaves extract keep its desired properties after formulation?
- Would Psidium guava leaves extract act as a preservative in these formulations?
- What is the minimum concentration of guava leaves extract that could be used as a preservative?
- Would guava leaves extract act as a preservative more effectively in one formulation of a certain product than other ones?

2.5 Hypothesis:

Psidium guava leaves extraction could be formulated in semi-solid dosage forms as a preservative / ingredient in topical drugs since it has intrinsic medical properties (antimicrobial, anti-inflammatory, anti-oxidant and wound healing). Chemical preservatives could be replaced by natural “antimicrobial” ones extracted from guava leaves.

Chapter Three

Instrumentation and Methodology

3. Materials and methods.

After extracting Guava leaves, they were examined as a pure raw material by preservative efficacy test at different concentration to determine the minimum concertation that could act as a preservative. Six types of semi-solid dosage forms (one shampoo, two gel, and three creams) were prepared with a natural preservative, chemical preservative (methyl paraben and propyl paraben), and with no preservative. Antimicrobial activity was estimated for the six prepared products. A full comparison was carried out among the passed pharmaceutical formulations (i.e., shampoo, gel, and cream) to evaluate the activity of guava leaves extracts as natural preservatives.

3.1 Materials and reagents :

Materials and reagents used in this study are described in Tables (3.1) and (3.2)

All materials used in the formulation of pharmaceutical preparations were of pharmaceutical reagents grade.

Table 3.1: List of materials used in the preparation of the six pharmaceutical formulations.

No.		No.	
1	Psidium guava leaves extract	17	Anhydrous Lanoline
2	Coconut oil	18	Permethrin
3	Texapon	19	Propylene Glycol
4	Loramide	20	Isopropyl Alcohol
5	PEG 6000	21	Isopropyl Myristate
6	Sodium Chloride Sigma	22	Sepigel
7	Nipagin M (methyl paraben)	23	Ibuprofen
8	Nipasol M (propyl p[paraben)	24	Cetostearyl alcohol
9	blue color	25	Emulgin B2
10	Kiwi mix	26	White Petrolatum
11	Cremophor (RH 40)	27	Paraffin Oil (9)
12	Sodium hydroxide (0.1 N)	28	Glycerin
13	Purified water	29	Clotrimazole
14	Ketoconazole	30	Indomethacin
15	Ibuprofen	31	Ethanol
16	Gentamycin	32	sepineo p600

These materials and reagents were purchased from trusted sources or donated by Beit Jala Pharmaceutical Co, Ltd/Bethlehem - Palestine.

Table 3.2: List of materials used for extraction, formulation, microbiological, Chemical - physical analysis, and stability testing.

No		No	
1	Psidium guava leaves	12	Letheen Broth, Letheen Agar
2	Magnesium ribbon	13	Broth media
3	Distilled water	14	Sterile saline
4	Acetic acid	15	Iron |(III) Chloride $FeCl_3$
5	Ethanol	16	Fluid Lactose Medium
6	Methanol	17	Phosphate buffer stock solution
7	Ethyl acetate	18	Chloroform
8	concentrated sulfuric acid	19	Concentrated hydrochloric acid
9	Soybean-Casein Digest Medium	20	Tween 80
10	Soybean-Casein Digest Broth	20	PET bottles, PP Caps
11	Casein Digest- soy Lecithin	21	Aluminum tubes (19mm, 22mm)

3.2 Equipment and Tools

Tools and equipment used for the extraction process, formulation of preparation, chemical- physical-microbiological, and stability testing are listed in Table (3.3).

All Beaker, Test tubes, Syringes, vials, pipettes, and cylinders were supplied by Beit Jala Pharmaceuticals Company.

Table 3.3: list of Tools and Equipment used in the study.

No.	Equipment and Tools
1	Sonication Device
2	Thermometer
3	pH meter
4	Filters: Whatman No.1 filter
5	HPLC Apparatus
6	Viscometer -Brookfield - De 28505
7	Extraction Tools
8	RADWAG PS.6000R2 Balance
9	Plates
10	Sterile petri dishes (diameter of 9 cm)
11	Manual blender(media)
12	Vortex
13	Laminar Flow Cabinet
14	Rotary evaporator

3.3 Sample Collection and preparation

The guava leaves samples were collected from a farm at Qalqilya City, Palestine. The leaves were washed with tap water and were dried at room temperature (25 ºC) for 30 days in a dry and airy environment. The dried samples were milled and sieved. The selected particle sizes for extractions were from 0.1 to 0.6 mm. The samples were packaged in plastic bags and kept in a dark and dry place until extraction.

Figure 3.1: green guava leaves before drying

3.4 Extraction of Psidium guava leaves using Sonication

Extractions with the Sonication method were performed using absolute (95%) ethanol as the extracting agent. 1500 g of leaves powder were mixed with 9000 mL of 95% ethanol the mixture was then sonicated for 3 hours at room temperature. Subsequently, the solvent was evaporated by a rotary evaporator, and the extraction yield was calculated. The recovered extracts were stored in a domestic freezer until needed for further analysis.

3.5 Preparation of semi-solid dosage forms containing Psidium guava leaves extract:

3.5.1 Preparation of Ketoconazole shampoo

Label claim: Ketoconazole 20 mg/g

Table 3.4: Illustration the formula for Ketoconazole shampoo with function for each component.

Item	**Percentage**	**Material Function**
Ketoconazole (API)	2%	Antifungal
Coconut oil	1.4%	Emulsifying agent
Texapon	20%	Surfactant
Loramide	3%	Foam booster
PEG 6000	3%	Solvent
Sodium Chloride Sigma	2%	Thickening agent
Psidium guava leaves extract	10%	Antimicrobial Preservative
blue color	0.00025%	Coloring agent
Kiwi mix	0.033%	Perfuming Agent
Cremophor (RH 40)	1%	Emulsifying agent
Sodium hydroxide (0.1 N)	0.25%	pH adjusting agent
Purified water	57.32%	Solvent

Preparation method

1. 40 % of purified water was transferred to vessel No. 1 and heated to 100 ℃

2. Cremophor RH 40 was added to vessel No. 1 and mixed for about 5 minutes using the manual blender

3. Texapon was added to vessel No. 1 with continuous mixing for 5 minutes using the manual blender

4. Loramide was added to mixing vessel No. 1 and mixed for 2 minutes using the manual blender

5. PEG 6000 was added to vessel No. 1 and mixed for 2 minutes using the manual blender

6. (8 %) of water was transferred to the mixing vessel No. 2, then Heated to about 80 ºC

7. Coconut oil was added to purified water in vessel No. 2 and mixed till dissolving

8. Coconut oil in step 7 was added to the mixing vessel No. 1 and mixed for 2 minutes using the manual blender

9. 35 % of purified water was transferred to vessel No. 4

10. Guava leaves powder was added to vessel No. 4 and mixed for 10 minutes using the manual blender, filtered the solution, then transferred to vessel No. 1 and mixed for 3 minutes using the manual blender

11. Ketoconazole was added to vessel No. 1 and mixed for 3 minutes using the manual blender

12. Sodium hydroxide (0.1 N) was added to vessel No. 1 and mixed for 2 minutes

13. (16.8 %) of purified water was transferred to vessel No. 3, then sodium chloride was added to it and mixed till dissolve.

14. Blue color was dissolved in about (0.2 %) of purified water, then was added it to vessel No. 3 and mixed for 2 min

15. The mixture in vessel No. 3 was added to vessel No. 1 and mixed for 3 minutes using the manual blender

16. The perfume was transferred to mixing vessel No. 1 and mixed for 10 minutes

3.5.2 Preparation of Ibuprofen gel

Label claim: Ibuprofen 5.0% w/w.

Table 3.5: Illustration the formula for Ibuprofen gel with function for each component.

Item	**Weight/100 g**	**Material Function**
Ibuprofen (API)	5 g	Nonsteroidal- Anti-inflammatory
Propylene Glycol	20 g	Solvent
Isopropyl Alcohol	23 g	Solvent
Isopropyl Myristate	1 g	Vehicle
Psidium guava leaves extract	10 g	Antimicrobial Preservative
Sepigel	3.0 g	Viscosity increasing agent
Purified Water	38 g	Solvent

Preparation method

1. Propylene Glycol was transferred to mixing vessel No. 1.

2. Isopropyl Alcohol was transferred to the mixing vessel No. 2.

3. Purified water was transferred to vessel No. 3.

4. Guava leaves powder was added to vessel No. 3 and mixed for 10 minutes using the manual blender, filtered the solution, then transferred to vessel No. 4 and mixed for 3 minutes using the manual blender.

5. Ibuprofen was added to vessel No. 2 and mixed for 5 minutes using the manual blender

6. The mixture in vessel No. 2 was slowly added to the Propylene Glycol in vessel No. 1 and mixed for 5 min using the manual blender.

7. Solution was transferred from vessel No. 4 to vessel No. 1 with continuous mixing.

8. Sepigel was transferred to vessel No. 1 with continuous mixing for 5 min.

9. Isopropyl Myristate was transferred to vessel No. 1 with continuous mixing for 2 min.

10. The preparation was mixed for 5 min using the manual blender.

3.5.3 Preparation of Clotrimazole Cream

Label claim: Clotrimazole 1%

Table 3.6: Illustration the formula for Clotrimazole Cream with function for each component.

Item	**Weight/100 g**	**Material Function**
Clotrimazole	2.00 g	Antifungal agent for intra vaginal application
Cetostearyl alcohol	8.80 g	Emulsifying agent
Emulgin B2	1.69 g	Emulsifying agent
White Petrolatum	13.00 g	Emollient
Paraffin Oil (9)	10.5 g	Emulsifying agent
Psidium guava leaves extract	10 g	Antimicrobial Preservative
Glycerin	4.31 g	Emollient
Purified Water	49.7 g	Solvent

Preparation method

1. White Petrolatum, Crystal oil 9, Lanette O and Emulgin B2 were transferred to mixing vessel No. 1, then heated to 80-85 ºC (oily phase).

2. Purified water was transferred to Vessel No. 2, then heated to a temperature 85 ºC.

3. guava leaves powder was added to vessel No. 2 and mixed for 10 minutes using the manual blender, filtered the solution, and then transferred to vessel No. 3.

4. Clotrimazole was added to the oil phase in vessel No.1, and mixed for 10 minutes using manual blender

5. Glycerin was added to Vessel No. 3, and mixed for 2 min. (aqueous phase)

6. Aqueous phase was transferred from No. 3 to the mixing vessel No. 1 with mixing

7. Vessel No. 1 was cooled at 35 ºC with continuous mixing.

3.5.4 Preparation of Permethrin Cream

Label claim: Permethrin 5% w/w.

Table 3.7: Illustration the formula for Permethrin Cream with function for each component

Item	**Weight/ 100 g**	**Material Function**
Permethrin (API)	5.00 g	Scabicide
Cetostearyl alcohol	6.49 g	Emulsifying
Emulgin B2	1.07 g	Emulsifying
Anhydrous Lanoline	1.80 g	Emulsifying
Topical light mineral oil (Crystal Oil 9)	4.49 g	Oleaginous Vehicle
Glycerin	8.95 g	Emollient
Psidium guava leaves extract	10 g	Antimicrobial preservative
Purified water	62.2 g	Solvent

Preparation method

1. Lanette O, Emulgin B2, and Lanoline anhydrous were transferred to vessel No. 1, then heated to 80-85 ºC (oily phase).

2. Crystal oil 9 was separately heated to about 50 ºC by using vessel No. 2.

3. Permethrin was added to the Crystal oil 9 and mixed for about 2 minutes until dissolved using the manual blender.

4. The (Permethrin / Crystal oil 9) mixture was transferred to vessel No. 1, and mixed for 5 minutes.

5. Purified water was separately heated to about 80-85 ºC, using mixing vessel No. 3.

6. guava leaves powder was added to vessel No. 3 and mixed for 10 minutes using the manual blender, filtered the solution, then transferred it to vessel No. 4 and mixed for 3 minutes using the manual blender.

7. Glycerin was added to vessel No. 4 and mixed for 3 minutes using the manual blender

8. Aqueous phase was added gradually from mixing vessel No. 4 to the mixture in vessel No. 1 and mixed for 5 minutes.

9. The cooling was operated with continuous mixing until temperature reached 35 ºC.

3.5.5 Preparation of Gentamycin Cream

Label claim: Gentamycin (as Sulfate) 0.1%

Table 3.8: Illustrates the formula for Gentamycin Cream with function for each component.

Item	Percentage	Material Function
Gentamycin (as Sulfate) (API)	0.1%	Aminoglycoside antibiotic
Lanette O	6.79%	Emulsifying agent
Emulgin B2	1.7%	Emulsifying agent
White Petrolatum	14.1%	Oil Phase
Crystal Oil 9	7.9%	Oil Phase
Psidium guava leaves extract	10%	Antimicrobial preservative
Purified water	59.41%	Solvent

Preparation method

1. Lanette O, Emulgin B2, White Petrolatum, and Crystal Oil 9 were transferred to vessel No. 1, then heat to 80-85 ºC (oily phase).

2. 5% from the total amount of purified water was transferred to vessel No. 2 and heated to 45 ºC.

3. Gentamycin was added to vessel No. 2, then mix for about 2 minutes until dissolved using the manual blender.

4. The purified water was transferred to vessel No. 3, and heated the purified water to about 80-85.

5. Guava leaves powder was added to vessel No. 3 and mixed for 10 minutes using the manual blender, filter the solution, then transferred it to vessel No. 4 and mixed for 3 minutes using the manual blender.

6. Aqueous phase was added from mixing vessel No. 4 to the mixture in vessel No. 1 and mixed for 5 minutes.

7. The cooling was operated with continued mixed until temperature was reached 45 ºC

8.The gentamycin mixture was transferred from vessel No. 2 to vessel No. 1, and mixed for 5 minutes using the manual blender.

9. The cooling was operated with continuous mixing until temperature reached 35 ºC.

3.5.6 Preparation of Indomethacin emulgel

Label claim: Indomethacin 1% w/w.

Table 3.9: Illustrates the formula for Indomethacin emulgel with function for each component.

Item	Weight/ 100 g	Material Function
Indomethacin (API)	1.0 g	Anti-inflammatory, Analgesic
Glycerin	1.0 g	Emollient
Sepineo P600	3.0 g	Gelling Agent
Ethanol	2.0 g	Solvent
Psidium guava leaves extract	10 g	Antimicrobial Preservative
Purified Water	83 g	Solvent

Preparation method

1. 50 % of purified was transferred water to vessel No. 1.

2. Indomethacin was added to vessel No. 1, and mixed for 10 minutes using the manual blender.

3. 50 % of purified was transferred water to vessel No. 2.

4. guava leaves powder was added to vessel No. 2 and mixed for 10 minutes using the manual blender, the solution was filtered, then transferred it to vessel No. 1 and mixed for 3 minutes using the manual blender.

5. Ethanol was added to vessel No. 1 and mixed for 3 minutes using the manual blender

6. Glycerin was added to vessel No. 1 and mixed for 2 minutes.

7. Sepineo P600 was added to vessel No. 1 and mixed for 5 minutes using the manual blender.

3.6 Chemical and physical analytical Test Methods

3.6.1 Determination of Extracted powder

3.6.1.1 Determination by HPLC

The Liquid chromatographic separation was performed at 40 degrees Celsius with analytical solvents consisting of methyl alcohol HPLC grade (A) and purified water (B) containing1.0% acetic acid (v/v). HPLC method was used with Purospher ® RP-18 Endcapped (5 µm), length 250 mm. Chromatography analysis was carried out for 45 minutes at a continuous flow-rate of 1.0 mL /min. The gradient program was as follows: 0–10 min, 100% B; 10–20 min, 70% B; 20–30 min, 10% B; 30–37 min, 70%B, and 37–45 min, 100% B. The UV chromatograms were acquired at 280 nm.

