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ROLE OF HERBAL NANOPARTICLES IN TREATMENT OF INFLAMMATION

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ABSTRACT

Inflammation is a response to an injurious stimulus, which is caused by a wide variety of noxious agents involving four basic principles i.e. calor, dolar, rubor and tumor. A distinctive feature of inflammatory response in relation to other facts of anti parasite defense is that damage to the self is unavoidable. Several synthetic pharmaceutical products in various dosages form are available in the market for Inflammation treatment but are less preferred because of their elevated allergic reactions, repeated therapy, and side effects. Herbal products provide relief with comparatively less side effects. Now a day, for effective treatment, more and more search is diverted towards herbals. Although a number of herbal products are available for topical admistration like creams, ointment, gel etc, and these conventional formulations have less effect to the body and have little percutaneous absorption. In this respect, the newer approaches like silver nano formulations are developed as these formulations are stable and with high drug loading capacity and increased percutaneous absorption. Alcoholic extract of peel of *Citrus lemon* were screened for the presence of phytochemical constituents. Silver nanogel of *Citrus lemon* and peel extract can overcome the problems related with conventional formulations such as low uptake, poor penetration and high cost.

KEYWORDS: Silver nano particles, Inflammation, *Citrus lemon*, Herbal products, polymer, Phytochemical Screening.

1. INTRODUCTION

Skin is the biggest organ of the body, with a covered surface area of about 1.5 to 2 m^2 in adults including glands, hair, and nails. Epidermis and dermis are two main layers of the skin. It is made up of areolar and adipose (fat) tissue. A waterproof layer that forms below the skin protects more profound and progressively fragile structures and also protects it from microorganisms, chemicals, physical agents, like- mild trauma, U.V light, and dehydration.

The tissue consists of a large number of cells and according to size, shape, and functions of cells it is classified as epithelial tissue/epithelium tissue, connective tissue, and muscular tissue. Osteocytes (bone cells) are surrounded by collagen fibers (inorganic salts especially calcium and phosphate). Bones are the physique of the body that allows movement of the body by forming joints between the cranial, thoracic and pelvic cavities. They also provide attachment to muscles and tendons. **Joel G. Hardman et al.**[31]

1.1 INFLAMMATION

Inflammation is a response to a destructive stimulus, which is caused by a large number of noxious agents (e.g.-infection, antibody, or physical injuries). Generally, four basic principles are involved in the mechanism of inflammation. First one is color (also known as warmth), the second is dolor (or pain), third is rubor (redness) and last is tumor (swelling).

There are three distinct temporal phases of inflammation. Each phase follows a different mechanism. First one is the intense phase described by increased capillary permeability and transient local vasodilation. Second is postponed subacute phase, characterized by invasion of leukocytes and phagocytic cells. Third, is the incessant proliferative phase, wherein tissue fibrosis and denaturation of tissue occur. **Joel G. Hardman et al.**[31], **Anne Waugh et al.**[8]

1.1.1 Type of inflammation

Inflammation is categorized on the basis of two different parameters. First is intensity (low grade versus high grade) and the second one is duration (acute versus chronic).

- Acute inflammation is depended upon the seriousness of the infection. It originates in a local area and spreads rapidly to the periphery for a short duration. Side effects of intense aggravations are swelling, redness, pain, heat, and loss of functions. It permits the exudation of inflammatory mediators like plasma proteins and leukocytes into the encompassing tissue.

- Chronic inflammation is characterized as concurrent dynamic aggravation and tissue decimation. The tumor is the best example of chronic inflammation and occurs for a long duration (a few days to a week).**Abdullatif Azab et al.**[2]

1.1.2 Mechanism of inflammation

Various inflammatory mediators are involved in the mechanism of inflammation. Such mediators are Prostaglandins (PGE_1 and PGE_2), Bradykinin and Leukotrienes (especially LTB_4) which produce pain. Pain occurs through the synergistic action of inflammatory mediators like Bradykinin and Prostaglandins. **Noah T. Ashley et al.**[53]

