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FORMULATION AND EVALUATION OF ANTIFUNGAL CREAM OF GRAPE SEEDS EXTRACT AND POMEGRANATE FRUIT EXTRACT

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ABSTRACT

Fungal infections are of serious public health concern. The incidence of fungal infections in patients with other diseases including Covid-19 is associated with life threatening mycoses and mortality. Oxygen uptake while breathing cause's free radical production and in addition to that environmental factors such as pollutants, smoke and certain chemicals also contribute to their formation. Antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. Pomegranate is a potent antioxidant. This fruit is rich in flavonoids, anthocyanins, punicic acid, ellagitannins, alkaloids, fructose, sucrose, glucose, simple organic acids. The antioxidant property of proanthocyanidin is because of the availability of the phenolic-hydrogens as singlet oxygen quenchers and hydrogen donating radical scavengers. Creams having less adverse effect than dosage form.

KEYWORDS: Antifungals, Antioxidants, pomegranate, Grape seed, Topical cream, DPPH activity.

INTRODUCTION

Over the last decades the treatment of illness have been accomplished by administrating drugs to human body via various roots namely oral, sublingual, rectal, parental, topical, inhalation etc. Topical delivery can be defined as the application of a drug containing formulation to the skin to directly treat cutaneous disorder or the cutaneous manifestations of a general disease (eg. psoriasis) with the intent of containing the pharmacological or the effect of drug to the surface of the skin or within the skin semisolid formulations in all their diversity dominate the system for topical delivery, but foams, spray, medicated powders, solutions and even medicated adhesive systems are in use.[1]

Fungal infections are of serious public health concern. The incidence of fungal infections in patients with other diseases including Covid-19 is associated with life threatening mycoses and mortality. Fungal infections can include superficial, cutaneous, sub-cutaneous, mucosal and systemic infections with varying degree of severity. Organisms such as *Candida* spp. are part of human microbiota that can cause opportunistic infections in individuals and life threatening infections (invasive candidiasis) in immuno-compromised patients such as HIV patients, cancer patients receiving

chemotherapy, and patients receiving immuno-suppressive drugs. Besides, opportunistic and systemic infections, fungal pathogens such as *Candida*, *Aspergillus*, *Fusarium*, Mucorales and molds can cause healthcare-associated infections (HAI) in patients with underlying diseases. In certain geographical areas, fungal pathogens cause prevalent life-threatening endemic mycoses such as Blastomycosis, Coccidiodomycosis, Histoplasmosis, Talaromycosis, Paracoccidiodomycosis and Sporotrichosis.[2]

Systemic fungal infections are often diagnosed lately increasing mortality rates. Centers for Disease Control and Prevention (CDC) has declared September 20–24, 2021 as fungal disease awareness week, to educate and to highlight the importance of early diagnosis of fungal infections to alleviate the debilitating effects (CDC website). This article provides an overview of the spectrum of fungal infections in humans, pathogenesis, immune evasion mechanisms, antifungal drugs along with their mode of action, resistance mechanisms and alternate antifungal approaches to combat fungal infections.[3]

Fungal Pathogens and routes of transmission-

The sub-kingdom Dikarya of fungi comprising of the phyla Ascomycota and Basidiomycota is the major contributor of all fungal pathogens and infections in humans. Ascomycota organisms are known for causing oropharyngeal, otolaryngeal, dermatological, ophthalmic, neuronal, genitourinary, cardiac, pulmonary and systemic infections. The organisms of Basidiomycota such as *Cryptococcus* and *Malassezia* are well-known for invasive meningitis and superficial skin infections, respectively.

