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ResearchArticle

DEVELOPMENT AND EVALUATION OF HERBAL TOPICAL GEL FOR ITS ANTI-OXIDANT AND ANTI-BACTERIAL EFFECT

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ABSTRACT

Introduction: Herbal medicine has become a thing of global importance for medicinal and economical. Herbal remedies are getting popular as they are avoiding typical side effects of allopathic medicines. **Objectives:** The present research has been undertaken with the aim to formulate and evaluate the herbal gels for its antioxidant and antibacterial effect. Method: The antioxidant potential was investigated by in vitro models like scavenging of DPPH radical, ABTS radical scavenging assay, lipid peroxidation assay and bleaching of pyrogallol red by peroxynitrite. Gel formulations were prepared by using Carbapol 940 as a gel base. The skin pH (6.8-7) was maintained by the dropwise addition of Tri-ethanolamine. The physical parameters of formulated gel, homogeneity, pH, viscosity and Spreadability were evaluated. The evaluation of the gels for antibacterial efficiency is done by agar diffusion method against some bacterial agents. **Result:** The herbal gels showed that formulations containing Punica extract and berberis extract have better antibacterial activity. Conclusion: The formulation similar to this kind of combination can be very useful and can make the herbal society to grow in a good way.

Keywords: Anti-oxidant, anti-bacteril, *Punica Grantum, Berberis Aristata*

INTRODUCTION

Oxygen is an element crucial for life. When cells use oxygen to produce energy, free radicals are created as a result of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally highly reactive species [1]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) collectively describing free radicals. A molecule having one or more unpaired electron in the outer shell is known as free radical. Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$),hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH)[2]. Free radicals are a natural by-product of aerobic cell metabolism. They are generated by a number of actions including infections, diseases, lifestyle and also when our body exposed to Ionizing radiations (from industry, sun exposure, cosmic rays, medical x-ray); Ozone and nitrous oxide (primarily from automobile exhaust); Heavy metals (such as mercury, cadmium and lead); Cigarette smoking (both active and passive); Alcohol; Unsaturated fat (this may create a strain on the natural antioxidants of the body) and other chemicals (pesticides) and compounds from food, water, drugs and air.

These species play a dual role as both toxic and favorable compounds. At low or moderate concentrations, ROS and RNS are indispensable for the maturation process of cellular structures and can act as weapons for the host defense system. When produced in surplus, free radicals and oxidants generate a phenomenon called oxidative stress, a venomous system that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA)[3].

The subtle balance between their two opposed effects is clearly an important feature of life. For cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases oxidative stress is the main cause. The human body is surprisingly furnished with antioxidants defense system to detoxify these dangerous agents but unfortunately, the body's defense system becomes less operative as we get older leading to accumulation of oxidative damage and the development of chronic degenerative diseases.

Free radicals are referred to as oxidants while the defense mechanisms against these free radicals are called antioxidants. Antioxidants are clearly important to human life. Antioxidants are substances that prevent tissue damage due to free radical action [4]. They are able to deactivate free radicals and break the vicious cycle. Antioxidants could act in several ways like the catalytic exclusion of free radical, as scavengers of free radicals or in the form of proteins that minimize the availability of pro-oxidants such as metal ions [5].

During normal metabolism in the body, various antioxidants including glutathione, ubiquinol, and uric acid are produced. Another defense mechanism is a system of antioxidant enzymes. These enzymes are endogenous (produced by the body) and these include superoxide dismutase (SOD), catalase, glutathione peroxidase.

ROS production also has an impact in the pathogenesis of many infectious diseases, and is somewhat of a double-edged sword. The body's natural defense mechanism for suppression of pathogens involves the production of ROS by immune cells. In this case, ROS serve a protective and beneficial role. Yet at the same time, overproduction of ROS by the immune system damages the immune cells themselves, as well as host tissues. Further, many infectious organisms establish pathogenicity by ROS production, to counteract the host immune system. Therefore, ROS may contribute to host tissue damage in infectious disease via several mechanisms. This suggests the potential for utilization of ROS scavengers.

A better understanding of free radical production and antioxidant mechanisms in both the host and pathogen is obligatory to control infectious diseases[6]. Due to the prescription antibiotics which to the development of antibiotic-resistant strains of bacteria, many medical experts are taking a new look at natural, safe antibiotic alternatives. There are many natural herbal antibiotics that should be studied. To treat any kind of infection or illness, medicines made from the natural herbs can be used which are relatively less expensive.