To prepare the HPLC sample 0.6 g of extracted powder was taken and transferred to a volumetric flask, 90 ml of ethanol was added and shaken for 30 min by using magnetic stirrer at 50 rpm then purified water was added up to volume, mixed and filtered through PTFE syringe filter (0.45 µm) before HPLC and spectroscopic determinations.

Identification and qualitative analyses were performed by comparing with standard spectra at each retention time. The compounds tested at 280 nm wavelengths were gallic acid, catechin, chlorogenic acid, caffeic acid, and, rutin hydrate.

3.6.1.2 Test for Phenols and Tannins.

Guava leaves extract was mixed with two mL of two percent solution of Iron (III) chloride. A black coloration indicating the presence of phenols and tannins.

3.6.1.3 Test for Terpenoids

Guava leaves extract was mixed with two mL of chloroform. Then two ml of concentrated H_2SO4 was added carefully and shaken gently. A reddish-brown coloration at interphase was formed indicating the presence of terpenoids.

3.6.1.4 Test for Flavonoids

Guava leaves extract was mixed with magnesium ribbon fragments and concentrated HCl was added dropwise. Red coloration indicating the presence of flavonoids.

3.6.1.5 Test for Glycoside.

Guava leaves Extract was mixed with two ml of glacial acetic acid (CH_3COOH) containing two drops of two percent Iron (III) chloride. The mixture was poured into another tube containing two mL of concentrated sulfuric acid (H_2SO_4). A brown ring at the interphase indicating the presence of glycosides.

3.6.2 Test for Ketoconazole Shampoo

For determination of Ketoconazole in shampoo formula

Chromatographic Conditions:

Mobile Phase: 35% ACN / 65%, buffer (5.7 (NH_4) H_2PO_4 / 1L H_2O)

Column: Rp-8, 125* 4 mm, 5 µm

Flow Rate: 1ml/min.

Wavelength: 240nm

Injection Volume: 20µl

Diluent: 25% Purified Water / 75% Ethanol

Retention Time: ~ 12 min.

Identification of Ketoconazole was based on retention times in comparison with the standard of Ketoconazole. The concentration of Ketoconazole was calculated using peak area and the calibration curves obtained from the standard solution. The amount of Ketoconazole was expressed as a percentage (Assay for Ketoconazole (0.1 g/5 g) 90.0 – 110.0%)

3.6.3 Test for Ibuprofen Gel

For determination of Ibuprofen in gel formula

HPLC Conditions:

Mobile phase: 62 % of 1% HAC: 38 % of Acetonitrile

Wavelength = 254 nm

Column: Lichrosphere RP-Select B, 125x4 mm, 5 um

Flow rate = 1.5 ml/min

Injection volume: 20 µl

Retention Time: 6.79-7.70 min

Diluent: 80% absolute ethanol (96%) / 20% purified water Identification of Ibuprofen was based on retention times in comparison with standard of Ibuprofen**.**

The concentration of Ibuprofen was calculated using peak area and the calibration curves obtained from standard solution. The amount of Ibuprofen expressed as a percentage (Assay for Ibuprofen (5%) 90.0 – 110.0%).

3.6.4 Test for Clotrimazole Cream

For determination of Clotrimazole in cream formula

Chromatographic Condition

Mobile Phase: 80% methanol/ 20% buffer (4.35 g of dibasic potassium phosphate in water to make 1000 mL of solution)

Column: 25cm*4.6mm, Rp 18-e (5µm)

Pump: Isocratic.

Flow Rate: 1.2 ml/min.

Wavelength: 254 nm

Injection Volume: 20µl

Retention Time: About 11.8 min.

Diluent: Ethanol (96%)

Identification of Clotrimazole was based on retention times in comparison with standard of Clotrimazole. The concentration of Clotrimazole was calculated using peak area and the calibration curves obtained from standard solution. The amount of Clotrimazole expressed as a percentage (Assay for Clotrimazole (1%) 95.0 – 105.0%)

3.6.5 Test for Permethrin 5% Cream

For determination of permethrin in cream formula

HPLC condition:

Wavelength : 240 nm

Column : Lichrospher, Rp – 18e , 125 X 4 mm , 5 µm

Flow rate : 1.5 ml / min

Mobile phase: 83% MeOH : 17% buffer(0.5% HAc/ 1000 mL purified water)

Injection volume : 20 µL

Inject for 30 minutes the (blank, standard, each sample solution) for assay calculation.

Active ingredient retention time:

Permethrin (Z) RT= 6 - 8 minutes

Permethrin (E) RT= 9 - 11 minutes

the % assay for Permethrin in the sample using the following equation:

$$\% \text{ Assay} = \frac{A_s \times C_{st}}{A_{st} \times C_s} \times 100\%$$

Where:

A_s =The mean response of Permethrin peaks obtained from sample solution (for two E and Z isomers).

A_{st} =The mean response of Permethrin peaks obtained from standard solutions (for two E and Z isomers).

C_{st} = Concentration of standard solution

C_s = Concentration of sample solution

Limits: for Permethrin: 90.0% - 110.0%

3.6.6 Test for Gentamycin cream

Assay for Gentamycin sulfate (Biologically) according below procedure

1. Procedure:

1.1 Preparations:

A. Preparation of Standard Stock Solution

Assay for Gentamycin sulfate (Biologically) according the procedure described below:

Limits: 90.0 – 135.0 %

1. Procedure:

1.1 Preparations:

A. Preparation of Standard Stock Solution

1. 12.5-100 mg was dissolved quantity of USP Reference Standard (RS) or working Standard of the tested antibiotic (accurately weighed) to prepare a stock solution in a certain concentration

2. a proper dilution was made in a solvent specified, to obtained the target concentrations Dilute to the required concentration.

3. The preparation stored in a refrigerator (where applicable) and use within the period indicated according to the validation method recommendations for each antibiotic

On the day of the assay: using the stock solution,

Prepared from the standard stock solution, three standard target concentrations

Standard low = lowest concentration

St. Median = median dose

St. High = highest concentrati**on**

B. Preparation of Sample

1. An assumed potency was assigned to the unknown sample per unit weight or volume, depending on the declared potency by the manufacturer

2. On this assumption, on the day of the assay: A stock solution and a test solution were prepared as specified for each antibiotic using the same final diluent as used for standard.

3. From a sample stock solution, three test sample target concentrations were prepared as well as standard concentrations:

Test low = lowest concentration

Test median = median dose

Test high = highest concentrati**on**

2. Microorganisms and inoculum:

A. Test Organism:

The test organism and the method of assay were given in table (3.10). A culture on slants of the medium was Maintained and under the incubation conditions specified in table (3.10) and transferred weekly to fresh slants.

B. Preparation of the Inoculum's:

1. 1 loopful of frozen culture (at – 80 ℃ or – 25 ℃) was taken and transferred to 2 ml SCDM or BHIM., mix gently on vortex

2. 1 ml from the SCDM or BHIM was pipetted and inoculate on SCDA or BHIA plate or one loopful from slant culture and stroke on SCDA or BHIA plate

3. The growth was removed from a recently grown slant or culture of the organism with 1 mL of sterile saline TS and sterile glass beads.

4. The removed growth was inoculated by streaking on a surface of BHIA or SCDA agar medium specified for that microorganism

5. Agar was incubated at the temperature and for the time indicated in table (3.10).

6.At the end of incubation, the stock suspension was prepared by collecting the surface growth in 10 - 20 ml of sterile saline TS.

7. The Transmittance % (T) was measured of the bacterial suspension using visible Spectrophotometer at wavelength = 580 nm. The transmittance of the suspension shall be around 23%-27%.

8. A portion of bacterial stock suspension was added to a sufficient amount of agar medium melted and cooled to 45^{o}C - 50^{o}C and swirl to homogenize

1.2. Preparation of the assay plates for cylinder plate method:

1. 13 ml of the prepared antibiotic medium was poured in each of the 5 petri dishes (90 mm) and canopy the plates.

2. The content to solidify was allowed, at temperature, into a smooth base layer of uniform depth.

3. 5ml of the seed layer inoculum was added (Refer to preparation of inoculum)

4. The plates were tilted back and forth to spread the inoculum evenly over the surface, and permit it to solidify.

1.3 Assay Steps:

1. 4 assay cylinders (holes) were prepared on the inoculated surface of every 5 plates using the neck of 5 ml Sterilin pipette (about 8 mm in diameter) slight pressure to immerse partially in agar

2.The remained agar was removed from each hole (holes) carefully to avoided agar damage round the assay cylinder.

3. The 4 holes in each plate were inoculated with 50µl of every Standard (S) and Sample (T) concentration.

4. The plates were covered to avoid contamination

5. The plates were left stand under LF for about half-hour so as to permit the antibiotic to diffuse into the agar layers, (to avoid formation of double inhibition zone).

6. The plates were incubated at 30-35 ºC for 18-24 hours.

7. The diameter of every inhibition zone were measured and recorded using digital caliber in keeping with USP equations as in point 2.0 below.

2. Calculations :(Calculation of potency estimate by three dose level)

For calculation the potency of antibiotic from the info obtained by the cylinder

plate method, the subsequent equations were used provided by USP

1.0 E = the difference in response between adjacent dose levels

$E = ¼ \times ((S3+T3) – (S1+T1))$

2.0 F= the difference in response between the 2 preparations (Standard and sample)

$F= ⅓ \times ((T3 + T2 + T1) – (S3 + S2 + S1))$

3.0 I= Log ratio of the doses (log ratio between two adjacent doses).

I= Log dilution factor.

4.0 b= the slope of response

b=E/I

5.0 M = the log of the potency ratio

M=F/b

6.0 Potency ratio = Anti log10 M X one hundred pc.

* Potency ratio = antilog10 MX100%

S1 = Standard low concentration T1 = Sample low concentration

S2 = Standard medium concentration T2 = Sample medium concentration

S3 = Standard high concentration T3 = Sample high concentration

Limits: 90.0 – 135.0 %

3.6.7 Test for indomethacin emulgel

Determination of indomethacin in indogel formula

HPLC Assay Conditions:

Wavelength = 254 nm .

Column : Rp – select B , 125 X 4 mm , 5 μm.

Flow Rate = 1.2 ml / min

Mobile Phase : 52 % MeOH / 48 % buffer (0.33g KH_2PO4 + 0.47 g K_2HPO_4/500 ml p.water)

Injection volume: 20 μl

Calculation : Calculate assay for results as follows:

$$\% \text{ Assay result} = \frac{\text{Response (Area) Sample}}{\text{Area of Standard}} \times \frac{\text{Concentration of Standard conc.}}{\text{Concentration of Sample}} \times 100\ \%$$

3.6.8 Determination of pH

pH of the prepared formula was measured using a pH meter (Mettler Toledo S220-Kit). it was calibrated according to the range which will be measured.

To measure the pH of permethrin cream, about 1.0 g of the cream was poured into a suitable beaker, 50 ml purified water was added, stirred to obtain a homogenous mixture, and the pH at 20 – 22 ˚C was measured using pH meter, the electrode dipped was let stranded with for a stable reading.

To measure, the pH of gentamicin cream, 2.5 g was weighed accurately and transferred to a 100 ml beaker, then a 50 ml of purified water was added and dissolved with warming for 5 min, then cooled and the pH of the solution was measured using a pH meter.

For the rest, preparations the electrode was Immersed into the product and the pH was measured.

All measurements were repeated three times and the average were calculated to give accurate readings.

3.6.9 Determination of Viscosity of ketoconazole shampoo and permethrin cream

Viscosity was measured by taking 50 ml of the preparations in 50 ml beaker then spindle No. 4, 100 rpm was used at about 20-22 ˚C, Brookfield DV2T Viscometer was used.

3.6.10 Determination of Ketoconazole Shampoo Density:

Thoroughly the outside and inside of the pycnometer was cleaned and dried and weighed individually with its corresponding cover.

A. The pycnometer was filled with a suitable sample of the shampoo.

B. The pycnometer was closed and again washed and dried the outside of the pycnometer.

C. The filled pycnometer was weighed together with its corresponding cover.

D. The difference between the two weights is the net weight of the sample.

E. The density was calculated by using the following equation:

$$\text{Density} = \frac{\text{Wt. (g.)}}{\text{Volume (ml)}}$$

Where: Wt. = the difference between the filled pycnometer and the unfilled pycnometer

Volume = volume of the pycnometer.

3.6.11 Physical appearance:

A few grams of preparations were placed on a white paper, then the observations were written.

3.6.12 Odor :

A sample of all preprations was smelled and the odor was recorded

3. 7 Evaluation of formulation as a preservative

3.7.1Antimicrobial Effectiveness Testing

This procedure was designed to measured and evaluated the antimicrobial effectiveness of antimicrobial preservatives used in topical products made with aqueous base or vehicles

3.7.1.1 General instructions

• The agar media was prepared to be ready to be used, during a liquid form at 45 degrees Celsius.

• The bacterial and yeast suspensions were used within two hours of harvest otherwise refrigerate the suspension for twenty-four hrs., while the fungal preparation may be stored undercooling for up to 7 days.

• The work was aseptically done under streamline flow for all the preparations.

3.7.1.2 Media:

For the cultivation of the test organisms, an agar medium was selected that's favorable to the rigorous growth of the respective stock culture. The suggested media were Soybean

Casein Digest Agar/Broth and Sabouraud Dextrose Agar/Broth. an appropriate inactivator (neutralizer) was added for the particular antimicrobial properties within the product to the broth and/or agar media used for the test procedure if required.

3.7.1. 3 Growth promotion of the media

Media used for testing must be tested for growth promotion by inoculating the medium with appropriate microorganisms. it's preferable that test microorganisms be chosen for growth promotion testing. Solid media tested for growth promotion is to be founded using pour plate method so as to determine a microbial plate count (CFU) which must be ≥ 70% of the microorganism inoculum's calculated value.

3.7.1.4 Test organisms, (NMT 5 passages from the lyophilized culture):

a. Candida albicans (ATCC # 10231)

b. Aspergillus brasiliensis (ATCC # 16404)

c. Escherichia coli (ATCC # 8739)

d. Pseudomonas aeruginosa (ATCC # 9027)

e. Staphylococcus aureus (ATCC # 6538)

3.7.1.5 Procedure:

Preparation of the inoculum

A. Preparatory to the test:

1. The surface of a suitable volume of solid agar medium was Inculcated from a recently revived stock culture of each of the specified microorganisms.
2. Culture conditions for the inoculums culture were described in Table (3.10)

B. Harvesting the bacterial and Candida albicans cultures:

1. The surface growth was washed by using sterile saline TS.

2. Collected in a suitable vessel

3. Sufficient sterile saline TS was added to obtain a microbial count of about $1X10^8$ CFU /mL.

4. The number of cells was counted by measuring the turbidity using a spectrophotometer at 650nm to obtain an O. D. of:

   A. 0.3-0.45 for *S.* aureus (~$1\text{-}3\ X10^8$ CFU).

   B. 0.2-0.3 for P. aeruginosa and E. coli (~$1\text{-}3\ X10^8$ CFU).

   C. $\leq$ 1.0 for c. albicans ($\simeq$ $1\text{-}3 \times 10^8$)

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

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

C. Harvesting the Aspergillus brasiliensis cultures:

1. The surface growth was washed using a sterile saline TS containing 0.05% of polysorbate 80.

2. Sufficient sterile saline TS was added to obtain a microbial count of about $1X10^8$ CFU/ mL.

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

4. Incubated between 2-4 days at 23 ± 2^oC and count the CFU (count plates having between 10-100 CFU).

D. Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e. Soy-bean Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in sterile saline TS to obtain a microbial count of about $1X10^8$ CFU per mL.