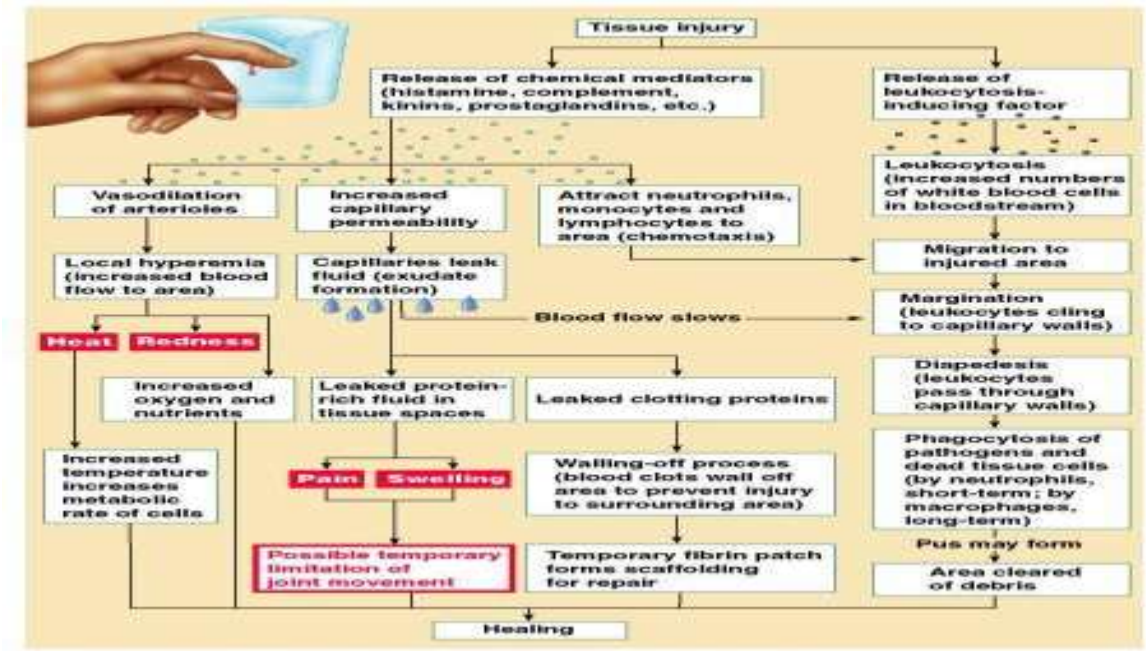

Fig.1.1: Mechanism of inflammation. Frederic H. Martini.[18]

1.1.3 Components of inflammation

Due to injury, various chemicals are released that cause inflammation. These chemicals are Prostaglandins (PGs), Leukotrienes (LTs), Histamines, Bradykinin, and migrating cells.

1.1.3.1 Prostaglandins

Prostaglandins consist of twenty carbons that contain unsaturated fatty acids with a cyclopentane ring. They act as a chemical messenger like a hormone but are immobile in nature and do not move to another site. They are synthesized from arachidonic acid. Cyclooxygenase (COX) enzyme activates the prostaglandins, which further activate the inflammatory response like the production of pain and fever.

1.1.3.2 Eicosanoids

Eicosanoids are chemical messengers called as "lipid mediators" which regulate the oxygenation of Arachidonic acid. They act as a cellular messenger as they carry information from one cell to another and have important physiological and pathological roles. They protect tissues from adverse conditions like- a bacterial infection**. William F. Ganong et al.**[90]

1.1.3.3 Leukotrienes

Leukotrienes has originated from the word leukocytes, and was given by Swedish Biochemist B. *Samuelson*. Arachidonic acid gets converted into epoxide leukotriene which further, by hydration, forms different intermediates like- Leukotriene A_4 (LTA_4) and LTB_4. Bailey and Martyn (1985) found that Leukotrienes were naturally produced by Eicosanoid lipid mediators, which are responsible for inflammation. LTB_4 is an intense chemotaxis for neutrophils which increases leukocyte adhesion to blood vessel walls.