With the continuous use of antibiotics, microorganisms have become resistant to bacteria. In this problem, antibiotics are

sometimes linked with adverse effects on the host which include hypersensitivity, depletion of the beneficial gut and mucosal microorganism, immunosuppressant, and allergic reactions. This has created a massive clinical problem in the treatment of infectious diseases. Plant materials having antioxidant and antimicrobial potential remain an important resource to battle against infectious diseases in the world[7].

Further to add a feather, an agent with anti-oxidant as well as antimicrobial can severe as potent wound healing medicines. This is because the healing of a wound is a natural process but microbial infection associated with various types of the wound is worrying concern. The wounds are the welcoming site for bacterial growth and thus pus formation. Therefore, the primary objective of wound healing is to control bacterial infection and inflammation

This is the reason why the search for natural compounds, rich in wound healing properties along with antioxidant, anti-microbial anti-inflammatory properties is on demand. Till date, very few natural agents are available in the market for wound healing which will comprise of all the above actions. For wound healing, a good effective topical formulation is required.

Topical drug administration is the easiest delivery anywhere in the body. Application of pharmaceutical products to the skin serve as localized delivery. Skin is the readily accessible organ of body. The topical formulations can be applied for various skin related pathological problems like wounds, skin disorder (microbial infection, psoriasis, skin cancer).

In the pharmaceutical field, the gel is most convenient and patient-friendly dosage form. Now self-image alert world, patients are like to use topical products which are safe, effective, cosmetically acceptable and easy to use.

Gels are relatively easier to prepare compared to emulsion-type creams and lotions. In general, a selected gelling agent, such as Carbomers and xanthan gum, can be dispersed in purified water or hydroalcoholic medium to form uniform lump-free dispersion and subsequently, an active and preservative phase can be added to the gel phase to form a medicated gel. Viscosity modification is an important part of semi-solid formulations [8].

Thus, in the present study we attempt to perform the in-vitro anti-oxidant study by four different methods, antimicrobial and formulate the topical dosage form of the best-combined dose of extracts of *Punicagranatum* peel and *Berberisaristata*root.

Pomegranate peel (*Punicagranatum*belonging to family Punicaceae) has been used for thousands of years to heal a wide range of diseases across different cultures and civilizations. It has great nutritional values and numerous health benefits. P. granatum is reported substantial amounts of polyphenols like ellagic tannins, ellagic acid, and gallic acid. Many researchers reported that Pomegranate peel plays a crucial role in treating various diseases such as cancer, osteoarthritis and throats, coughs, urinary infections, digestive disorders, skin disorders, arthritis, and to expel tapeworms[9].

Berberis (Berberidaceae) includes about 500 species that occur in most areas of Europe, the northeastern region of the United States and in South Asia including the in Pakistan. The main characters isolated in different species of *Berberis*, are berberine and berbamine. Phytochemical evaluation of various species of this genus shows the presence of alkaloids, tannins, phenolic compounds, sterols, and triterpenes. Medicinal uses of different parts of the plants is studied as tonic, antimicrobial, antiemetic, anti-pyretic, antioxidant, anti-inflammatory, hypotensive, anti-arrhythmic, sedative, anti-nociceptive, anticholinergic, cholagogic, and have been employed in cholecystitis, cholelithiasis, jaundice, dysentery, leishmaniasis, malaria, gall stones, hypertension, ischemic heart diseases (IHDS), cardiac arrhythmias and cardiomyopathies.

MATERIALS AND METHOD

COLLECTION OF PLANT MATERIAL

From Amsar Goa, Pvt. Ltd. *Punica granatum peel* extract and *Berberis aristata* root extract were procured as a gift sample.

CHEMICALS

1,1-Diphenyl-2-picrylhydrazyl(DPPH),2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), Allopurinol, Bovine Brain Extract (BBE), Bovine Serum Albumin (BSA), were purchased from Sigma Chemical Co. (USA). From Himedia Ltd. Mumbai, India. Xanthine oxidase, Nitrobluetetrazoliumchloride(NBT) and xanthine were purchased. Folin-Ciocalteusolution,Pyrogallol Red, Potassium dihydrogen phosphate and thiobarbituric acid, Dipotassium hydrogen phosphate, trichloroacetic acid, Anhydrous sodium carbonate, Ascorbic acid, Potassium persulfate, Ascorbate, Ethylenediaminetetraacetic acid (EDTA), were purchased from S.D.Fine Chemicals, Mumbai, India.