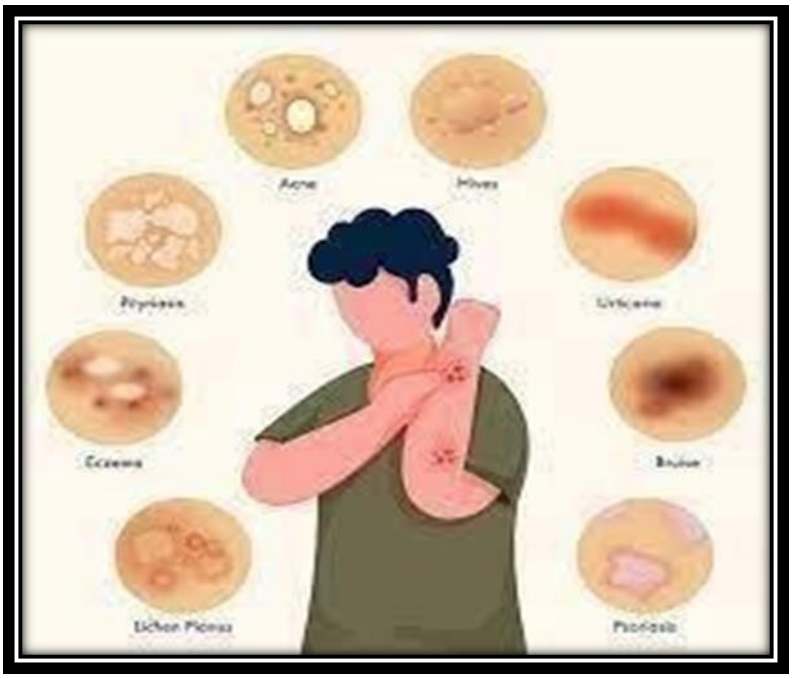

Fig. 01: Fungal infections.

Fungal pathogens primarily use direct contact and/or inhalation route for transmission. Dermatophytic fungi belonging to the genera of *Microsporum*, *Epidermophyton* and *Trichophyton*, *Sporothrix* and *Malassezia* spp. infect the damaged skin by direct contact. They produce various proteolytic enzymes to cause superficial mycoses in keratinized tissues. The other predominant route for transmission is by inhalation of spores/ conidia that instigates pulmonary infections. *Blastomyces dermatitidis* (Blastomycosis), *Paracoccidioides brasiliensis* and *P. lutzii* (Paracoccidiodomycosis),

Histoplasma capsulatum (Histoplasmosis), *Pneumocystis jirovecii* (Pneumocystis pnuemonia), *Aspergillus fumigatus* and *A. flavus* (Aspergillosis), *Coccidioides immitis* and *C. posadasii* (Coccidioidomycosis), *C. neoformans* and *C. gattii* (Cryptococcosis) are mainly transmitted through inhalation.

While, *Talaromyces marneffei* (talaromycosis) uses both direct contact and inhalation route for transmission.[4,5]

Antifungal agents and their mechanism of action

Antifungal drugs are the drugs which act against fungal species. Antifungal medications usually work either by killing the fungal cells or stopping them from growing and multiplying. Parts of the cell that the antifungal drugs target include the fungal cell membrane and the fungal cell wall. These are both protective parts of the cell that can cause the cell to leak and die when damaged.

Human bodies do not have these structures, meaning antifungal drugs can target the fungi without harming the body's cells. Antifungals can be grouped into three classes based on their site of action: azoles, which inhibit the synthesis of ergosterol (the main fungal sterol); polyenes, which interact with fungal membrane sterols physicochemically; and 5-fluorocytosine, which inhibits macromolecular synthesis.[6]