1.1.3.4 Histamines

Histamine causes aggravation of antigen-activated mast cells that increase the penetrability of vessels, which are involved in many allergic reactions. Histamine is produced by decarboxylation of histidine. Allergy is caused by an innocuous substance like- pollen dust, which produces an immune response when coming in contact with lymphocytes and antigens. **Stephen J. McPhee et al.**[79]

1.1.3.5 Bradykinin

Bradykinin is a biologically active oligopeptide composed of nine amino acids, and is synthesized from kininogen (a precursor protein) by kallikrein enzymes (1950). It activates two distinct membrane receptors namely B_1 and B_2 receptors. Both activate the phospholipase C pathway that leads to the metabolism of membrane phospholipids, phosphatidylinositol bisphosphate (PIP_2), and release of two resulting fragments, inositol triphosphate (IP_3) and diacylglycerol (DAG).**K.D. Tripathi.**[32]

1.1.3.6 Platelet-activating factor

PAF is a glyceride-derived substance called acetyl–glyceryl–ether –phosphorylcholine. It acts between numerous leukocytes like- platelet aggregation, anaphylaxis, and inflammation. A number of cells are responsible for the synthesis of PAF like neutrophils, monocytes,

macrophages, and platelets. The main function of PAF is to mediate intracellular interactions, by binding to its specific receptors. **DiPiro Robert L. Talbert et al.[15]**

1.2 *CITRUS LEMON*

The fruit rind (peel) of citrus lemon is used as a stomachic, carminative, febrifuge, and vermifuge. The crude extract or constituents from the plant also exert anti-inflammatory, anti-arthritic, anti-microbial and anti-cancer activity in the *in-vitro* and *in-vivo* models **Sriparna Knudsen et al.[78], Galati E. M et al.[20]**

"Pati" and "kagzi" two kinds of lemon are found in the Indian market, which are cultivated in northern India and belong to family Rutaceae. **P. Singh et al.[58]**

1.3 Herbal Medicines

Herbal medicine is the use of plants and plant extracts to treat disease, something mankind has always done. Herbal medicine exists in many local varieties depending on the regional flora. Many modern drugs were originally extracted from plant sources, even if they are now made synthetically, and many other drugs are descended from plant substances.[1,2] The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair which are aimed at host defense and usually activated in most disease condition. For instance, Aspirin, the original non-steroidal anti-inflammatory drug (NSAID).[3,4] Currently much interest have been paid in the searching of medicinal plants with anti-inflammatory activity which may lead to the discovery of new therapeutic agent that is not only used to suppress the inflammation but also used in diverse disease conditions where the inflammation response in amplifying the disease process.[5,6] *Citrus lemon* (Rutaceae) or its constituents exhibited various biological activities such as antibacterial, antifungal, antiviral, antitumor and antidiabetic effects.[5,6] The greatest interest, however, has been focused on their anti-cholesterolemic and antithrombotic activities. In this work the various extracts of *Citrus lemon* peel were studied for its *in vitro* anti-inflammatory activities.

2.2 POLYMER PROFILE

2.2.1 Carbopol

Non-Proprietary Names: Carbomer

Synonym – Polyacrylic acid, carboxy polymethylene

Chemical Name – Carbomer

Empirical Formula – $(C_3H_4O_2)_n$

Functional Category: Bioadhesive, suspending agent, emulsifying agent, reaction-modifying agent, tablet binder, viscosity-increasing agent.

Description: Carbopol is white colored, acidic, hygroscopic, fluffy powders with a slight characteristic odor.

Melting point – 260^{o}c

Specific gravity – 1.4

Solubility – Soluble in water, glycerin and after neutralization in ethanol (95%).

Application in pharmaceutical formulation and technology: used as viscosity or suspending increasing agents in various formulations like- gels, creams, and ointments for use in an ophthalmic, rectal and topical preparation. **Rowe et al.[68]**

1.4 SILVER NANOPARTICLES

Nanoparticles are very small sized material in the size scope of 1-100 nanometer (one nanometer is one-millionth of a millimeter). Due to their nanosize, they can work more efficiently. It has fewer side effects and can reach the target organ in the desired concentration but its low therapeutic dose can give better treatment. **V. Kumar et al.[87]**

Silver nanoparticles are most imperative and captivating nonmaterial that assume a significant job in nanomedicine (nanotechnology and nanoscience) and have been centered on the potential application in malignancy determination and treatment. In nanosize, silver shows better wound healing effect and has other multifunctional bio-applications like- anti-microbial, anti-inflammatory, anti-angiogenic, and anti-cancer activities. **David L et al.[13]**

1.4.1 Characterization of silver nanoparticles

Silver nanoparticles are largely described by their shape, size, surface area and polydispersity index by different techniques like UV-visible spectrometry, DLS, SEM, TEM, XRD, EDS, and FTIR. **Ahmed S et al.[4], Anandalakshmi K et al.[7], Bakner A et al.[11]**