MICROORGANISMS

For anti-bacterial study gram-positive bacteria such as *Staphylococcus aureus*(MTCC 902),*Bacillussubtilis*(MTCC 121) and gram-negative bacteria like *Pseudomonas aeruginosa*(MTCC 424), *Klebsiella Pneumonia*(MTCC 109), *Escherichia coli* were used in the study

INSTRUMENTS

The instruments used for the study were UV spectrophotometer (Jasco, V-630), laboratory centrifuge (Remi motors, R4C) and Digital pH meter (Equip-Tronics, EQ-610), Brookfield viscometer(DV-I PRIME, USA).

IN-VITRO ANTIOXIDANT ASSAY

SCAVENGING ACTION OF 2, 2-DIPHENYL-1-PICRYLHYDRAZYL

A stock solution of DPPH was prepared by dissolving 150 mg of DPPH in 500ml methanol. Working solutions were prepared by taking different concentrations of extract with DPPH and ethanol. The change in deep violet color of the solution was then analyzed at 520 nm spectrophotometrically and compared with reference standard compound being used was ascorbic acid[10].

ABTS RADICAL SCAVENGING ASSAY

ABTS solution (2mM) and potassium persulphate solution (17mM) were prepared separately, mixed and allowed to stand in dark for about 12-16 hrs. This results in ABTS cation radical formation. Different concentrations of extract solution were reacted with the above ABTS solution and Absorbance was recorded at 750 nm compared with ascorbic acid as standard reference [10].

BLEACHING ACTION ON PYROGALLOL RED BY PEROXYNITRITE NITRATE

Peroxynitrite was prepared by reacting hydrogen peroxide, nitric acid, and sodium nitrite (2M each) followed by addition of 4 M sodium hydroxide in frozen conditions (at -70^0 C). Pyrogallol red solution (100µm) was prepared using 100 mM phosphate buffer (pH 7.4). Peroxynitrite solution was then reacted with a mixture of extract solution and pyrogallol solution with immediate vortexing for 15 min. Absorbance was measured at 540 nm and Ascorbic acid was used as a standard antioxidant for comparison [11].

ASCORBATE IRON INDUCED LIPID PEROXIDATION

Bovine Serum Albumin (BSA) suspension in phosphate buffer (pH 7.4) was prepared by ultra-sonication. Ferric chloride solution (1mM), extract solution, phosphate buffer (pH 7.4) and ascorbate solution (1mM) were mixed together and incubated at 37^0C for 1 hr. After incubation, trichloroacetic acid(10%) was mixed and centrifuged at 1800rpm for 10 min. Equal amount supernatant liquid and thiobarbituric acid (0.67%), were treated by boiling at 100^0C for 20 min.

Rapidly cooled and measured the absorbance at 532nm. A tube containing all the reaction mixture except the plant extract was used as a control. Blank was phosphate buffer. The percent inhibition was calculated with the formula Percentinhibition (%) = (Abs of control – Abs of the sample) X 100 / (Abs of control)[12].

DEVELOPMENT OF GEL[13,14,15]

Five different combinations of gel were formulated by varying the concentration of excipients. The weighed amount of Carbopol 940P was soaked in distilled water overnight. The alcoholic extract of Punica peel and *Berberis aristata* was accurately weighed and uniformly suspended in water. Soaked carbopol and the suspension is mixed. The mixture was stirred using magnetic stirrer for around 1-1.5 hr until a uniform suspension was obtained. Care was taken to prevent air entrapment during stirring. This was followed by neutralization of the gelling agent by 50% Tri-ethanolamine, pH was adjusted to 7.3-7.5.(table no. 1)

Table1: Development of gel

Ingredients	Formulation code			
	A	B	C	D
Carbopol	1%	1.5%	2%	2.5%
Extract	5%	5%	5%	5%
Triethnolamine(50%)	-	-	P^H 7.3-7.5	P^H 7.3-7.5
Sodium hydroxide(10%)	P^H 7.3-7.5	P^H 7.3-7.5	-	-
Water q.s.	100 g	100 g	100 g	100 g

EVALUATION OF GEL[13,14,15]

Formulations were evaluated for various parameters such as appearance, color, pH, viscosity, homogeneity, spreadability, Franz diffusion study,content of gallic acid and berberine.