E. The number of CFU per mL in each suspension was determined, by using the condition of media and microbial recovery incubation times listed in Table (3.10) to confirm the initial CFU per mL estimate.

3.7.1.6 Test Procedure

1. The test was conducted in five sterile, capped bacteriological containers of suitable size into which a sufficient volume of product has been transferred. (20ml sample/each container).
2. Each container was inoculated with one of the prepared and standardized inoculum, and mix:
   A. The initial concentration of viable 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.
   B. The volume of the suspension inoculum used was between 0.5% and 1% of the volume of the product
   C. The concentration of test microorganisms that was added to the product shall reach a final concentration in the test preparation after inoculation between $1X10^5$ and$1X10^6$ CFU per mL of the product (For example, when 100 to 200µl inoculum of $1X10^8$ CFU/ ml are inoculated in 20ml sample, the final concentration will be 0.5 to $1X10^6$ CFU/ ml respectively).
3. The inoculated was incubated containers at 23 $\pm$ 2 ^oC.
4. Each container was sampled at the appropriate intervals: on time 0,7,14 and28 days as follow: Make several dilutions 1:10, pipette 1ml from each container separately (containing test microorganism) and transferred it to a test tube contains 9ml Letheen broth medium so that 10^{-1}….10^{-5} dilutions are achieved according to test conditions
5. Pipette 1 ml from each dilution of each microorganism and transfer to a sterile petri dish 90mm, and then pour about 15ml of Letheen agar medium (at 48 ^oC) in each plate.

6. The plates were lifted under LF cabinet till solidified and then incubated as follow:

   - For Bacteria plates; at 35 $\pm$ 2 ^{o}C for 2-3 days

   - For Fungal plates: at 23$\pm$ 2 ^{o}C for 5-7 days

7. The plate-count method was used to determine the number of CFU present in each test preparation for the applicable intervals.

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 is defined as the difference between the log 10-unit value of the starting concentration of CFU/ml in the suspension and the log 10-unit value of CFU/ml of the survivors at that time point).

3.7.1.7 Criteria for antimicrobial effectiveness:

The requirements for antimicrobial effectiveness are achieved if the following are met. (For category topically products).

Bacteria: Not less than 2.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.

Yeast and Molds: No increase from initial calculated count at 14, and 28 days.

Table 3.10: Culture Conditions for Inoculum Preparation (USP)

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

3.7.2 Total Aerobic Microbial Count Testing Method for Creams, Gels, and Shampoo

3.7.2.1 Purpose:

To provide qualitative and quantitative estimation of microbial (TAMC and total combined yeast and mold (TYMC) in finished products in the form of gels, creams, and shampoo.

3.7.2.2 General Instructions:

1. Physical inspection was performed for any damage in the product package, or products color change …. etc.

2.For Water immiscible gels, creams, and shampoo: a suspension was prepared with the aid of an emulsifying agent using a blender. If needed, the sample preparation was warmed to a temperature not to exceed 45 ºC. You can try warming to ≤ 45 ºC without adding an emulsifying agent for some products.

3.Test controls: At the time of the test, air plates controls, buffer/broth negative controls, and media negative controls were performed.

4. Sample size: 10 tubes (if each tube contains > 10 g)

5 All plates, tubes, pipettes….. etc. used for testing and identification of microorganism growth were autoclaved and disposed of according to standard operation.

3.7.3.3 Procedure:

A. Preparation of the Sample:

1. The tubes were placed under an operating Laminar Flow Hood (LF).

2. The surface of the caps was washed with Ethanol 70%. Uncap the tubes and wash the mouth of the tubes with Ethanol 70%.

3. The surface of the mouth of the tubes was swept with wet tissue paper (wetted with Ethanol 70%) to remove the excess of ethanol.

4. The aluminum seal of the tubes were opened using the caps pre-washed with ethanol 70%, and squeeze a portion of the product to be eliminated in a waste beaker.

5. The tube was Squeezed to transferred as much as possible of the product to a sterile bottle (100-200 ml).

6. The bottle was closed, and transferred to a water bath at ~ 45^{o}C. Wait until the product becomes suspension, mix by vortex, and transferred the bottle under the LF for testing.

Note: If the product does not dissolve, add an emulsifying agent (Polysorbate) to facilitate the process or warm to $\underline{\sim}$ 45 °C in the water bath.

B. Direct Transfer and Plate Testing Method for Bacterial Count

1. Samples were transferred aseptically to sterile bottles as follows:

10 ml of the dissolved specimen was transferred to a sterile bottle using a sterile 10 ml pipette, repeated with another sterile bottle.

90 ml of SCDM (SCDB) or phosphate buffer of pH 7.2 was added in 1 bottle, so that the final dilution is 1:10. The bottles was closed and mixed gently by hand.

2. The SCDM (SCDB) bottle or phosphate buffer bottle was opened, and with a sterile 2 ml pipet 1 ml of the sample was drew and transferred to a petri dish and the petri dish was covered. Repeated with another plate:

Either the petri dish contains already solidified SCDA.

Or the dish is empty, and 15-20 ml of sterile liquid SCDA (around 45ºC) is poured on the 1 ml sample and then mixed.

3 Each plate was gently rotated so that 1 ml of the sample was covered all the agar surface of the plate, or if liquid SCDA is added, that the agar mixes completely with the sample and mixes with the entire agar and solidifies at room temperature.

4. Settling for at least 10 minutes was allowed.

5. The dishes were inverted, and incubated at 30 to 35 ºC for 3 to 5 days

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

If growth: Average number of microorganisms in each plate /g or /ml (taking into account, the initial dilution 1:10).

If no growth: Express, the result as $\leq$ 10 CFU/g or /ml.

C. Plate Method for Total Aerobic Yeast and Molds Count

1. The same procedure was followed as for bacterial count, (refer to point B) but use Sabouraud Dextrose Agar and Borth instead of SCDA, SCDM.

2. The SAB Agar plates SABM were incubated at 20 to 25 ºC for 5-7 days.

3.7.2.4 Acceptance criteria for finished products sample.

Table 3.11: Acceptance criteria for Cutaneous finished products (USP/BP)

Route of Administration	TAMC CFU/g	TYMC CFU/g	Absence of*
Cutaneous	≤200 (10^2)	≤20(10^1)	S. aureus and P. aeruginosa

** USP/ BP acceptance criteria interpretation: -
10^1 CFu: maximum acceptable count =20
10^2 CFu: maximum acceptable count =200

3.8 Stability Test

The stability testing was done for the formulations that contained guava leaves extract as a natural preservative (Ketoconazole shampoo, Ibuprofen gel, Clotrimazole Cream) that passed the preservative efficacy test and met the chemical and physical specifications., several tests were done at end of every month for three months. Antimicrobial tests, chemical test for the active ingredient within the formulation, Physical test like physical appearance, color, pH, odor tests were done monthly for three months on accelerated conditions (40 ± 2 °C / 75% ± 5% RH). Three months of accelerated stability test is equivalent to two-year stability test at a normal storage condition.

Chapter Four

Results & Discussion

4. Results & Discussion

All raw materials and reagents, which were used in this research, were fully tested since they were used in the production process in Beit Jala pharmaceutical company.

All media used in the microbiology test were fully tested (growth promotion test) and the results demonstrate that they met all specifications.

All direct packaging materials used in this research were fully tested since they were used in the packaging process in Beit Jala pharmaceutical company. No interaction was detected between preparations (with chemical preservative) and direct packaging material, so it is safe to use them in our preparations.

4.1 Determination of Extracted powder

4.1.1 Yield Percentage of Extracted Guava Leaves

The percentage yield of guava leaves extract was 20% (300g, combined) and the dried extract was fine, soft, and clear brown powder.

Figure 4.1: powder extract of guava leaves

4.1.2 Determination of Extracted Powder by HPLC

Standard phenolic compounds for HPLC chromatograms were gallic acid, caffeic acid, chlorogenic acid, catechin, and rutin. Absorbance was measured at 280 nm. Standard solutions concentrations for the phenolic compound were 1mg/ml for gallic acid, 0.3 mg/ml for catechin, 0.25 mg/ml for chlorogenic acid, 0.3 mg/ml for caffeic acid, and 0.15 mg/ml for rutin (see Figure 4.2).

The major phenolic constituents determined by HPLC analyses of the ethanol extracts included all the previous phenolic standard, and other compounds which couldn't be identified because some of the standard phenolic compounds were not available (see Figure 4.3).

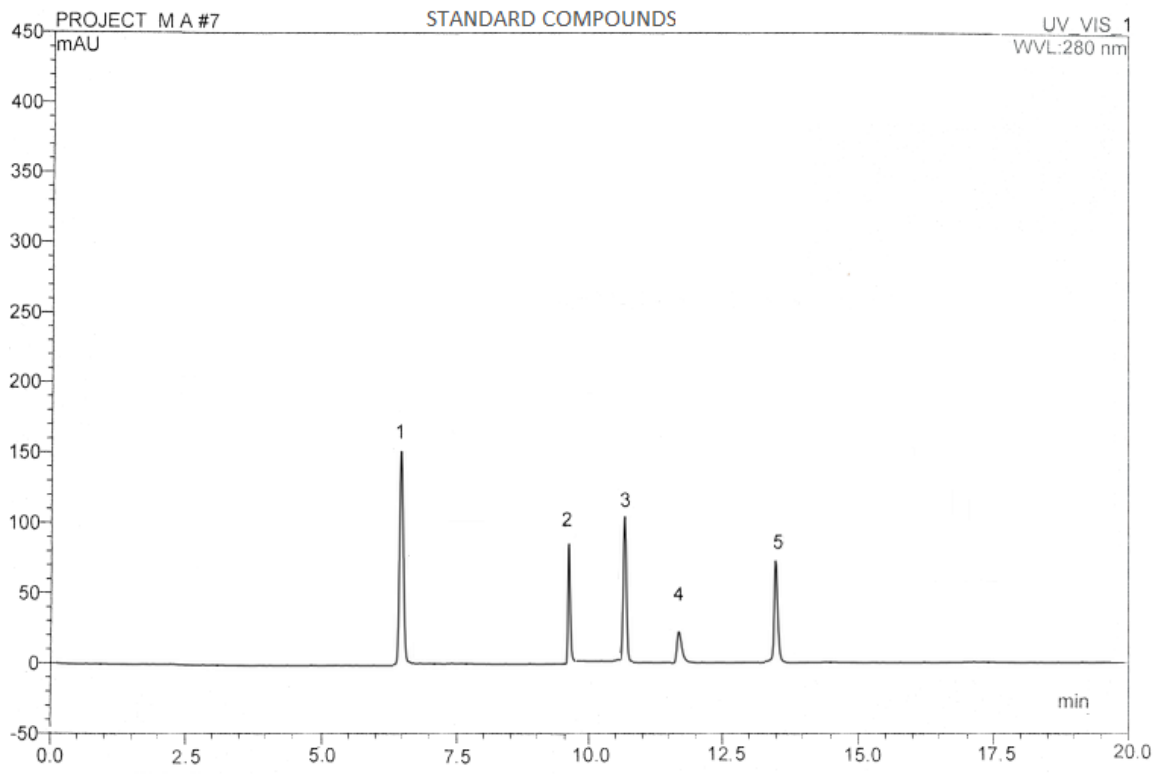


Fig. 4.2: UV-vis Chromatogram of a standard solution contains five phenolic compounds (1: gallic acid (Rt = 6.52); 2: catechin (Rt = 9.725); 3: chlorogenic acid (Rt = 10.624); 4: caffeic acid (Rt = 11.731); and 5: rutin (Rt = 13.524).

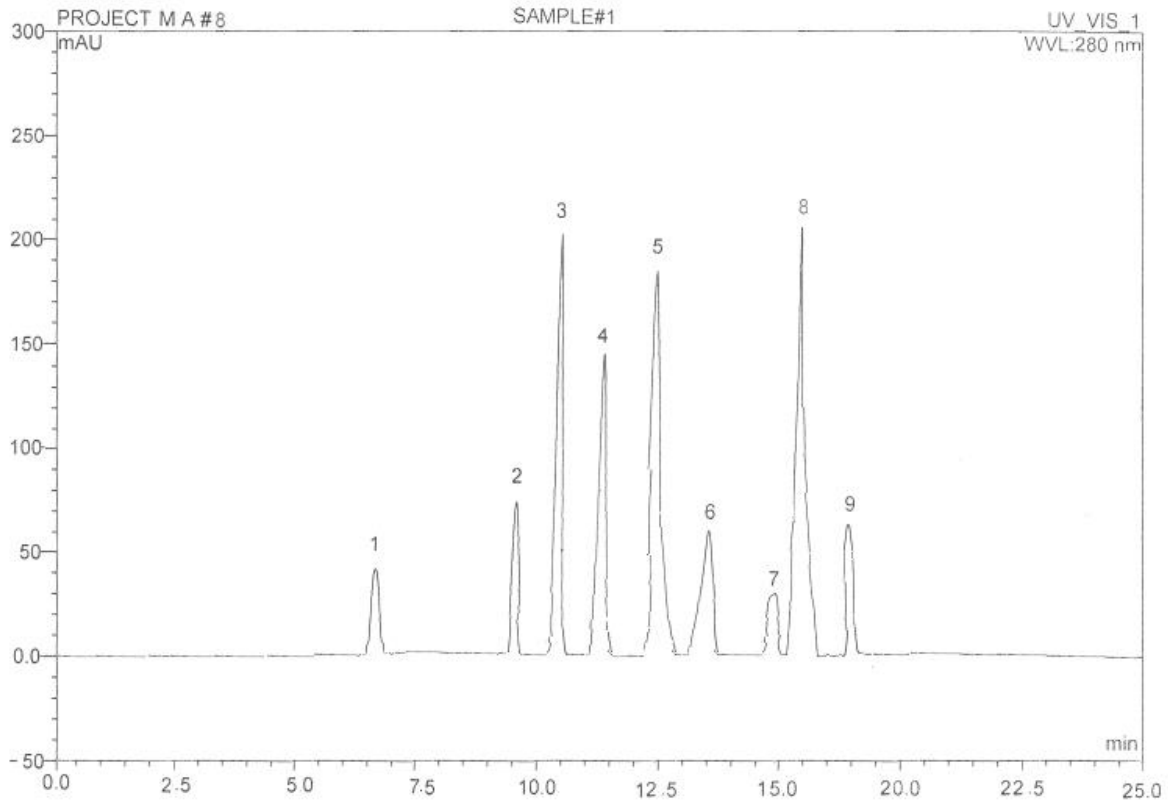


Fig. 4.3: HPLC-UV-vis chromatogram of ethanol extract of guava leaves. Peaks: 1- gallic acid (RT = 6.61); 2-catechin hydrate (RT =9.61); 3- chlorogenic acid (RT =10.61), 4-caffeic acid (RT =11.49); 6 rutin hydrate (RT = 13.62); (5,7,8,9 are unknown).

Table 4.1: Chemical concentrations of phenolic compounds in the ethanol extracts of guava leaves.