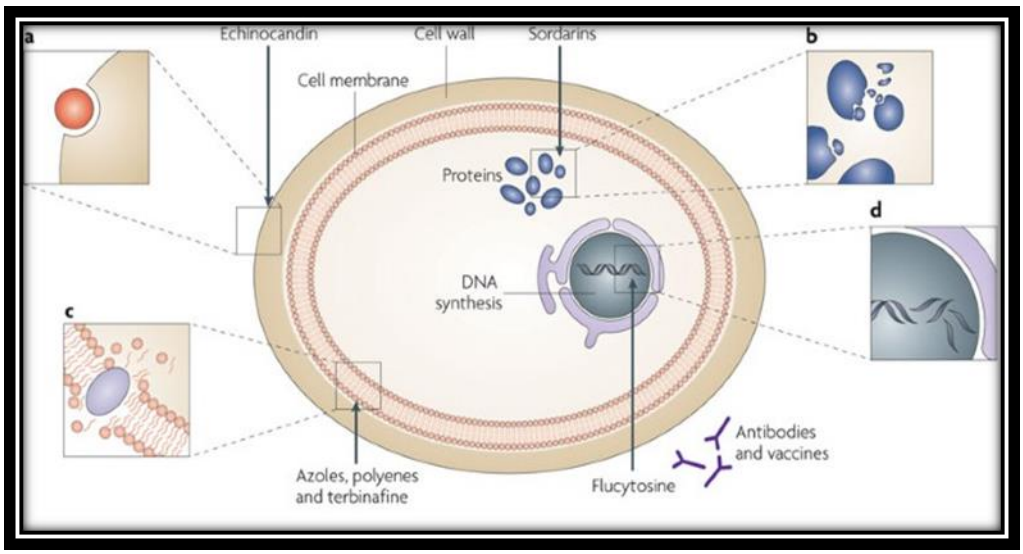

Fig. 02: Mechanism of action of Antifungal agent.

Antioxidants and Their Mechanism of Action

Antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. This is a broader definition encompassing many vulnerable macromolecules (e.g. DNA, lipids and proteins) that can be affected by oxidation. Antioxidants can also be defined as substances that trap harmful forms of oxygen and prevent them from damaging cells. Mechanistic definitions of antioxidants are usually focused on the ability to be a hydrogen donor or an electron donor.[6] Antioxidants block the process of oxidation by neutralizing free radicals. In doing so, the antioxidants themselves become oxidized. The two possible pathways are chain-breaking and preventive.[7]

Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gain the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken.

After donating an electron an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without becoming reactive.[8]

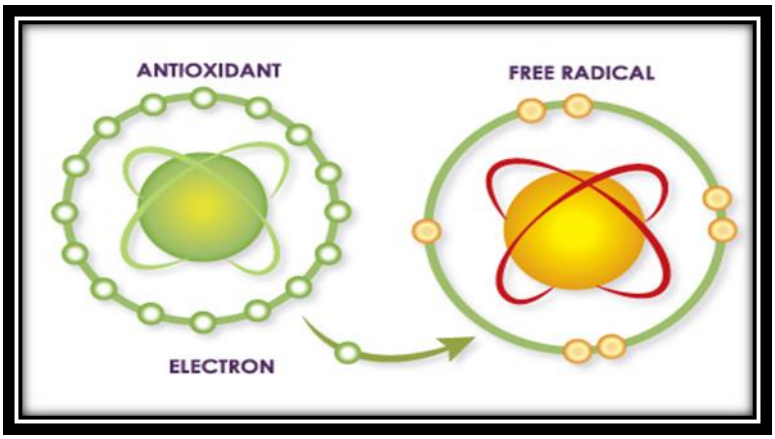

Fig. 03: Antioxidant Process.

Creams

Creams are the topical preparations which can be applied on the skin. Creams are defined as "viscous liquid or semi-solid emulsions of either the oil-in-water or water in-oil type" dosage forms which consistency varies by oil and water. Creams are used for cosmetic purposes such as cleansing, beautifying, improving appearances, protective or for therapeutic function. These topical formulations are used for the localized effect for the delivery of the drug into the underlying layer of the skin or the mucous membrane. These products are designed to be used topically for the better site specific delivery of the drug into the skin for skin disorders. Creams are considered as a pharmaceutical product as they are prepared based on techniques developed in the pharmaceutical industry; unmedicated and medicated creams are highly used for the treatment of various skin conditions or dermatoses.