VISCOSITY

The viscosity of the formulations was checked using a Brookfield Viscometer (DV-I PRIME, USA).

HOMOGENEITY

Homogeneity of formulated gels was examined by visual inspection for the presence of any aggregates.

SPREADABILITY

Spreadability is expressed in terms of time in seconds taken by two slides to slip off from the gel when placed in between the slides under the direction of a certain load. The gel was sandwich between the two glass slides, and a known amount of weight was placed on these glass slides to press the glass slides of uniform thickness. By adding a weight of 70 g the time to separate the two slides was determined. Spreadability was calculated using the formula S = ML/T, where, M =wt tied to upper slide, L = length of glass slides, T = time taken to separate the slides.

PRIMARY DERMAL IRRITATION INDEX (PDII)

This test is done by applying the gel onto the skin and then observed for any reversible change on the skin within 4 h. Based on their PDII score, the formulation can be graded as irritating or non-irritating.

STABILITY STUDY

The formulated gel was filled in the collapsible tubes and stored at different temperatures and humidity conditions, that is 25^0 C ± 2^0C/ 60% ± 5% RH, 30^0 C ± 2^0C/ 65% ± 5% RH, 40^0 C ± 2^0C/ 75% ± 5% RH for a period of three months and studied for appearance, pH, and spreadability.

ANTIBACTERIAL ASSAY [16,17]

Agar well diffusion technique was used for in vitro antibacterial activity. Nutrient agar was used as the medium. The sterile agar was inoculated with the bacteria culture for 48 h, at 37°C and formulated gel was placed into wells bored by the borer.

Plates were kept for 2 h in the refrigerator to enable pre-diffusion of the extracts into the agar. Next, the plates were incubated overnight (24 h) at 37°C.

RESULTS AND DISCUSSION

IN-VITRO ANTIOXIDANT ASSAY

SCAVENGING ACTION OF 2, 2-DIPHENYL-1-PICRYLHYDRAZYL

In free radical scavenging activity, DPPH has accepted an electron or hydrogen radical to become a stable diamagnetic molecule. The efficacy of antioxidants is measured by their capability to converting stable DPPH to yellow color eddiphenylpicrylhydrazine. As the action of antioxidant increases, there is a decrease in absorbance of DPPH radical at 517 nm. This occurs because the antioxidants scavenge the radicals by donating hydrogen.

Alcoholic extract of *Berberisaristata* is effective to scavenge DPPH radical (IC_{50}=207.93 µg/ml) comparable to *Punica granatum*peel (IC50 =46.5µg/ml).

ABTS RADICAL SCAVENGING ASSAY

In this assay stable ABTS+• radical cation is produced and then the extract was reacted with ABTS+•Chromophore (blue/ green) is generated by the reaction of ABTS and potassium persulfate and then it can be reduced by an antioxidant.

The IC50 values scavenging 50% of ABTS+• were estimated. Ascorbic acid was used as standard antioxidants for comparison. All the tests were carried out in triplicate and the results were expressed as mean ± SD.

Alcoholic extract of *Berberisaristata* is effective to scavenge ABTS radical(IC_{50}=711.61µg/ml) comparable to *Punicagranatum*peel(IC50 =244.79µg/ml).

BLEACHING OF PYROGALLOL RED BY PEROXYNITRITE

Pyrogallol Red is a dye which gets bleached by peroxynitrite radical and the intensity of dark red color decreases. The bleaching of pyrogallol red is prevented as Antioxidants scavenge the peroxynitrite radical. In this assay, the increase in absorbance is proportional to the inhibition of pyrogallol red bleach by peroxynitrite. Berberis extract inhibited bleaching of peroxynitrite at IC_{50} 213.33µg/ml and Punica inhibited bleaching of peroxynitrite with the IC_{50} value was found to be 736.56µg/ml as compared to standard ascorbic acid.