Polyphenolic compound	**Content (mg/100 g of dry extract)**
Gallic acid	555.49
Catechin hydrate	821.11
Caffeic acid	39.54
Rutin hydrate	300.84
Chlorogenic acid	42.04

The results showed that the extract contained the major phenolic compounds which are promising sources of antimicrobials. Phenolic compounds act by inhibiting microbial cell wall outgrowth, perturbing, degrading, impairing biogenesis, suppressing deoxyribonucleic acid replication and transcription, impairing ATP production, suppressing bacterial toxins, and generating reactive oxygen species. (Mickymaray S., 2019).

4.1.3. Phytochemical Analysis.

Table (4.2) shows the phytochemical screening of chemical constituents of ethanol guava leaves extract. The results revealed the presence of active compounds in ethanol extract. As shown in the table, the ethanol extract indicated the presence of phenols and tannins, terpenoids, flavonoids, and glycosides.

The presence of phytochemicals which are known to offer medical and physiological activities. For example, tannins are polyphenolic compounds that link to the proline-rich protein that interferes with protein synthesis (Sanches et al., 2005; Tsuchiya et al., 1996; and Ulubelen A., 2003) showed to have antibacterial activity (Akiyama et al., (2001); and Min et al., 2008).

Flavonoids are hydroxylated polyphenolic compounds that are produced by plants in response to microbial infections; an aspect that was widely studied. Flavonoids were characterized to have antimicrobial activity against a range of microorganisms in vitro (Cowan M., 1999). Their antimicrobial activity refers to their ability to form complexes with extracellular and soluble proteins and bacterial cell walls (Trease et al., 1989).

Terpenoids were mainly used for their aromatic qualities, however, they were also used as agents against inhibiting bacteria (Tsuchiya et al., 1996).

Glycosides were found to have inhibitory effects on gram-positive bacteria, staphylococcus aureus. As it's shown, that phytochemical analysis revealed that the ethanol extract has chemical compounds that were found to possess antibacterial activities Figure (4.4). (a) to (d) showed the colorimetric results for the ethanol extracts.

Table 4.2: Phytochemical constituents of Psidium guava extracts.

Phytochemical tests	Phenols and tannins	Terpenoids	Flavonoids	Glycosides
Ethanol extract	+	+	+	+

+: presence of the constituent; −: absence of the constituent

a b c d

Figure 4.4: (a) Existence of phenols and tannins. (b) Existence of terpenoids (c) Existence of flavonoids (d) Existence of glycosides.

4.2 Determination of antimicrobial activity of guava leaves extract by Antimicrobial Effectiveness Testing:

4.2.1 Antimicrobial Effectiveness Test for guava leaves extract powder as a pure material

To use guava leaves, extract as a preservative in semi-solid pharmaceutical preparations, preservative efficacy of guava leaves as pure (raw material) was tested at different concentrations using purified water as solvent (0.5, 2.5, 5, 7.5, 9.5, 10) % w/w. Table (4.3) to table (4.8) show preservative efficacy results for powdered guava extract at the previously mentioned concentrations.

For this test panel, the following five representative microorganisms were used:

• Pseudomonas aeruginosa (non-fermentative Gram-negative bacillus) (ATCC 9027)

• Escherichia coli (Gram-negative bacillus) (ATCC NO. 3739)

• Staphylococcus aureus (Gram-positive coccus) (ATCC NO. 6538)

• Candida albicans (yeast) (ATCC NO.10231)

• Aspergillus (Niger) brasiliensis (mold) (ATCC NO. 16404)

To use the antimicrobial compound as a preservative, it should be effective against all tested microorganisms. Results of preservative efficacy for guava leaves extract at 0.5% indicated that it was ineffective for all microorganism (refer to Table 4.3); at 2.5% it was effective (6 log reduction) for Gram-positive coccus (Staphylococcus aureus) only (no activity on other microorganisms was observed) (refer to Table 4.4). At a concentration of 5%, guava leaves extract was very effective (complete inhibition) against Staphylococcus aureus. However, the observed count for the gram-negative (Pseudomonas aeruginosa,

Escherichia coli) at 5% was still ineffective (1 log reduction), it was better than that observed at lower concentrations (0.5% (0.3 log reduction)) and (2. 5% (0.6 log reduction)). Moreover, no activity against yeast and mold was observed at this concentration (i.e., 5%; refer to Table 4.5). Increasing the concertation of guava leaves extracts to 7.5% resulted in improving the antibacterial activity against the three tested bacteria species, where a complete inhibition of all three bacterial strains was observed after 28 days. Nevertheless, guava leaves extract at 7.5% was still ineffective against yeast and mold (refer to Table 4.6). At 9.5% satisfactory results (no increase from the initial calculated count at 14, and 28 days) were obtained for Aspergillus (Niger) brasiliensis(mold). For Candida albicans the result became better but it was still ineffective (refer to table 4.7). Increasing the concentration to 10% yielded the best result ever. A complete inhibition of all three bacteria strains after 7, 14 and 28 days was observed, there was no increase in the yeast and mold counts from the initial time at 14 and 28 days refer to Table (4.8), Figure (4.6), so 10% was the best concentration to be used as a preservative.

A B C

Figure 4.5: guava leaves extract powder dissolve in purified water solvent at concentrations (A) 2.5%, (B) 5 %, (C) 10%.

Table 4.3: Antimicrobial activity of guava leaves extract powder (0.5% w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			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	918	658			492	62	
		7	>>10^3	820	640	650	440				>>10^3	>10^3
		14	>>10^3	680	580	480	89					>10^3
		28	>>10^3	900	480	320	65					452
P. aeruginosa	9027	0	>>10^3	>>10^3	>10^3	985	800			70	4	
		7	>>10^3	980	854	650	652			>>10^3		>1000
		14	>>10^3	850	740	480	212					>1000
		28	>>10^3	985	800	320	80					622
E. coli	8739	0	>>10^3	>>10^3	>10^3	918	658			1096	85	
		7	>>10^3	931	654	650	365				>>10^3	>1000
		14	>>10^3	852	585	480	189					>1000
		28	>>10^3	995	360	340	98					384
Candida albicans	10231	0	>>10^3	>>10^3	>10^2	918	658		888	73	6	
		7	>1000	800	900	800	658		>>10^3	>>10^3	>1000	
		14	>>10^3	>1000	879	584	458				>1000	
		28	>>10^3	>1000	687	465	100				210	
A. brasiliensis	16404	0	>>10^3	>>10^3	>10^3	918	658		280	42	10	
		7	652	8	6	0	0		>1000	49	5	
		14	>>10^3	680	580	480	89		380	42	9	
		28	>>10^3	900	480	99	7		>1000	255	30	

Table 4.4: Antimicrobial activity of guava leaves extract powder (2.5 % w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			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	>1000	>1000	500			492	62	
		7	>1000	852	300	0	0				>>10^3	>10^3
		14	652	13	0	0	0					>10^3
		28	18	0	0	0	0					452
P. aeruginosa	9027	0	>>10^3	>>10^3	>10^3	985	400			70	4	
		7	>1000	815	954	632	200			>>10^3		>1000
		14	>>10^3	808	352	214	93					>1000
		28	>>10^3	684	48	320	70					622
E. coli	8739	0	>>10^3	>>10^3	>10^3	862	258			1096	85	
		7	>>10^3	690	454	250	118				>>10^3	>1000
		14	>>10^3	700	602	352	100					>1000
		28	>10^3	801	360	340	17					384
Candida albicans	10231	0	>>10^3	>>10^3	>10^2	918	658		888	73	6	
		7	>1000	1000	800	400	100		>>10^3	>>10^3	>1000	
		14	>1000	>1000	852	485	16				>1000	
		28	>>10^3	>1000	314	302	0				210	
A. brasiliensis	16404	0	>>10^3	>>10^3	>10^3	918	658		280	42	10	
		7	1000	963	6	97	0		>1000	49	5	
		14	>>10^3	652	526	325	85		380	42	9	
		28	>>10^3	>10^3	685	65	26		>1000	255	30	

Table 4.5: Antimicrobial activity of guava leaves extract powder (5 % w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			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	>1000	>1000	150			492	62	
		7	0	0	0	0	0				>>10^3	>10^3
		14	0	0	0	0	0					>10^3
		28	0	0	0	0	0					452
P. aeruginosa	9027	0	>>10^3	>>10^3	>10^3	214	98			>1000	200	
		7	>1000	652	562	99	32					>1000
		14	685	578	468	65	26					>1000
		28	512	432	232	5	0					540
E. coli	8739	0	>>10^3	>>10^3	>10^3	198	98			336	40	
		7	>1000	474	362	91	20					>1000
		14	578	400	221	40	11					>1000
		28	300	220	141	8	0					>1000
Candida albicans	10231	0	>>10^3	>>10^3	>10^3	932	684		779	73	6	
		7	>1000	947	614	214	13			>1000	320	
		14	>1000	>1000	312	110	0			>1000	391	
		28	>>10^3	>1000	358	65	0				>1000	
A. brasiliensis	16404	0	>>10^3	>>10^3	>10^3	918	658		280	42	10	
		7	580	145	88	65	0		>1000	49	5	
		14	>1000	845	580	180	11		380	42	9	
		28	>1000	>1000	685	75	0		>1000	255	30	

Table 4.6: Antimicrobial activity of guava leaves extract powder (7.5 % w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			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	>1000	>1000	150				62	7
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					310
P. aeruginosa	9027	0	>>10^3	>>10^3	>1000	155	13				79	
		7	252	92	23	5	0				>1000	>1000
		14	98	0	0	0	0					>1000
		28	0	0	0	0	0					444
E. coli	8739	0	>>10^3	>>10^3	>1000	650	25				56	
		7	259	53	16	10	0				>1000	>1000
		14	52	7	3	0	0					>1000
		28	0	0	0	0	0					295
Candida albicans	10231	0	>>10^3	>>10^3	>10^3	932	684		>1000	125	11	
		7	>1000	947	614	214	13			>1000	245	
		14	>1000	>1000	312	110	0			>1000	385	
		28	>>10^3	>1000	358	65	0			>1000	467	
A. brasiliensis	16404	0	>>10^3	>>10^3	>10^3	518	158		215	19	1	
		7	580	145	88	65	0		370	37	4	
		14	>1000	845	580	180	11		>1000	240	32	
		28	>1000	>1000	685	75	0		>1000	228	21	

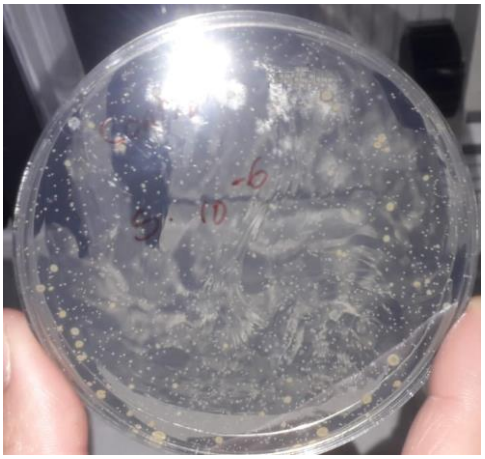
Table 4.7: Antimicrobial activity of guava leaves extract powder (9.5 % w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	$>>10^3$	$>>10^3$	>1000	>1000	18				62	7
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					310
P. aeruginosa	**9027**	**0**	$>>10^3$	$>>10^3$	>1000	96	2				79	
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					444
E. coli	**8739**	**0**	$>>10^3$	$>>10^3$	>1000	111	5				56	
		7	100	18	0	0	0				>1000	>1000
		14	17	0	0	0	0					>1000
		28	0	0	0	0	0					295
Candida albicans	**10231**	**0**	$>>10^3$	$>>10^3$	519	59	12		>1000	125	11	
		7	>1000	83	614	1	13			>1000	245	
		14	>1000	53	312	3	0			>1000	385	
		28	1154	200	120	29	0			>1000	467	
A. brasiliensis	**16404**	**0**	$>>10^3$	$>>10^3$	$>10^3$	32	2		215	19	1	
		7	>1000	440	111	20	0		370	37	4	
		14	>1000	385	57	2	0		>1000	240	32	
		28	18	0	0	0	0		>1000	228	21	

Table 4.8: Antimicrobial activity of guava leaves extract powder (10% w/w) in purified water against Microorganisms.

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			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	>1000	0	0				62	7
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					310
P. aeruginosa	**9027**	**0**	>>10^3	>>10^3	>1000	12	0				79	
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					444
E. coli	**8739**	**0**	>>10^3	>>10^3	>1000	11	0				56	
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					295
Candida albicans	**10231**	**0**	>>10^3	>>10^3	314	160	13		>1000	125	11	
		7	>1000	480	268	112	7			>1000	245	
		14	914	312	153	43	1			>1000	385	
		28	200	100	50	29	0			>1000	467	
A. brasiliensis	**16404**	**0**	>>10^3	>>10^3	>10^3	200	42		215	19	1	
		7	>1000	365	118	35	0		370	37	4	
		14	652	200	84	20	0		>1000	240	32	
		28	219	112	57	1	0		>1000	228	21	

A



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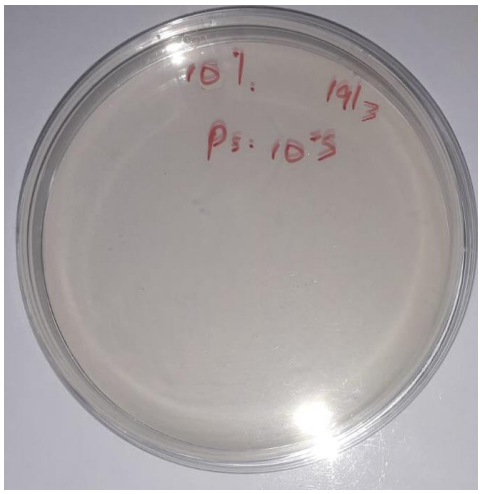

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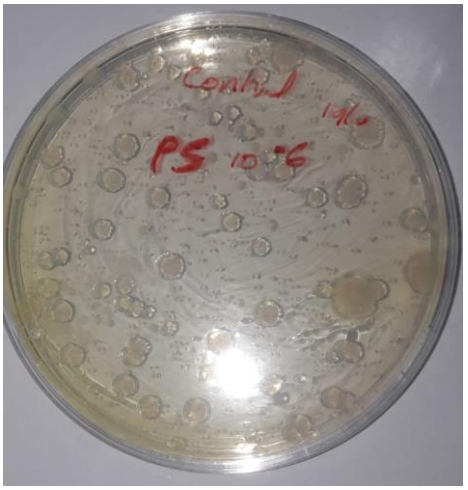

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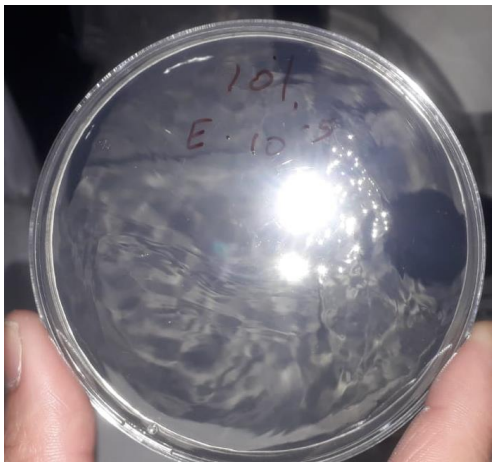