Creams can be ayurvedic, herbal or allopathic which are used by people according to their needs for their skin conditions. They contain one or more drugs substances dissolved or dispersed in a suitable base. Creams may be classified as o/w or w/o type of emulsion on the basis of phases. The term 'cream' has been traditionally applied to semisolid formulated as either water-in-oil (e.g.: cold cream) or oil-in-water (e.g.: vanishing cream).[9]

Advantages of topical Cream

1. Avoidance of first pass metabolism
2. Convenient and easy to apply.
3. Avoid of risk.
4. Inconveniences of intravenous therapy and of the varied conditions of absorption like P^H changes presence of enzymes gastric emptying time etc.
5. Achievement of efficacy with lower total daily dosage of drug by continuous drug input.
6. Avoid fluctuation of drug levels inter and intra patent variations.

Disadvantages of topical Cream

1. Can be used only for drugs which require very small plasma concentration foraction.
2. Skin irritation or dermatitis may occur due to the drug or excipients.

3. It can be used only for those drugs which need very small plasma concentrationfor action.
4. Can be used only for drugs which require very small plasma concentration foraction.
5. Possibility of allergic reactions.
6. Drugs of larger particle size not easy to absorb through the skin.
7. Most drugs have a high molecular weight and are poorly lipid soluble, so are not absorbed via skin or mucous membranes.[10,11]

Fig. 04: Formulated cream.

Physiology of Human Skin

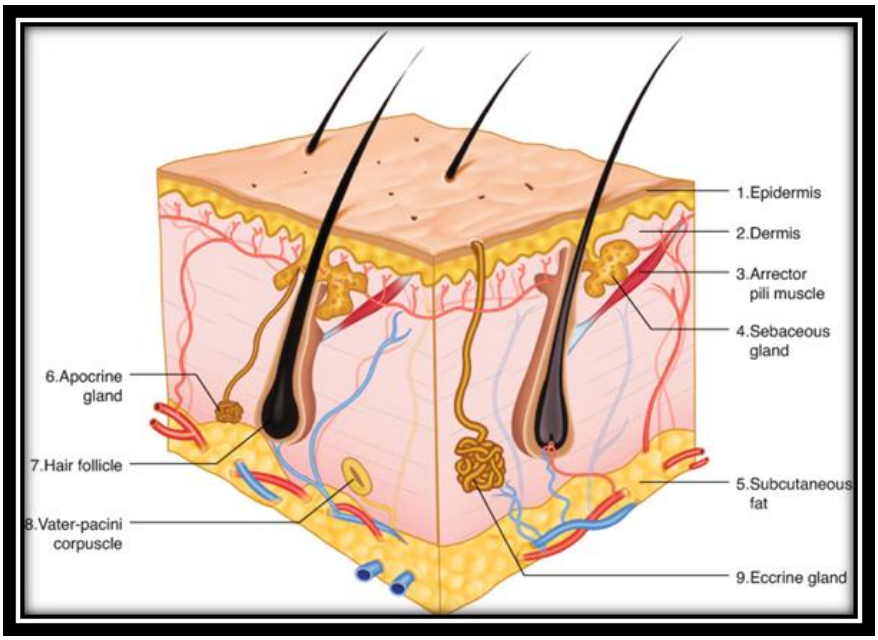

Fig. 05: Physiology of Human Skin.

Epidermis

The epidermis is the most superficial layer of the skin and is composed of stratified keratinized squamous epithelium, which varies in thickness in different parts of the body. It is thickest on the palms of the hands and soles of the feet. There are no blood vessels or nerve endings in the epidermis, but its deeper layers are bathed in interstitial fluid from the dermis, which provides oxygen and nutrients, and drains away as lymph.[12]

Dermis

The dermis is tough and elastic. It is formed from connective tissue and the matrix contains collagen fibres interlaced with elastic fibres. Rupture of elastic fibres occurs when the skin is overstretched, resulting in permanent striae, or stretch marks, that may be found in pregnancy and obesity. Collagen fibres bind water and give the skin its tensile strength, but as this ability declines with age, wrinkles develop. Fibroblasts, macrophages and mast cells are the main cells found in the dermis. Underlying its deepest layer there is areolar tissue and varying amounts of adipose (fat) tissue.