The spectrophotometric absorption measurement is in the wavelength ranging from 300-800 nm of and has a size range of 2-100 nm. Surface charge and size distribution are characterized by using DLS. Morphological characterization at the nanometer range is characterized by SEM and TEM. Crystal structure of silver nanoparticles is identified by XRD**. Umoren S.A et al.[84] Nazeruddin G.M et al.[53], Kanmani P et al.[34]**

1.4.2 Application of silver nanoparticles

The silver nanoparticles have a broad-spectrum of anti-microbial activity against animal and human pathogens, and are widely used as anti-microbial agents in commercial medical and consumer products. Anti-microbial activity includes anti-bacterial, anti-fungal, and anti-viral activity.

Other applications of silver nanoparticles include anti-inflammatory, anti-arthritic and anti-cancer. They are also used in the preparation of cosmeceuticals. Due to their longer stability and nanosize range, they are used in various topical preparations like- creams, gel, and ointment. **Firdhouse M. et al.[19], Nasrollahzadeh M et al.[51]**

1.4.3 Method of synthesis of silver nanoparticles

Generally, two methods -"top-down" and "top up" methods are used for the synthesis of silver nanoparticle on the basis of size reduction of a suitable starting material. Various physical and chemical treatments used for size reduction are microwave method, sonication method, and high-speed stirring method. **Shakeel A et al.[72], Milad Torabfam et al.[46]**

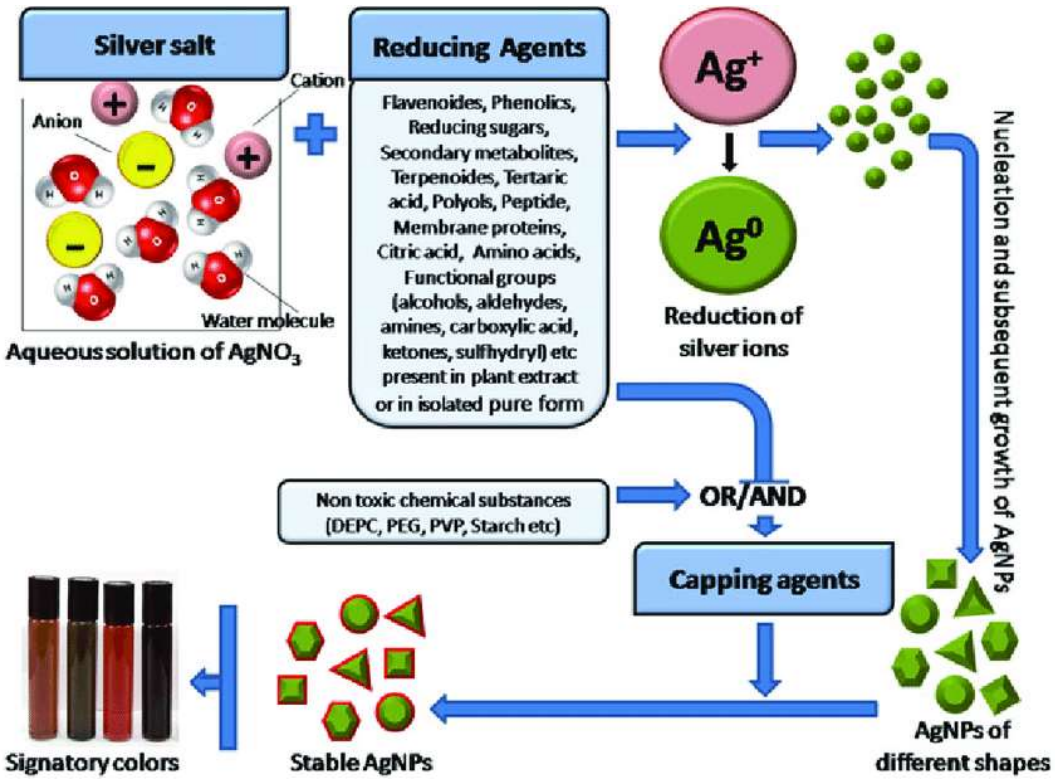

Fig.1.3: Synthesis of silver nanoparticles. Poonam Patel et al.[60]