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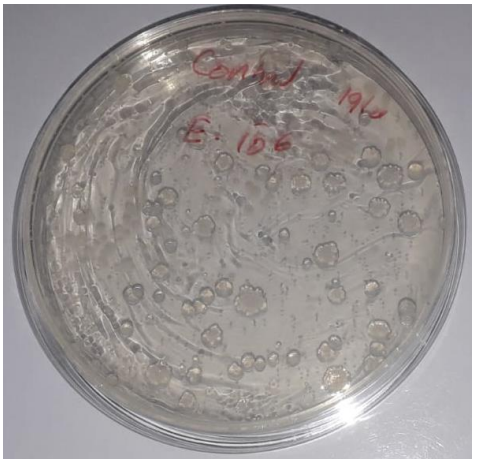
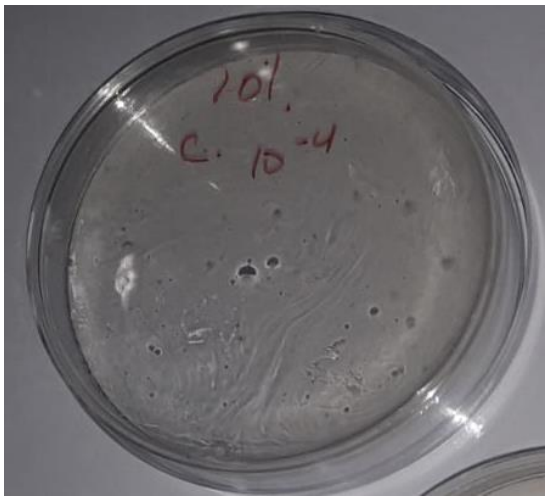


F



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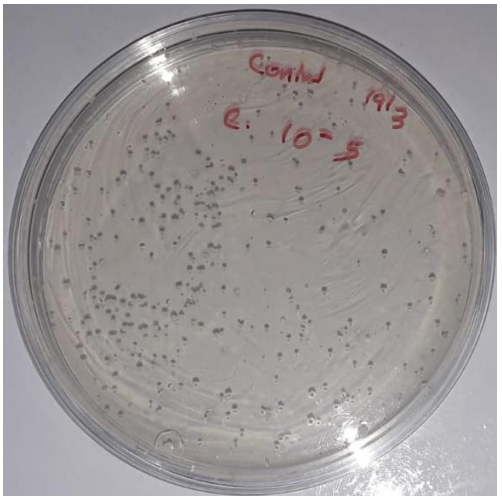

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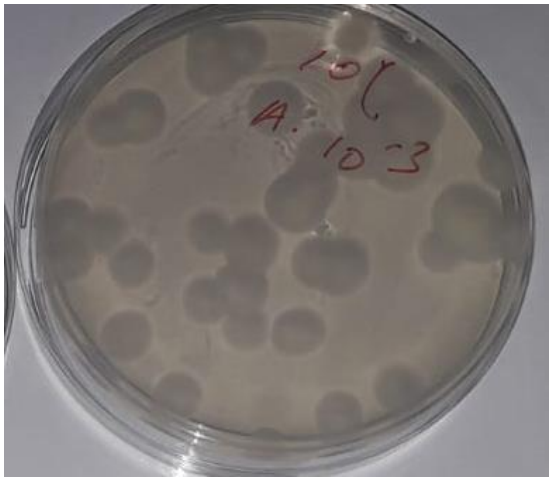
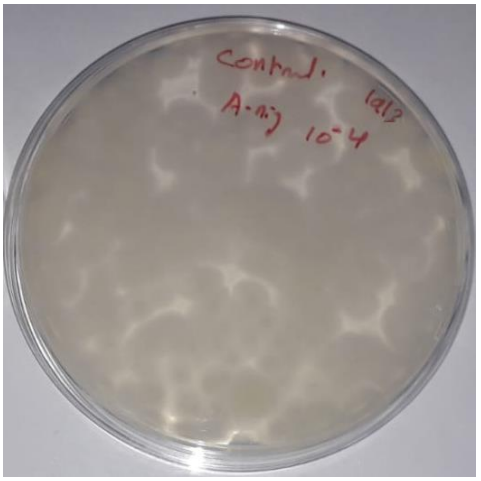


I



J

Figure 4.6: Plates show preservative efficacy test results shown by Ethanolic extracts of guava leaves (10% concentration) at 28 days' count, against: (A) Staphylococcus aureus (10^{-3}); (B) Staphylococcus aureus control (10^{-6}); (C) Pseudomonas aeruginosa (10^{-5}); (D) Staphylococcus aureus control (10^{-6}); (E) Escherichia coli (10^{-5}); (F) Escherichia coli (10^{-6}); (G) Candida Albicans (10^{-4}); (H) Candida Albicans control (10^{-5}); (I) A. brasiliensis (10^{-3}); and (J) A. brasiliensis control (10^{-4}).

4.2.2 Antimicrobial Effectiveness Test for guava leaves extract powder in semi-solid pharmaceutical preparations.

Guava leaves extract was tested as a natural preservative (to replace conventional chemical preservatives) in pharmaceutical preparations at a concentration of 10% (w/w). Six pharmaceutical semi-solid preparations were included in this test, namely: one shampoo preparation (Ketoconazole shampoo), three cream preparations (Clotrimazole Cream, Permethrin Cream, Gentamycin Cream), and two gel preparations (Ibuprofen gel, and Indomethacin emulgel).

All six preparations that contain guava leaves extract powder as a natural preservative were tested by preservative efficacy test to evaluate the efficiency of the natural preservative in pharmaceutical preparation.

The results of the preservative efficacy tests for guava extract powder as natural preservatives in semi-solid preparations are shown in tables 4.9 – 4.14.

The results of the preservative efficacy tests for Ketoconazole shampoo were impressive because they passed with complete inhibition for microorganism strains (Escherichia coli, Staphylococcus aureus Candida albicans, Aspergillus (Niger) brasiliensis) at 14 and 28 days, see Table (4.9). Satisfying results (2.1 log reduction) were observed for Pseudomonas aeruginosa. Similarly, Clotrimazole Cream results passed with complete inhibition for microorganism strains (Escherichia coli, Pseudomonas aeruginosa Staphylococcus aureus Candida albicans, Aspergillus (Niger) brasiliensis); moreover, it registered good reduction (2.9 log reduction) for Escherichia coli (refer to Table 4.10). For Permethrin Cream, Gentamycin Cream, and Indomethacin emulgel, test results passed for all microorganism strains (Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus), and no increase from the initial calculated count at 14, and 28 days was measured

for Aspergillus (Niger) brasiliensis). However, the test results failed for Candida albicans on day 28 (see Tables 4.11 through 4.13) For ibuprofen gel, it passed with complete inhibition for Escherichia coli, Staphylococcus aureus), there was an acceptable reduction for Pseudomonas aeruginosa, and there was no increase from the initial calculated count at 14, and 28 days for Candida albicans and Aspergillus (Niger) brasiliensis, (see Table 4.14).

All six formulations passed the three tested types of bacteria (Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa) with impressive inhibition. For Candida albicans (yeast), Aspergillus brasiliensis (mold), they were completely inhibited by Ketoconazole shampoo, and Clotrimazole Cream. These results might be obtained because two formulations contained antifungal compound (i.e., Ketoconazole, Clotrimazole respectively, as active material). The ibuprofen gel formula contained Propylene Glycol and Isopropyl Alcohol which may improve the effectiveness of guava extract as a preservative

The preparations that passed the test (Ketoconazole shampoo, Clotrimazole Cream, and Ibuprofen gel) continued with several tests including (microbial limit test, drug content, all chemical test, stability test) to ensure that it would be safe to use guava leaves extract powder in pharmaceutical drugs as a preservative.

Table 4.9: Preservative efficacy testing for Ketoconazole shampoo containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	979	424	83	0	0				58	7
		7	0	0	0	0	0				>>10^3	>1000
		14	0	0	0	0	0					452
		28	0	0	0	0	0					219
P. aeruginosa	**9027**	**0**	>>10^3	>>10^3	>1000	990	230				70	
		7	>1000	600	98	9	0				>>10^3	>1000
		14	580	99	22	2	0					>1000
		28	27	12	0	0	0					622
E. coli	**8739**	**0**	>1000	388	18	5	1				250	
		7	250	2	0	0	0				>>10^3	>1000
		14	0	0	0	0	0					884
		28	0	0	0	0	0					435
Candida albicans	**10231**	**0**	>1000	>1000	247	121	8		>1000	137	14	
		7	0	0	0	0	0		>>10^3	>1000	944	
		14	0	0	0	0	0			>1000	575	
		28	0	0	0	0	0				196	
A. brasiliensis	**16404**	**0**	>1000	>1000	269	52	7		366	43	7	
		7	0	0	0	0	0		>>10^3	>1000	237	
		14	0	0	0	0	0		>>10^3	>1000	179	
		28	0	0	0	0	0		>>10^3	>1000	230	

Table 4.10: Preservative efficacy test for Clotrimazole Cream containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	>1000	>1000	673	50	5		>1000	850	60	7
		7	276	34	8	0	0				>1000	>1000
		14	0	0	0	0	0				>1000	>1000
		28	0	0	0	0	0				735	75
P. aeruginosa	**9027**	**0**	>1000	>1000	800	116	16		>1000	877	212	
		7	600	12	8	1	0				>1000	>1000
		14	0	0	0	0	0				>1000	473
		28	0	0	0	0	0				>1000	>1000
E. coli	**8739**	**0**	$>>10^3$	>1000	>1000	800	280		>1000	1548	212	
		7	>1000	630	110	8	0				>1000	>1000
		14	600	112	26	1	0				>1000	>1000
		28	96	42	18	0	0				>1000	478
Candida albicans	**10231**	**0**	>1000	600	50	5	0		120	17	2	
		7	0	0	0	0	0	210		4	0	
		14	0	0	0	0	0	>1000	>1000	966	91	13
		28	0	0	0	0	0				>1000	478
A. brasiliensis	**16404**	**0**	HG	HG	47	6	1		70	16	2	
		7	>1000	204	54	7	0	>1000	>1000	592	69	7
		14	0	0	0	0	0					
		28	0	0	0	0	0					

Table 4.11: Preservative efficacy test for Permethrin Cream containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	6538	0	>1000	>1000	>1000	1005	112		>1000	690	69	7
		7	0	0	0	0	0				>1000	2550
		14	0	0	0	0	0				>1000	1120
		28	0	0	0	0	0				111	17
P. aeruginosa	9027	0	>1000	>1000	320	56	0		>1000	375	43	
		7	900	212	3	0	0				>1000	1460
		14	0	0	0	0	0				>1000	1500
		28	0	0	0	0	0				1354	110
E. coli	8739	0	>1000	>1000	1400	252	29		>1000	560	63	
		7	712	71	9	0	0				>1000	1528
		14	0	0	0	0	0				>1000	1620
		28	0	0	0	0	0				>1000	1768
Candida albicans	10231	0	>1000	>1000	679	420	132		610	125	6	
		7	>1000	900	600	325	130		>1000	608	68	
		14	>1000	800	500	200	69	>1000	>1000	720	81	
		28	$>>10^3$	1000	411	112	12			396	37	
A. brasiliensis	16404	0	>1000	960	113	19	3		162	19	1	
		7	>1000	440	100	18	3		85	5	0	
		14	800	344	99	11	1	290	37	4	2	
		28	600	327	33	1	0		100	12	1	

Table 4.12: Preservative efficacy test for Gentamycin Cream containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	6538	0	>1000	900	213	181	47	>1000	>1000	>1000	654	7
		7	0	0	0	0	0				>1000	402
		14	0	0	0	0	0				>1000	585
		28	0	0	0	0	0				>1000	1000
P. aeruginosa	9027	0	>1000	0	0	0	0	>1000	>1000	>1000	80	
		7	0	0	0	0	0				>1000	910
		14	0	0	0	0	0				>1000	>1000
		28	0	0	0	0	0				>1000	>1000
E. coli	8739	0	>1000	100	4	11	2	>1000	>1000	>1000	245	
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0				>1000	>1000
		28	0	0	0	0	0				>1000	>1000
Candida albicans	10231	0	>1000	>1000	979	520	232	>1000	350	36	5	
		7	>1000	1000	900	485	199		900	131	21	5
		14	>1000	920	518	300	87		>1000	625	82	15
		28	$>>10^3$	380	511	105	22		>1000	310	34	6
A. brasiliensis	16404	0	>1000	>1000	571	110	19	>1000	>1000	150	18	
		7	>1000	500	200	100	58		151	25	3	
		14	900	451	125	98	31		124	14	3	
		28	700	265	122	65	12		130	9	3	

Table 4.13: Preservative efficacy test for Indomethacin emulgel containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	>1000	287	30	3	0		731	76	7	>1000
		7	800	96	0	0	0			>1000	>1000	>1000
		14	28	3	0	0	0					>1000
		28	0	0	0	0	0					>1000
P. aeruginosa	**9027**	**0**	344	36	4	0	0		>1000	697	61	
		7	48	32	0	0	0			>1000	>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					>1000
E. coli	**8739**	**0**	112	10	0	11	0		384	19	3	
		7	0	0	0	0	0			>1000	>1000	>1000
		14	0	0	0	0	0					>1000
		28	0	0	0	0	0					>1000
Candida albicans	**10231**	**0**	>1000	581	55	6	1		>1000	>1000	188	
		7	914	250	312	3	0			658	65	>1000
		14	300	53	25	1	0			>1000	900	89
		28	>1000	300	80	62	5					11
A. brasiliensis	**16404**	**0**	>1000	>1000	900	700	122		HG	HG	1	
		7	>1000	900	600	112	58			100	10	1
		14	752	600	354	100	45			122	10	0
		28	700	584	321	125	12				21	23

Table 4.14: Preservative efficacy test for Ibuprofen gel containing 10% w/w of guava leaves extract powder as a natural preservative

Micro-organism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					Control Dilution				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	>1000	>1000	>1000	0	0			>1000	>1000	
		7	0	0	0	0	0				700	205
		14	0	0	0	0	0				76	81
		28	0	0	0	0	0				758	
P. aeruginosa	**9027**	**0**	>1000	>1000	>1000	1200	99			>1000	140	
		7	600	450	300	11	0				>1000	>1000
		14	390	200	112	8	0				>1000	>1000
		28	116	65	32	5	0					>1000
E. coli	**8739**	**0**	>1000	>1000	1200	200	0			>1000	620	
		7	0	0	0	0	0				>1000	>1000
		14	0	0	0	0	0				>1000	800
		28	0	0	0	0	0					741
Candida albicans	**10231**	**0**	>>10^3	>>10^3	714	22	18		244	35	3	
		7	>1000	500	132	23	11		>1000	390	63	
		14	>1000	400	126	20	9		>1000	853	151	
		28	1000	350	112	7	2			730	132	
A. brasiliensis	**16404**	**0**	>>10^3	>1000	>1000	112	32		272	36	3	
		7	>1000	400	365	128	22		>1000	965	11	
		14	921	398	355	118	19		>1000	785	119	
		28	800	221	118	87	14			>1000	952	

4.2.3 Pharmaceutical Preparations with natural preservative (guava leaves extract 10% w/w) VS. Pharmaceutical Preparations with Chemical Preservatives (methyl and propyl paraben) VS. Pharmaceutical Preparations without any preservative.