Subcutaneous gland

These consist of secretory epithelial cells derived from the same tissue as the hair follicles. They secrete an oily substance, sebum, into the hair follicles and are present in the skin of all parts of the body except the palms of the hands and the soles of the feet. They are most numerous in the skin of the scalp, face, axillae and groins. In regions of transition from one type of superficial epithelium to another, such as lips, eyelids, nipple, labia minora and glans penis, there are sebaceous glands that are independent of hair follicles, secreting sebum directly onto the surface.[13]

Diseases of Skin

1) Vitiligo

Vitiligo is a condition in which areas of skin lose their normal pigment and so become white. It is common, and affects about 1% of the world's population. The pigment that gives your skin its normal colour is melanin, which is made by cells known as melanocytes.

2) Scabies

Scabies is a common and very itchy skin condition caused by human scabies mites. It can affect people of any age but is most common in the young and the elderly. The mites that cause scabies are tiny parasites, smaller than a pinhead. The rash of scabies is a mixture of scratch marks and red scaly areas; later it can become infected and develop small pus spots.

3) Rosacea

Rosacea is a common rash, found on the central part of the face, usually of a middle aged person. A tendency to flush easily is followed by persistent redness on the cheeks, chin, forehead and nose. The cause of rosacea is not fully understood, but many think that the defect lies in the blood vessels in the skin of the face, which dilate too easily.

4) Psoriasis

Psoriasis is a common skin problem affecting about 2% of the population. It occurs equally in men and women, at any age, and tends to come and go unpredictably. It is not infectious, and does not scar the skin. The skin is a complex organ made up of several different layers.[14]

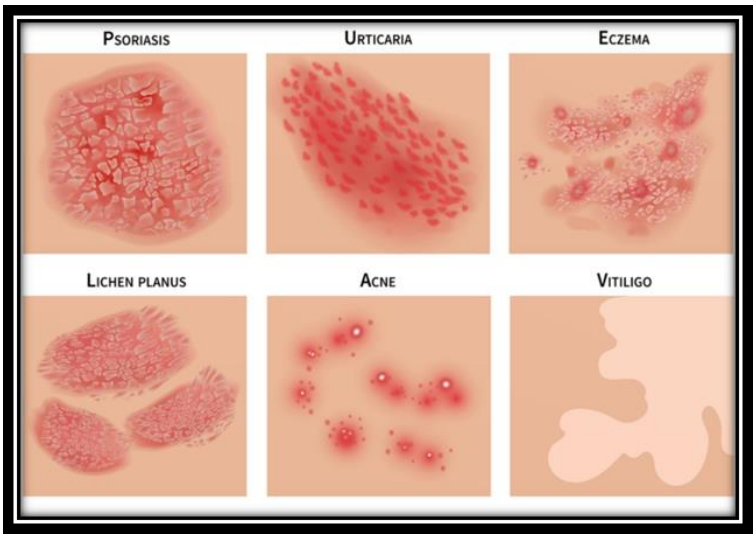

Fig. 06: Diseases of Human Skin.

Antioxidant and antifungal effect of pomegranate and grape seed extract

Punica granatum (Pomegranate) is a small tree which measures between five and eight meters tall and mainly found in Iran, the Himalayas in northern India, China, USA and throughout the Mediterranean region. The fruit of the Pg has extensively been used as a traditional remedy against acidosis, dysentery, microbial infections, diarrhea, helminth infection, hemorrhage and respiratory pathologies. Pg seeds have also been shown to contain the estrogenic compounds, estrone and estradiol. Furthermore, the dried pericarp and the juice of the fruit are considered beneficial for treatment of colic, colitis, menorrhagia, oxyuriasis, headache, diuretic, acne, piles, allergic dermatitis and treatment of oral diseases.

Pomegranate is a potent antioxidant. This fruit is rich in flavonoids, anthocyanins, punicic acid, ellagitannins, alkaloids, fructose, sucrose, glucose, simple organic acids, and other components and has antiatherogenic, antihypertensive, and anti-inflammatory properties.[15]

Fig. 07: Pomegranate.