INHIBITION OF LIPID PEROXIDATION

The results of the effect of extracts of pomegranate peel to prevent lipid peroxidation are shown in Fig.1 MDA is a very reactive species and takes part in cross-linking of DNA with proteins and also damaging the liver cells. The main product of lipid peroxidation and it is used for study the lipid peroxidation process in rat liver homogenate. Lipid peroxide content was carried out by means of derivatizing MDA with TBA at high temperature and acidic conditions. Punica extract and Berberis extract produces inhibition with IC_{50} 180.67 and 216.69 respectively.

DEVELOPMENT OF GEL

The topical gels were prepared by varying concentration of excipients. The formulation code D which gave the most significant results for preliminary evaluation parameters was selected.

Table 2: Development of gel

Ingredients	Formulation D
Carbopol	2.5%
Extract (1:1)	5%
Triethanolamine(50%)	P^H 7.3-7.5
Water q.s.	100g

EVALUATION OF FORMULATION

The herbal gel was brownish in color and translucent in appearance and had a cool and smooth feeling on application. pH also maintained constant throughout the study and the gel was non-irritant upon application on the skin. Spreadability was also precise and found to be less change than the firstly prepared gel after performing a stability study. The evaluation results are been displayed in table no.3.

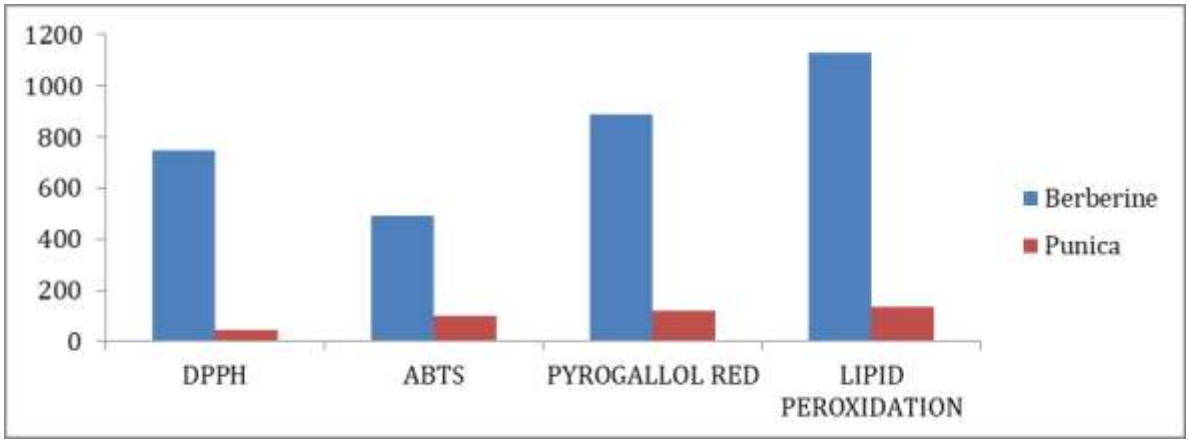

Fig. 1: Comparative graph of IC50 values of *Punicagranatum*and Berberisaristata.

Table 3: Evaluation of formulation

Appearance	Lustrous
Color	Pale yellow
P^H	7.3-7.5
Viscosity	5412±112 cps
Spreadability	5.01 sec

STABILITY STUDY

Further stability test for three months has been carried out and results revealed gel containing 2.5% extract showed better stability than 5%.

ANTIBACTERIAL STUDY OF EXTRACT AND GEL

The antimicrobial study of the alcoholic extract was observed at five concentrations. Among them, 5-15mg concentrations were found to be significantly effective. Thus, for gel 30mg of the dose was studied. Each sample was analyzed in triplicate.

Table 4: Antibacterial assay

Organisms	Zone of inhibition(mm) Volume added(µl)						
	Punica extract			Berberine extract			Gel
	5mg	10mg	15mg	5mg	10mg	15mg	30 mg
B. subtilis	12	15	20	15	19	19	18
S.aureus	21	25	29	23	29	31	27
P.auregenosa	15	18	22	19	23	27	23
E.coli	19	24	27	23	25	25	26

CONCLUSION

The combination of the bioactive extracts in the gel form showed excellent anti-oxidant and anti-bacterial effect. The combination proved to be a potential agent to evaluate for its wound healing effect. The formulation similar to this kind of combination can be very useful and can make the herbal society to grow in a good way.

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