Each of Pharmaceutical Preparations (Ketoconazole shampoo, Clotrimazole Cream, and Ibuprofen gel) was prepared three times:

- First preparations were prepared with natural preservative (guava leaves extract 10% w/w)
- Second preparations (Positive control) were prepared with chemical Preservatives (methyl and propyl paraben)
- Third Preparations (Negative control) were prepared without any preservative

Antimicrobial tests were performed for the three preparations. Results for preparations with natural preservative and chemical preservative were very similar in all conducted tests. Small difference was noted for ibuprofen gel preparation where chemical preservatives completely inhibited the growth of Candida albicans and A. brasiliensis at 14 and 28 days, while the powder guava leaves extract as a natural preservative did not yield a complete inhabitation. However, its results complied with the tests criteria, and passed the tests.

The Third preparations were tested without any preservative (chemical or natural) to investigate if they remain safe against contamination, the antimicrobial tests results confirmed that these preparations failed, and proved that a preservative must be added to these preparations to ensure the consumers safety.

Table 4.15: Anti-microbial test for ketoconazole shampoo with: (natural preservative, chemical preservative, and no preservative)

Micro-organism 10^6 CFU/ml	ATCC No.	Day	With extract 10% w/w As a preservative		Positive control (chemical preservative) Methyl Paraben: 0.12 % Propyl paraben: 0.08 %		Negative control (without preservative)		Control Dilution	
			10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-5}	10^{-6}
S. aureus	6538	0	979	0	>1000	0	>1000	60	58	7
		7	0	0	0	0	985	658	$>>10^3$	>1000
		14	0	0	0	0	>1000	418		452
		28	0	0	0	0	>1000	112		219
P. aeruginosa	9027	0	$>>10^3$	230	>1000	0	>1000	60	70	
		7	>1000	0	984	0	>1000	665	$>>10^3$	>1000
		14	580	0	110	0	>1000	954		>1000
		28	27	0	0	0	>1000	500		622
E. coli	8739	0	>1000	1	>1000	11	>1000	111	250	
		7	250	0	118	0	885	452	$>>10^3$	>1000
		14	0	0	0	0	>1000	500		884
		28	0	0	0	0	>1000	700		435
Candida albicans	10231	0	>1000	8	979	0	$>>10^3$	1	14	
		7	0	0	0	0	>1000	15	944	
		14	0	0	0	0	999	14	575	
		28	0	0	0	0	312	0	196	
A. brasiliensis	16404	0	>1000	7	>1000	1	>1000	16	7	
		7	0	0	0	0	>1000	0	237	
		14	0	0	0	0	952	0	179	
		28	0	0	0	0	111	0	230	

Table 4.16: Anti-microbial test for Clotrimazole Cream with: (natural preservative, chemical preservative, and no preservative)

Micro-organism 10^6 CFU/ml	ATCC No.	Day	With extract 10% w/w As a preservative		Positive control (chemical preservative) Methyl Paraben: 0.2% Propylparaben: 0.07%		Negative control (without preservative)		Control Dilution	
			10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-5}	10^{-6}
S. aureus	6538	0	>1000	5	>1000	0	>1000	60	>1000	>1000
		7	276	0	0	0	>1000	985	>1000	>1000
		14	0	0	0	0	>1000	365	735	75
		28	0	0	0	0	>1000	>1000	>1000	>1000
P. aeruginosa	9027	0	>1000	16	>1000	0	>1000	60	212	
		7	600	0	0	0	>1000	>1000	>1000	>1000
		14	0	0	0	0	112	854	>1000	473
		28	0	0	0	0	>1000	400	>1000	>1000
E. coli	8739	0	$>>10^3$	280	>1000	11	>1000	111	212	
		7	>1000	0	0	0	654	954	>1000	>1000
		14	600	0	0	0	>1000	852	>1000	>1000
		28	96	0	0	0	>1000	900	>1000	478
Candida albicans	10231	0	>1000	0	>1000	0	$>>10^3$	6	2	
		7	0	0	0	0	>1000	2	0	
		14	0	0	0	0	112	0	91	13
		28	0	0	0	0	22	0	>1000	478
A. brasiliensis	16404	0	HG	1	>1000	0	>1000	16	212	
		7	>1000	0	654	0	965	0	514	13
		14	0	0	0	0	0	0	65	218
		28	0	0	0	0	0	0	>1000	

Table 4.17: Anti-microbial testing for Ibuprofen gel with (natural preservative, chemical preservative, and no preservative)

Micro-organism 10^6 CFU/ml	ATCC No.	Day	With extract 10% w/w As a preservative		Positive control (chemical preservative) Methyl Paraben: 0.1% Propyl paraben: 0.01%		Negative control (without preservative)		Control Dilution	
			10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-1}	10^{-5}	10^{-5}	10^{-6}
S. aureus	6538	0	>1000	0	>1000	0	>1000	147	>1000	
		7	0	0	0	0	118	325	700	205
		14	0	0	0	0	>1000	25	76	81
		28	0	0	0	0	>1000	12	758	
P. aeruginosa	9027	0	>1000	99	>1000	0	>1000	60	>1000	>1000
		7	600	0	0	0	>1000	985	>1000	>1000
		14	390	0	0	0	>1000	623		>1000
		28	116	0	0	0	>1000	257	>1000	>1000
E. coli	8739	0	>1000	0	>1000	11	>1000	111	620	
		7	0	0	0	0	125	452	>1000	>1000
		14	0	0	0	0	587	846	>1000	800
		28	0	0	0	0	>1000	112		741
Candida albicans	10231	0	$>>10^3$	18	>1000	0	$>>10^3$	6	3	
		7	>1000	11	>1000	0	>1000	187	63	
		14	>1000	9	145	0	>1000	251	151	
		28	1000	2	0	0	>1000	156	132	
A. brasiliensis	16404	0	$>>10^3$	32	>1000	0	>1000	16	3	
		7	>1000	22	865	0	915	112	11	
		14	921	19	0	0	>1000	512	119	
		28	800	14	0	0	>1000	620	952	

4.3 Ketoconazole shampoo containing 10% w/w powered-guava leaves extract

4.3.1 Preparation Specifications of Ketoconazole Shampoo

Table 4.18: Specifications of Ketoconazole Shampoo.

Test	preparation with chemical preservative	Preparation with guava leaves extract as a preservative
Physical appearance (blue clear thick shampoo)	blue clear thick shampoo	Dark green (oily color) clear thick shampoo
Odor (Fresh kiwi odor)	Fresh kiwi odor	Kiwi
pH (as it is) (5.5 – 7.5)	6.6	6.8
Density 1.02 – 1.05 g/ml	1.03 g/ml	1.03 g/ml
Viscosity (20-22 °C) (800-1700 cp)	1450 cp	1500 cp

A B

Figure 4.7: (A) ketoconazole shampoo after mixing, (B) ketoconazole shampoo in direct packaging material

Odor, Density, Viscosity and pH results of Ketoconazole shampoo containing 10% w/w of guava leaves extract powder as a natural preservative were comparable to the results of Ketoconazole shampoo with a chemical preservative. Thus, it is safe to declare that the natural preservative did not affect the specification of odor, density, viscosity and pH of Ketoconazole shampoo. The physical appearance changed from blue in chemical preservative preparation to green with using powdered guava leave extract as a preservative. This change occurred because the ketoconazole shampoo without coloring agent is transparent, so adding a blue color would transfer it to blue clear shampoo. On the other hand, when we added a natural preservative which has a brown color. Mixing these two colors together in the formula yielded gave a green color, which was beautiful and acceptable. Active ingredient (ketoconazole) was determined by HPLC and the natural preservative was checked-up in the preparation (in terms of its effectiveness as antimicrobial natural preservative) for three months. The three shampoo preparations were stored at accelerated conditions (40 ± 2 °C / 75% ± 5% RH) to test the stability of the shampoo (concentration of active ingredient (ketoconazole), odor, density, viscosity, and pH).

HPLC analysis for the active ingredient (ketoconazole) for three months was 99.5 % at zero days decreased to 98.5 % in the first month, 97% in the second month, and to 96 % in the third month of storage (Figure 4.9). The results indicated that the ketoconazole shampoo was stable and wasn't affected by the addition of guava leave extract.

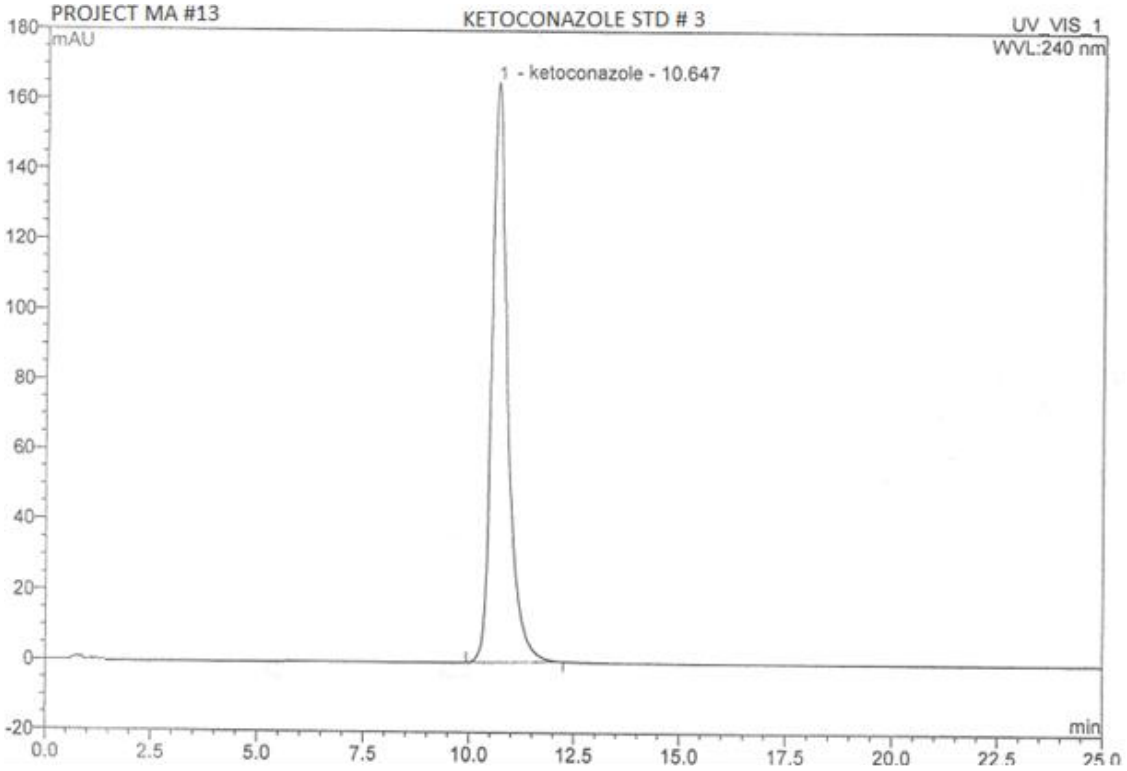


Figure 4.8: HPLC-UV-vis Chromatogram of standard ketoconazole

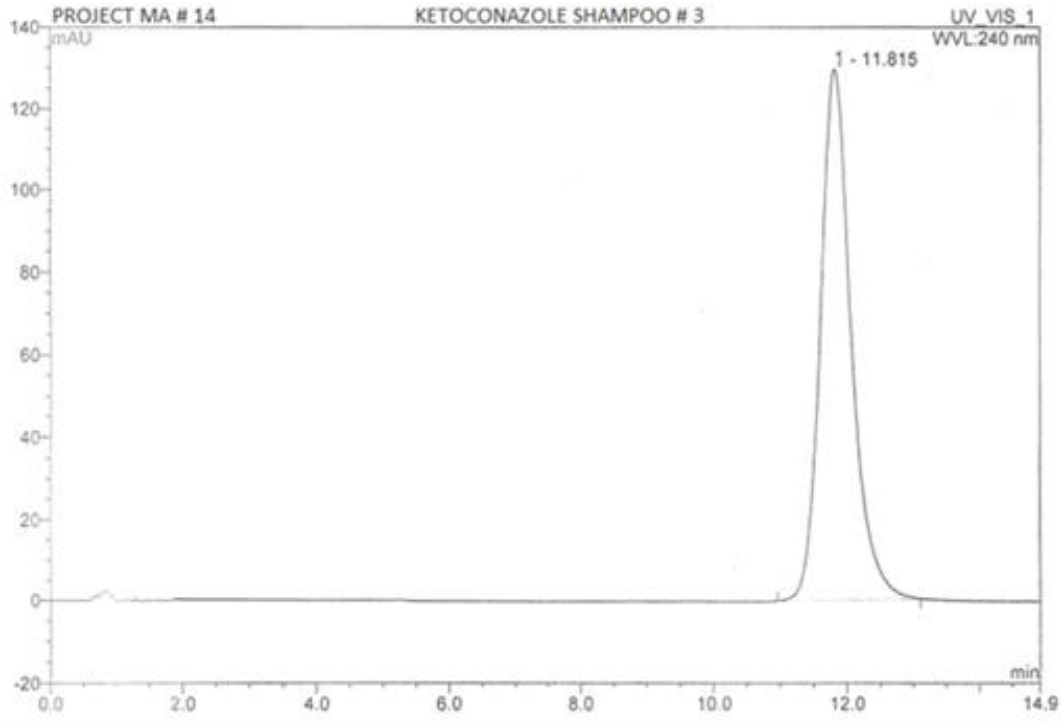


Figure 4.9: Chromatogram of ketoconazole in shampoo preparation, stored at accelerated Condition (40 ± 2 °C / 75% ± 5% RH) for three months.

4.3.2. Antimicrobial test of Ketoconazole shampoo:

4.3.2.1 Microbial limit test – Direct transfer (broth media) for Ketoconazole shampoo containing 10% w/w of guava leaves extracts powder:

Direct transfer (broth media) tests were conducted at zero days, and at the first, second, and third month of storage, and compared to the positive control (Ketoconazole shampoo with methyl paraben and propylparaben).

As shown in table (4.19), two media were used (Tryptic soy Broth, and Sabouraud dextrose broth) to test microbial growth. The results showed no microbial growth activity was observed in ketoconazole shampoo containing 10% w/w of guava leaves extract. These results agreed with these obtained for the positive control ((Ketoconazole shampoo with chemical preservative (methyl paraben and propylparaben). Comparing between results of natural- and chemical- preservative confirmed that our natural preservative was good enough to be used as alternative to conventional chemical preservatives. Results of negative control (Without Preservative) showed bacterial growth, but no fungal growth. This might be attributed to the fact that the formula contained ketoconazole which is an antifungal.

Table 4.19: Microbial limit test–Direct transfer (broth media) test for Ketoconazole shampoo.

Medium	**Results for Ketoconazole shampoo**		
	With 10% w/w of guava leaves extract as a natutral preservative	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy Broth	Clear	Clear	Turbid
Sabouraud dextrose broth	Clear	Clear	Clear

4.3.2.2 Microbial limit test –Total count for Ketoconazole shampoo:

The results of the total count test for Ketoconazole shampoo were illustrated in table (4.20). They were identical for both Ketoconazole shampoo containing 10% w/w of guava leaves extract and Ketoconazole shampoo with chemical preservative (methyl-paraben and propylparaben), Result of negative control (Without Preservative) showed that the bacteria number were very high compared to the preparations that contained natural and chemical preservatives.

Table 4. 20: Microbial limit test –Total count test for Ketoconazole shampoo.