Antioxidant properties Oxidative stress (OS) produces toxic metabolites which can initiate and promote cancers.consumption of polyphenoles and flavonoids are beneficial for the prevention of cardiovascular, inflammatory, and other diseases by preventing OS that induces lipid peroxidation in arterial macrophages and in lipoproteins. The presence of antioxidants has been reported in Pg juice. Pg contains some species of flavonoids and anthocyanidins (delphinidin, cyaniding and pelargonidin) in its seed oil and juice and shows antioxidant activity three times greater than green tea extract.

Pg fruit extracts exhibit scavenging activity against hydroxyl radicals and superoxide anions, which could be related to anthocyanidins. The antioxidant action of Pg is observed, not only through its scavenging reactions, but also by its ability to form metal chelates . Studies have indicated that methanolic extracts from the peel of Pg has a broad spectrum of antioxidant activities which were evaluated by 1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavengiphomphosolybdenum, Ferric(Fe3+) Reducing Antioxidant Power (FRAP), and Cupric (Cu2+) Reducing Antioxidanrt Capacity (CUPRAC) assays.[16]

Grape-seed extract

Grape (Vitis vinifera) belongs to family Vitaceae. Grapes itself is widely consumed all over the world. Major producers of grapes worldwide are USA, China, Italy and Europe. In Europe, grapes, its leaves and the sap have been used in traditional treatment for ages. There are many categories of grapes with respect to their uses like wine grapes, table grapes, seedless, edible seed and raisin grapes. Seeds of grapes can be collected as a byproduct from any wine manufacturing industry.

Fig. 08: Grape Seeds.

Chemical constituents of GSE

GSE is having abundant source of polyphenols. Polyphenols and flavonoids present in the GSE have been shown remarkable interest based on positive reports of their antioxidant properties and ability to serve as free radical scavengers Grape seed polyphenols have a higher antioxidant activity as compared to other well-known antioxidants

(such as vitamin C, vitamin E, and b-cotene). Beside their antioxidant activity, it also contains some enzymes that catalyze the release of histamine during inflammation and allergies. The amount of oil present in grape seed depends on the variety of the grape (usual range 10–16% of dry weight). Grape seed oil also contains a high amount fatty acids (unsaturated) ranging from 85 to 90% such as a-linolenic acid (x - 3) and c-linolenic acid (x - 6). These fatty acids are related to a reduction of cardiovascular disease, cancer, hypertension, and autoimmune disorders.

Pharmacological activity GSE shows anti-inflammatory, anti-apoptotic, anti-necrotic, cardiovascular and anti-carcinogenic activity and have beneficial effects against several diseases including skin aging). It also shows a positive effect on wound healing. Presence of antioxidant property in GSE has shown to exert a much stronger oxygen free radicalscavenging effect. Compounds those are having antioxidant properties able to protect the cells against oxidative stress. The antioxidant property of proanthocyanidin is because of the availability of the phenolic-hydrogens as singlet oxygen quenchers and hydrogen donating radical scavengers. Small quantity of free radicals helps in signal transduction process.

ROS (reactive oxygen species) and oxidative stress work as a physiological regulator of vascular gene expression of Vascular Endothelial Growth Factor (VEGF) Antimicrobial activity The GSE have shown an effective antimicrobial property; they are efficiently used against Grampositive bacteria (Bacillus cereus, Staphylococcus aureus, Bacillus coagulans and Bacillus subtilis), but are more effective against Gram-negative bacteria likePseudomonas aeruginosa or Escherichia coli. Resveratrol shows a positive antimicrobial activity against many pathogens, such as Staphylococcal aureus, Psedomonas aeruginosa and Enterococcus faecalis. In normal skin, when resveratrol applied topically increases production of cathelicidin, which inhibits the growth of Staphylococcal aureus and induces antimicrobial peptides.[17,18]

MATERIALS AND METHOD

Materials

API: Pomegranate and Grape-seed extract (Vedha Oils India LTD)

Excipient: Propylene glycol, bees wax, stearyl alcohol, cetyl alcohol, triethanolamine, propyl paraben, methyl paraben, liquid paraffin.

Methods

2) Formulation of Cream

1) Preparation of oil phase

White Bees Wax, stearic acid, stearyl alcohol, cetyl alcohol were melted in a stainless steel vessel. To this mixture Liquid paraffin were added and allowed to melt. The temperature of oil phase maintained between 65 – 70°C.

2) Preparation of Aqueous phase

Water was heated to 65 – 70°C. In this weighed propylene glycol, triethanolamine, methyl paraben and propyl paraben were added the temperature of the phase was maintained at 65 – 70°C.

3) Development of Cream formulation

Oil portion was then slowly incorporated into the aqueous phase at 65-70°C and mixed for 10 to 15 Minutes.

When the water and oil phase were at the same temperature, the aqueous phase was slowly added to the oil phase with moderate agitation and was kept stirred until the temperature dropped to 40°C and garlic oil was added to it. The emulsion was cooled to room temperature to form a semisolid cream base pH of cream kept between 4.5 – 6.[19]

Table 01: Formulation Table of Cream.

Part A (Oily Phase)		Part B (Aqueous Phase)	
Ingredient	**Quantity**	**Ingredient**	**Quantity**
Stearic Acid	2.5%	Propylene glycol	5%
White Beeswax	1.5%	Triethanolamine	2%
Stearyl alcohol	5%	Methyl paraben	0.01%
Cetyl alcohol	6.5%	Propyl paraben	0.04%
Mineral oil	5%	Water	Upto 100%
Pomegranet extract	2.5%	Grape-seed extract	2.5%

3) Evaluation of Cream

1) Physical examination

The prepared topical creams were inspected visually for their color, homogeneity, consistency, spreadability and phase separation. The pH was measured in each cream, using a pH meter, which was calibrated before each use with standard buffer solutions at pH 4, 7, 9. The electrode was inserted into the sample 10 min priors to taking the reading at room temperature. The pH of a topical preparation should be within the pH range corresponding to the pH of the skin, namely, 4.5- 6.5.[20]

2) Viscosity

The viscosity of formulated creams was measured by Brook field Viscometer LVD using spindle S 94 at varying speed and shear rates. The measurements were done over the range of speed setting from 0.10, 0.20, 0.30, 0.40 and 0.50 rpm in 60 s between two successive speeds as equilibration with shear rate ranging from 0.20 s-1 to 1.0 s-1. Viscosity determinations were performed at room temperature.

3) Tube Extrudability

In the present study, the method adopted for evaluating cream formulation for extrudability was based upon the quantity in percentage cream extruded from tube on application of finger pressure 7. More quantity extruded better was extrudability.

The formulation under study was filled in a clean, lacquered aluminium collapsible 5 gm tube with a nasal tip of 5 mm opening and applied the pressure on the tube by the help of finger. Tube extrudability was then determined by measuring the amount of cream extruded through the tip when a pressure was applied on a tube.

4) Spreadability Test

An important criteria for semisolids is that it possess good spreadability. “Spreadability is a term expressed to denote the extent of area to which the cream readily spreads on application to the skin”. The therapeutic efficacy of a formulation also depends on its spreading value.

A special apparatus has been designed to study the spreadability of the formulations. Spreadability is Expressed in terms of “time in seconds” taken by two slides to slip off from the formulation placed between, under the application of a certain load. Lesser the time taken for the separation of the two, better the spreadability. Two glass slides of standard

dimensions were selected. The formulation whose spreadability had to be determined was placed over one of the slides. The other slide was placed on top of the formulations was sandwiched between the two slides acrossthe length of 5 cm along the slide.