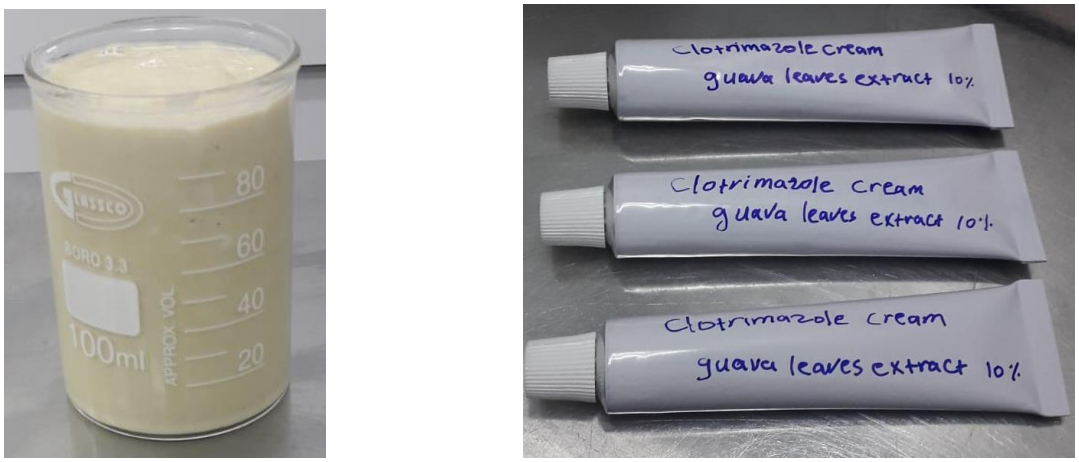
Medium	**Results of Ketoconazole shampoo CFU #/ml**			
	Limits on agar CFU #/ml	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy agar	Bacteria $\leq 200(10^2)$	< 10	< 10	< 210
Sabouraud dextrose agar	yeasts and molds $\leq 20\ (10^1)$	< 10	< 10	< 10

4. 4 Clotrimazole Cream containing 10% w/w guava leaves extract powder

4.4.1 Preparation Specifications of Clotrimazole Cream

Table 4.21: Specifications of Clotrimazole Cream.

Test	preparation with chemical preservative	Preparation with guava extract as preservative
Physical appearance (White, thick homogeneous cream)	White, thick homogeneous cream	Beige, thick homogeneous cream
Odor (Odorless Cream)	Odorless	Odorless
pH (5.0 g in 20 ml H_2O) 5.0 – 7.0	5.9	6.1



A B

Figure 4.10: (A) Clotrimazole Cream after mixing, (B) Clotrimazole Cream in direct packaging material

Odor and pH results of Clotrimazole Cream containing 10% w/w of guava leaves extract powder as a natural preservative result were so similar to the results of Clotrimazole Cream with chemical preservative so the natural preservative did not affect the specification of odor, pH of Clotrimazole Cream. However physical appearance was changed from white in chemical preservative preparation to beige in natural preservative preparation this change in color occurred because the natural preservative color was brown. The final color (beige) was beautiful and acceptable color. Active ingredient (Clotrimazole) was determined by HPLC and the natural preservative checked-up in the preparation in terms of its effectiveness as antimicrobial for three months. The three cream preparations were stored at accelerated conditions (40 ± 2 °C / 75% ± 5% RH) to test the stability of the cream (assay for active ingredient (Clotrimazole), odor, and pH, microbial limit).

HPLC analysis of the active ingredient (Clotrimazole) for three months was 99.9 % at zero days decreased to 97.5 % in the first month, 96% in the second month, 95 % in the third month of storage see Figure (4.12). The results indicated that the Clotrimazole cream was stable and wasn't affected by the addition of guava leave extract.

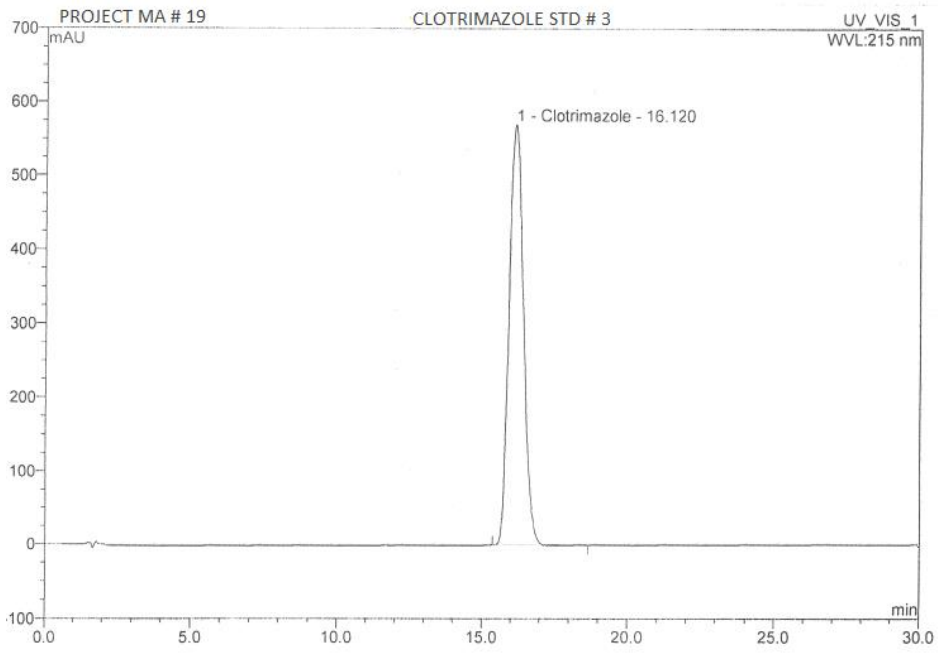


Figure 4.11: HPLC-UV-vis Chromatogram of standard Clotrimazole

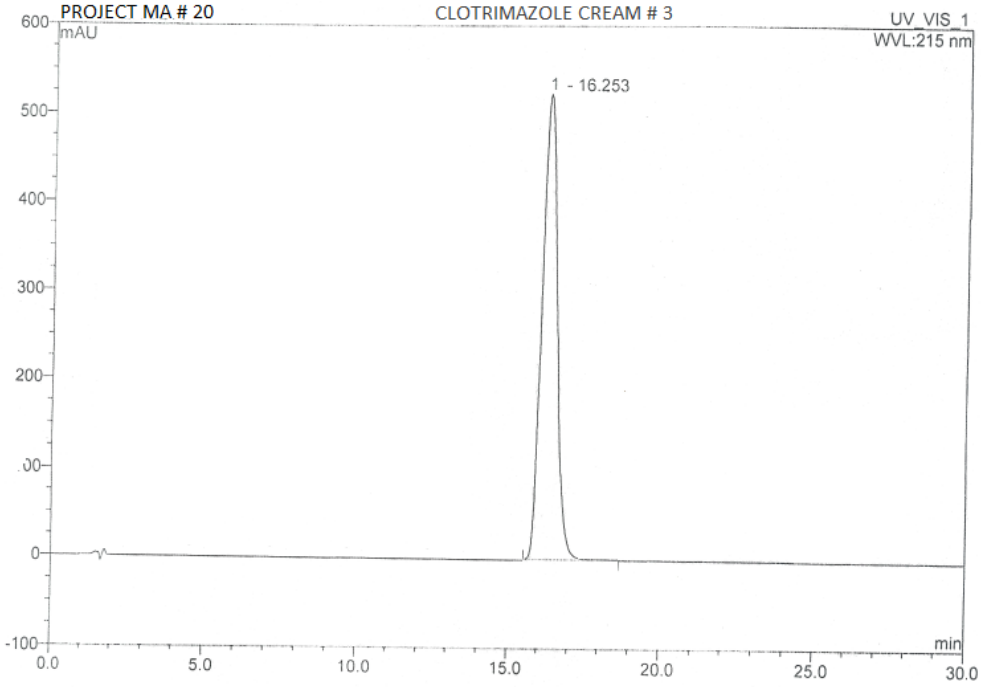


Figure 4.12: HPLC-UV-vis Chromatogram of Clotrimazole in cream preparation, stored at accelerated Condition (40 ± 2 °C / 75% ± 5% RH) for three months.

4.4.2 Microbial limit test

4.4.2.1 Direct transfer (broth media) for Clotrimazole Cream:

Microbial limit test for Clotrimazole Cream containing 10% w/w of guava leaves extracts powder was done at zero-day and in the first, second, and third month of storage, and compared to the positive control (Clotrimazole Cream with methyl paraben and propylparaben) and negative control (Without Preservative).

As shown from table (4.22) two medium were used (Tryptic soy Broth, Sabouraud dextrose broth) to test microbial growth and the results showed that no microbial growth occurred in Clotrimazole Cream containing 10% w/w of guava leaves extract which complied the results that obtained from the positive control (Clotrimazole Cream with chemical preservative (methyl paraben and propylparaben)), conversion between results of natural and chemical preservative confirm our natural preservative is good enough to be used. Results of negative control (Without Preservative) showed bacterial growth, but no fungal growth, this may attribute to the fact that the formula contained Clotrimazole which is an antifungal.

Table 4.22: Microbial limit test–Direct transfer (broth media) test for Clotrimazole Cream.

Medium	**Results for Clotrimazole Cream**		
	With 10% w/w of guava leaves extract as a natutral preservative	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy Broth	Clear	Clear	Turbid
Sabouraud dextrose broth	Clear	Clear	Clear

4.4.2.2- Microbial limit test –Total count for Clotrimazole Cream:

The total microbial count results of Clotrimazole Cream are illustrated in table (4.23). Results of both Clotrimazole Cream containing 10% w/w of guava leaves extract and Clotrimazole Cream with a chemical preservative (methyl paraben and propyl paraben) were identical for all tested microorganisms: Bacteria < 10 (Limits on agar ≤ 200), yeasts and molds < 10 (Limits on agar ≤ 20), Result of negative control (Without Preservative) showed that the bacteria number were high compared to the preparations that contained natural and chemical preservatives.

Table 4.23: Microbial limit test –Total count test for Clotrimazole Cream.

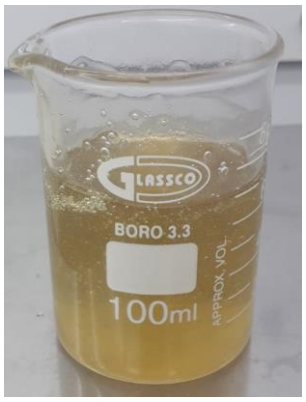
Medium	**Results of Clotrimazole Cream CFU #/ml**			
	Limits on agar CFU #/ml	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy agar	Bacteria ≤ 200(10^2)	< 10	< 10	< 180
Sabouraud dextrose agar	yeasts and molds ≤ 20 (10^1)	< 10	< 10	< 10

4.5 Ibuprofen gel containing 10% w/w guava leaves extract powder

4.5.1 Preparation Specifications for Ibuprofen gel

Table 4.24: Specifications of Ibuprofen gel.

Test	preparation with chemical preservative	Preparation with guava extract as preservative
Physical appearance (White, consistent thick Emulgel, no crystals appear)	White, consistent thick Emulgel, no crystals appear	Brown (oily), consistent thick Emulgel, no crystals appear
Odor (Isopropyl Alcohol odor)	Isopropyl Alcohol odor	Isopropyl Alcohol odor
pH (5.0 g Emulgel in 30 ml H_2O) (3.0 – 6.0)	4.1	4.5



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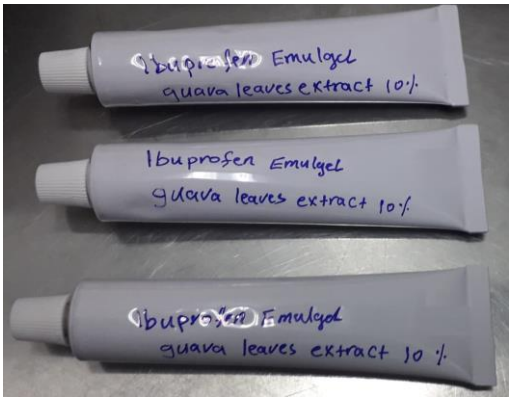

B

Figure 4.13: (A) Ibuprofen gel after mixing, (B) Ibuprofen gel in direct packaging material.

Odor and pH results of Ibuprofen gel containing 10% w/w of guava leaves extract powder as a natural preservative were so close to the results of Ibuprofen gel with a chemical preservative. So, it is safe to say the natural preservative does not affect the specification of odor and pH for Ibuprofen gel. However physical appearance changed from white in chemical preservative preparation to oily in natural preservative preparation because the natural preservative color was brown. The final color was still beautiful and acceptable. Active ingredient (Ibuprofen) was determined by HPLC and the natural preservative checked-up in the preparations in terms of its effectiveness as antimicrobial for three months. The three emulgel preparations were stored at accelerated conditions (40 ± 2 °C / 75% ± 5% RH) to test the stability of the emulgel (assay for the active ingredient (Ibuprofen), odor and pH).

HPLC analysis of the active ingredient (Ibuprofen) for three months was 97.5 % at zero days decreased to 95.5 % in the first month, 94% in the second month, 92 % in the third month of storage see Figure (4.15). The results indicate that the Ibuprofen is stable and not affected by the addition of guava leave extract.

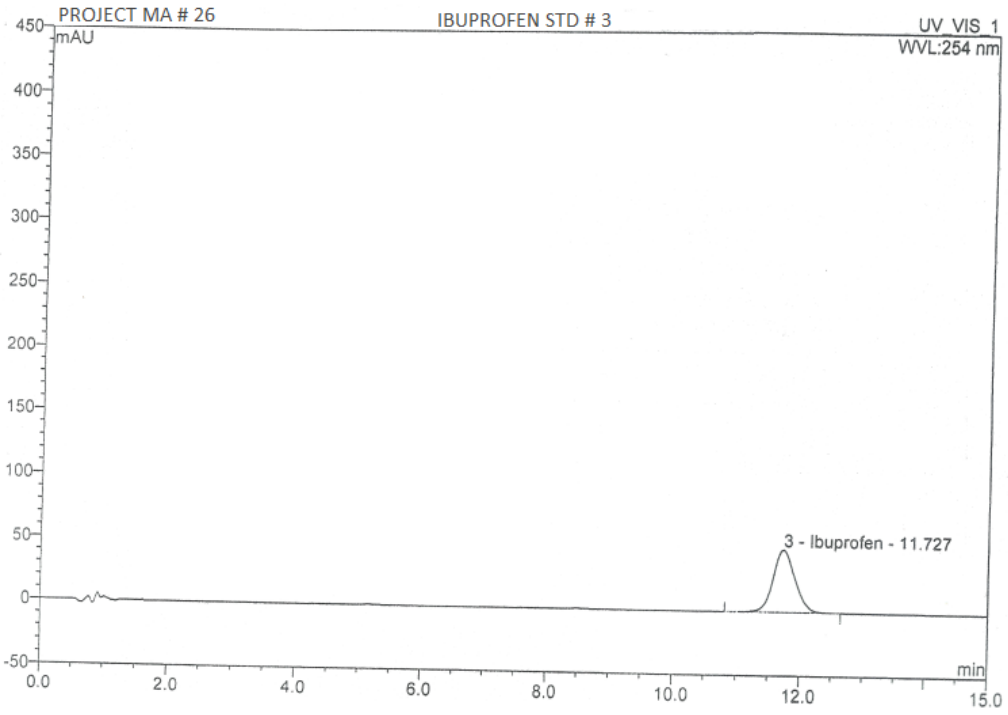

Figure 4.14: HPCL-UV-Vis chromatogram of standard Ibuprofen

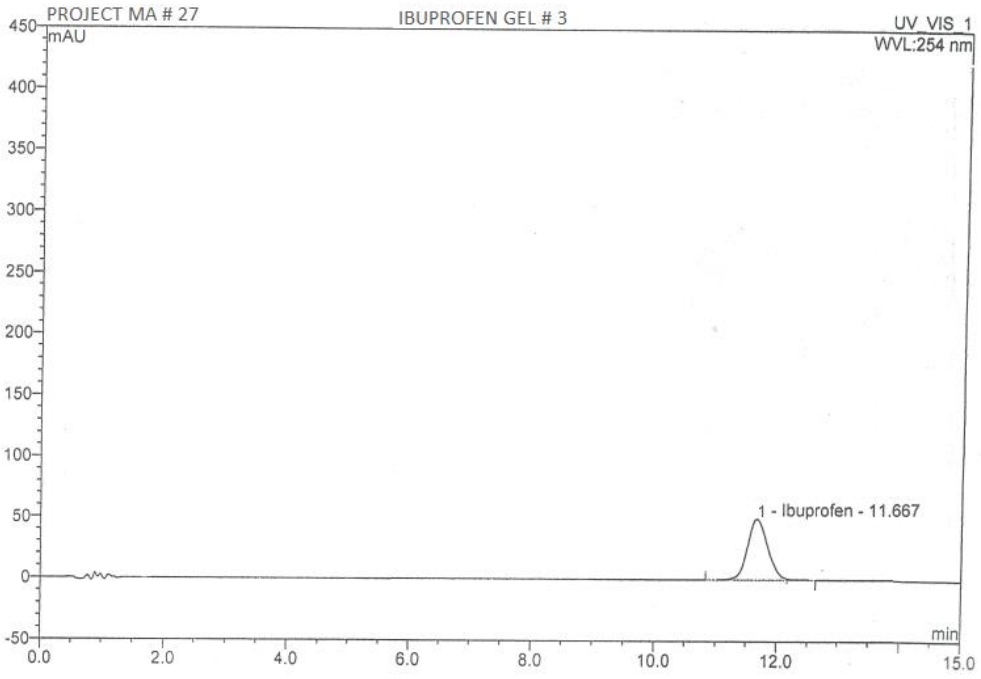

Figure 4.15: HPLC-UV-VI Chromatogram of Ibuprofen in emulgel preparation, stored at accelerated Condition (40 ± 2 °C / 75% ± 5% RH) for three months.