10 g weight was placed up on the upper slide so that theformulation between the two slides was pressed uniformly to form a thin layer. The weight wasremoved and the excess of formulation adhering to the slides was scrapped off. One of the slides was fixed on which the formulation was placed. The second movable slide was placed over it, with one end tied to a string to which load could be applied by the help of a simple pulley and apan.

A 3g weight was put on the pan and the time taken for the upper slide to travel the distanceof 5.0cm and separate away from the lower slide under the direction of the weight was noted.The spreadability was then calculated from the following formula:

$$\text{Spreadability} = m \times l / t$$

Where,

m = weight tied to the upper slide (3g)

l =length of glass slide (5cm)

t =time taken in seconds.[21]

5) 01, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical scavenging activity

An Aliquot of 3ml of 0.004% DPPH solution in methanol and 0.1 ml of plant extract at various concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200ug/ml) were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 minutes. Decolorization of DPPH was determined by measuring the absorbance at 517nm. A control was prepared using 0.1 ml of respective vehicle in the place of plant extract/ ascorbic acid. The %inhibition activity was calculated by using the following formula.[22]

Formula for DPPH activity

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbane of GSE/Ascorbic Acid}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

1. Physical Evaluation

The cream is white, appealing appearance and smooth texture, and they were all homogenous with no signs of phase separation.

2. pH measurement

The pH of the cream was found to 6.2. The pH should not be too acidic as it may cause skin irritation and should not be too alkaline as it may cause scaly skin.

3. Viscocity measurement

Viscocity was measured by Brookfield viscometer and it was found to be 67540 cps.

4. DPPH radical scavenging Activity

The IC_{50} value and correlation coefficient (R^2) of GSE by DPPH radical scavenging activity were calculated from the graph and is represented in Table no. 35. GSE had an IC_{50} value of 46.01 and R^2 0.950 which was comparable with ascorbic acid which had an IC_{50} 38.01 and R^2 0.907. GSE, thus, showed significance enhancement of DPPH radical.

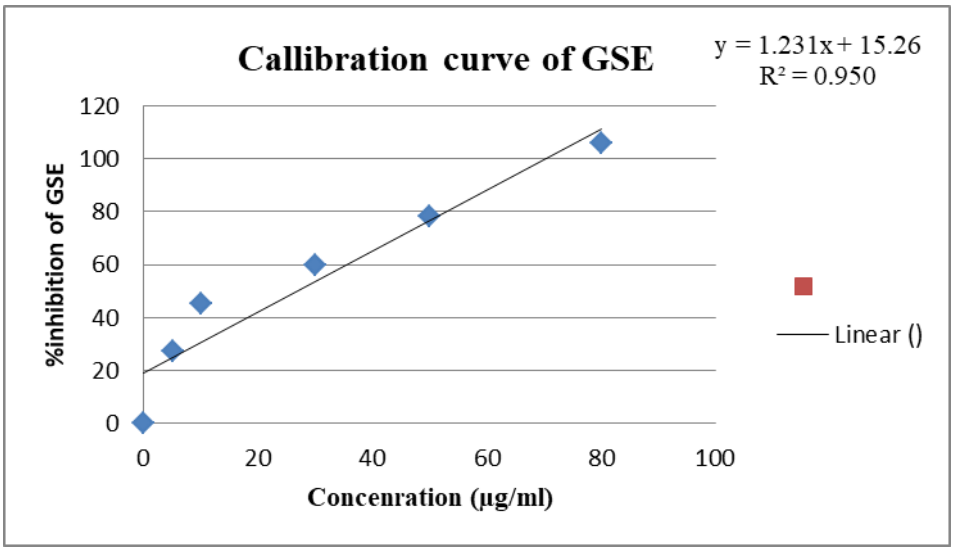

Fig. 09: DPPH scavenging radical activity of GSE.

CONCLUSION

The formulation of antimicrobial agents along with Grape seed & pomegranate exhibited enhanced rate of diffusion and extract antibacterial activity. The results of different chemical and physical tests of cream showed that it could use topically in order to protect against skin infections caused by fungus or bacteria.

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