4.5.2 Microbial limit test

4.5.2.1 Direct transfer (broth media) for Ibuprofen emulgel:

Microbial limit test for Ibuprofen emulgel containing 10% w/w of guava leaves extract powder was performed at zero-days, the first, the second, and the third month of storage. Results were compared to that of the positive control (Ibuprofen emulgel with methyl paraben and propylparaben), and negative control (Medium only).

As shown in table 4.25, two media were used to test microbial growth, Tryptic soy Broth, and Sabouraud dextrose broth. The results revealed no microbial growth was observed for Ibuprofen emulgel containing 10% w/w of guava leaves extract. This was in agreement with the results obtained in the positive control ((i.e., Ibuprofen emulgel with chemical preservative (methyl paraben and propylparaben)). Comparison n between results of antibacterial activity obtained with natural and chemical preservative confirmed that powdered guava leaves extract could be used as a natural preservative and replace conventional chemical preservatives. It should be noted that bacterial growth activities were observed for the negative control medium (i.e., without preservative).

Table 4.25: Microbial limit test – Direct transfer (broth media) for Ibuprofen emulgel**.**

Medium	**Results for Ibuprofen Emulgel**		
	With 10% w/w of guava leaves extract as a natutral preservative	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy Broth	Clear	Clear	Turbid
Sabouraud dextrose broth	Clear	Clear	Turbid

4.5.2.2 Microbial limit test –Total count for Ibuprofen emulgel:

The total microbial count results of Ibuprofen emulgel are shown in table 4.26. Results for both Ibuprofen emulgel containing 10% w/w of guava leaves extract and Ibuprofen emulgel with chemical preservative (methyl paraben and propylparaben) were identical for all tested microorganism (Bacteria < 10 (Limits on agar ≤ 200), yeasts and molds < 10 (Limits on agar ≤ 20)). Result of negative control (Without Preservative) revealed that the bacterial and fungal numbers were very high compared to the preparations that contained natural and chemical preservatives.

Table 4.26: Microbial limit test –Total count test for Ibuprofen emulgel.

Medium	**Results of Ibuprofen emulgel CFU #/ml**			
	Limits on agar CFU #/ml	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Tryptic soy agar	Bacteria ≤ 200(10^2)	< 10	< 10	< 220
Sabouraud dextrose agar	yeasts and molds ≤ 20 (10^1)	< 10	< 10	< 20

4.6 Stability Test of Pharmaceutical Preparations

4.6.1 Chemical Stability Test of Pharmaceutical Preparations

The Specifications (Physical appearance, odor, pH, and Assay of active ingredient) of preparations (Ketoconazole Shampoo, Clotrimazole cream, Ibuprofen gel) were tested for three months at accelerated conditions (40 ± 2 °C / 75% ± 5% RH). The results for all preparations complied with the accepted limit and passed the tests; such results confirmed that our preparations were stable

Table 4.27: Finished Preparation Specifications of Ketoconazole Shampoo at Accelerated Condition (40 ± 2 °C / 75% ± 5% RH).

Time	Physical appearance	Odor	PH	Density g/ml	Viscosity cp	Assay of Ketoconazole
Zero time	Dark green (oily color) clear thick shampoo	Kiwi	6.8	1.03	1500	99.5%
First month	Dark green (oily color) clear thick shampoo	Kiwi	6.8	1.03	1509	98.5 %
Second month	Dark green (oily color) clear thick shampoo	Kiwi	6.5	1.04	1508	97%
Third month	Dark green (oily color) clear thick shampoo	Kiwi	6.3	1.04	1520	96 %

Table 4.28: Finished Preparation Specifications for Clotrimazole Cream at Accelerated Condition (40 ± 2 °C / 75% ± 5% RH).

Time	Physical appearance	Odor	PH	Assay of Clotrimazole
Zero time	Beige, thick homogeneous cream	Odorless	6.1	99.9 %
First month	Beige, thick homogeneous cream	Odorless	6.0	97.5 %
Second month	Beige, thick homogeneous cream	Odorless	6.0	96%
Third month	Beige, thick homogeneous cream	Odorless	6.0	95%

Table 4.29: Finished Preparation Specifications for Ibuprofen Gel at Accelerated Condition (40 ± 2 °C / 75% ± 5% RH).

Time	Physical appearance	Odor	PH	Assay of Ibuprofen
Zero time	Brown (oily), consistent thick Emulgel, no crystals appear	Isopropyl Alcohol odor	4.5	97.5 %
First month	Brown (oily), consistent thick Emulgel, no crystals appear	Isopropyl Alcohol odor	4.4	95.5 %
Second month	Brown (oily), consistent thick Emulgel, no crystals appear	Isopropyl Alcohol odor	4.4	94 %
Third month	Brown (oily), consistent thick Emulgel, no crystals appear	Isopropyl Alcohol odor	4.4	92 %

4.6.2 Microbial Stability of Pharmaceutical Preparations

For microbial stability of our pharmaceutical preparations, direct transfer test and total count test were conducted for three months at accelerated condition (40 ± 2 °C / 75% ± 5% RH). The results of direct transfer test (Tryptic soy Broth Medium and Sabouraud dextrose broth Medium) were clear (not turbid), while total count for Tryptic soy agar Bacteria was ≤ 200(10^2), and for Sabouraud dextrose agar yeasts and molds was ≤ 20 (10^1).

All results of the preparations with a natural preservative (guava leaves extract 10% w/w) were within the accepted limit (see tables 4.30 to 4.35). The results of negative control confirmed the necessity of the presence of preservatives in all tested preparations. The final result of preparations confirmed that our preparations were microbial stable.

Table 4.30: Antimicrobial limit (Direct transfer test) for Ketoconazole Shampoo at accelerated conditions (40 ± 2 °C / 75% ± 5% RH).

Time	**Direct transfer (broth media)**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
First month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
Second month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
Third month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear

Table 4.31: Antimicrobial limit (Total count test) for Ketoconazole shampoo at accelerated conditions (40 ± 2 °C / 75% ± 5% RH)**.**

Time	**Total count**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy agar	< 10	< 10	< 120
	Sabouraud dextrose agar	< 10	< 10	< 10
First month	Tryptic soy agar	< 10	< 10	< 180
	Sabouraud dextrose agar	< 10	< 10	< 10
Second month	Tryptic soy agar	< 10	< 10	< 180
	Sabouraud dextrose agar	< 10	< 10	< 10
Third month	Tryptic soy agar	< 10	< 10	< 210
	Sabouraud dextrose agar	< 10	< 10	< 10

Table 4.32: Antimicrobial limit (Direct transfer test) for Clotrimazole Cream at accelerated conditions (40 ± 2 °C / 75% ± 5% RH)**.**

Time	**Direct transfer (broth media)**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
	Tryptic soy Broth	Clear	Clear	Turbid

First month	Sabouraud dextrose broth	Clear	Clear	Clear
Second month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
Third month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear

Table 4.33: Antimicrobial limit (Total count test) for Clotrimazole Cream at accelerated conditions (40 ± 2 °C / 75% ± 5% RH).

Time	**Total count**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy agar	< 10	< 10	< 30
	Sabouraud dextrose agar	< 10	< 10	< 10
First month	Tryptic soy agar	< 10	< 10	< 30
	Sabouraud dextrose agar	< 10	< 10	< 10
Second month	Tryptic soy agar	< 10	< 10	< 30
	Sabouraud dextrose agar	< 10	< 10	< 10
Third month	Tryptic soy agar	< 10	< 10	< 180
	Sabouraud dextrose agar	< 10	< 10	< 10

Table 4.34: Antimicrobial limit (Direct transfer test) for Ibuprofen gel at accelerated conditions (40 ± 2 °C / 75% ± 5% RH)).

Time	**Direct transfer (broth media)**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Turbid
First month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Turbid
Second month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Turbid
Third month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Turbid

Table 4.35: Antimicrobial limit (Total count test) of Ibuprofen gel at accelerated conditions (40 ± 2 °C / 75% ± 5% RH)**.**

Time	**Total count**	**With 10% w/w of guava leaves extract as a natutral preservative**	**positive control** with chemical preservative (methyl and propyl paraben	**Negative control** (Without Preservative)
Zero time	Tryptic soy agar	< 10	< 10	< 100
	Sabouraud dextrose agar	< 10	< 10	< 20
First month	Tryptic soy agar	< 10	< 10	< 100
	Sabouraud dextrose agar	< 10	< 10	< 20

Second month	Tryptic soy agar	< 10	< 10	< 110
	Sabouraud dextrose agar	< 10	< 10	< 20
Third month	Tryptic soy agar	< 10	< 10	< 220
	Sabouraud dextrose agar	< 10	< 10	< 20

Chapter Five: Conclusion and Future work

5. Conclusion and Future work

5.1 Conclusions

This research intended to incorporate herbal antimicrobial agent (preservative) instead of using a chemical preservative. The natural preservative was extracted from guava leaves simply and easily by using sonication with ethanol as extracting agent. The efficiency of guava leaves extract against microorganisms was studied with the optimum concentration at 10% w/w, which was a concentration that could be used as a preservative in pharmaceutical preparations.

Guava leaves extract cannot serve as a preservative in every pharmaceutical preparation so it is important to conduct a preservative efficacy tests for any preparation that will contain it as a preservative to ensure that it is effective.

The results of three out of six preparations passed all tests that were carried out to evaluate efficiency as a preservative. Specifications (odor, pH, and assay of active ingredient) of preparations weren't affected by using natural substances to pharmaceutical formulations as an alternative to conventional chemical preservatives. Stability tests for passed preparations at accelerated condition for three months passed the stability criteria and confirmed that they were stable and effective during the entire storage period of three months. The results of this

research will help researchers to pay more attention to the potential importance of natural herbal ingredients as natural preservative in pharmaceutical formulations instead of conventional chemicals

5.2 Future work:

1. To test the feasibility of using other solvents to extract guava leaves and to investigate if we can reduce the concertation less than 10% with passed results.

2. To investigate that sided incorporating guava leaves with other natural extracts to enhance activity as antimicrobial and try to use lower concentration if it gives acceptable results.

3. To implement guava leaves extract in cosmetic preparations as an active ingredient since it has intrinsic properties (antimicrobial, anti-inflammatory, anti-oxidant).

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تحضير أشكال دوائية شبه صلبة تحتوي على مستخلص أوراق الجوافة كمادة حافظة مضادة للميكروبات

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الملخص

كما هو معروف، فإن مستخلص أوراق الجوافة له خصائص طبية عديدة، فهو مضاد للميكروبات، مضاد للأكسدة، مضاد للسرطان، ومضاد للالتهابات. تم طرح العديد من الدراسات لاستخدام مستخلص أوراق الجوافة في العديد من المستحضرات. ومع ذلك، فإن استخدام مستخلص أوراق الجوافة كمادة حافظة في منتج صيدلاني لم يتم تناوله في الدراسات السابقة. في البحث الحالي، نحاول استخدام مادة حافظة طبيعية بدلاً من المادة الكيميائية (ميثيل وبربيل برابين) حيث أن العديد من الدراسات كانت تحذر من تأثيرها وأنه يجب استخدامها بتراكيز محدودة. تم استخلاص أوراق الجوافة بمحلول إيثانول 95.5٪ كمذيب استخلاص. كانت النسبة المئوية للناتج هي 20٪ وهي نسبة مقبولة. تم اختبار هذا المستخلص بواسطة جهاز HPLC وتم الكشف عن العديد من المركبات الفينولية.

لتحديد إمكانية استخدام مستخلص ورق الجوافة كمادة حافظة، تم إجراء اختبار الفعالية المضادة للميكروبات (اختبار فعالية المواد الحافظة) للمسحوق المستخلص حيث تم اذابته في الماء النقي بتراكيز مختلفة (0.5، 2.5، 5، 7.5، 9.5، 10) ٪ وزن / وزن، ضد ثلاثة أنواع من البكتيريا، واحدة موجبة الجرام(Staphylococcus aureus) واثنتان سالبة الجرام (Escherichia coli and Pseudomonas aeruginosa) واثنتين من الفطريات: خميرة (Candida albicans) ، وعفن واحد (Aspergillus (Niger) brasiliensis) . أظهرت النتائج أن التركيز الوحيد الذي اجتاز الاختبار

كان 10٪. تم استخدام هذا التركيز في المنتجات الصيدلانية شبه الصلبة. لهذا الغرض، تم استخدام ست مستحضرات مختلفة (كيتوكونازول شامبو، كلوتريمازول كريم، بيرميثرين كريم، جنتامايسين كريم، ايبوبروفين جل، إندوميثاسين إيملجل). تم استبدال المادة الحافظة الكيميائية بمواد طبيعية (مسحوق مستخلص أوراق الجوافة) لتكون بمثابة مادة حافظة. ثلاثة فقط من أصل ست مستحضرات (كيتوكونازول شامبو، كلوتريمازول كريم، ايبوبروفين جل) اجتاز اختبار فعالية المواد الحافظة. تم فحص هذه المستحضرات التي نجحت بشكل كامل عن طريق الاختبار الكيميائي (تركيز المادة الفعالة)، الفيزيائي (الرائحة، المظهر الفيزيائي) والاختبار البيولوجي لمدة ثلاثة أشهر على ظروف التسريع. أكدت النتائج أن المستحضرات الصيدلانية كانت مستقرة وفعالة.