1.5 NANOGEL

Nanogel term was first introduced in 2008 by Alexander and Serguei. Nanogels are hydrogel polymeric systems with a scattered molecule that is estimated in the nanometer range. Basically, nano gel is a bearer framework for conveying medications or they can be artificially altered with different ligands for focused medication conveyance and activated medication release. **Abd El-Rehim et al.[1], Hamidi M et al.[22]**

1.5.1 Advantages of Nanogels

Nanogel-based systems have different advantages like- higher drug loading capacity, high biocompatibility and biodegradability, rapid contracting/swelling capacity, better stability over the surfactant micelles, exhibition of lower critical micelle concentrations, slower rate of dissociation, viability in maintaining a strategic distance from the fast renal rejection however enough to stay away from the take-up by the reticuloendothelial system. **Kohli E. et al.[36], Keshavarz M et al.[35]**

Nanogels also show quicker response as compared to the conventional hydrogels. They are great bearers of intracellular conveyance and improve cellular accumulation significantly. **Look M et al.[40]**

1.6.2 Methods of preparation of Nanogels

Three basic methods used in the formulation of nanogels are photopolymerization, substance cross connection by emulsion polymerization, and pulse radiolysis method.

1.6.1 Photopolymerization

It involves the synthesis of nanogel directly in the presence of U.V, surfactant, initiator, an indicator, and pure nanogels can be obtained. In this method, nanogels is obtained from monomer molecules initiated directly with U.V.

1.5.2 Chemical cross-linking by emulsion polymerization

It includes expansion of cross-connected species with monomers, natural dissolvable surfactant, and water. Cross-linked nanogel is obtained by irradiation and purification of the organic phase.

1.5.3 Pulse radiolysis method

In this method, the polymer is treated with ionizing radiations in dilute aqueous solutions to promote intramolecular recombination of polymer radicals to frame nanogel. Mangalathillam S et al.[44]

Other methods additionally utilized for planning of nanogel are- physical self-get together of intuitive polymers, chemical cross-linking of preformed polymers, heterogeneous controlled/living radical polymerization and nanofabrication of nanogel particles. Ryu, J H et al.[69], Mohammed N et al.[48]

1.5.4 Applications of Nanogels

Nanogels are built up with incredible viability for the treatment of various types of disease like inflammatory disorders, a neurodegenerative disorders, autoimmune diseases, and diabetes. They are novel drug delivery system and very promising carrier vehicles to be used in various therapeutics, diagnostics, as macromolecules, and others. **Samah et al.[70], Nukolova et al.[55]**

They show a significant effect in intracellular delivery and have versatile application in cancer treatment.

For example, paclitaxel (PTX) loaded nanogels have more cytotoxicity in HEPG-2 cells. They have greater solubility and stability as compared to free drugs. Doxorubicin has a higher concentration of drug at cancer sites than the free drug. **Vinogradov et al.[89], Zabihian et al.[93]**

METHODOLOGY

Preliminary Phytochemical screening

Preliminary phytochemical screening were executed to evaluate the phytochemical constituents of the extracts. The extract acquired a dark brown, aromatic/pleasant, semisolid mass. The crude extracts of peel were subjected to different chemical tests separately, for the identification of various phytoconstituents. The ethanolic extract of *Citrus lemon* showed the presence of photochemical constituents like alkaloids, steroids, tannins, flavonoids, phenolic compounds, carbohydrates, glycosides and proteins. Negative results were founds for saponin and fixed oil.

3.3 Preliminary Phytochemical Studies of extract

Table 3.1: Phytochemical screening of *Citrus lemon* speel extract.

S.No	Phytochemical Constituents	Name of the test	Observation
1	Alkaloid	**Dragendroff's Test**	The test solution was mixed with Dragendroff's reagent (solution of potassium bismuth iodide). It gives reddish brown precipitate indicating the presence of alkaloids.
		Wagner test	The test solution was mixed with Wagner reagent (Iodine Potassium Iodide). Formation of reddish brown precipitate confirmed the presence of alkaloids.
		Hager test	The test solution was mixed with sodium hydroxide solution. Formation of yellow color precipitate confirmed the presence of alkaloids.
2	Steroids	**Salkowski Test**	The test solution was mixed with 1mL of chloroform. Then carefully added 1mL of concentrated sulphuric acid and shaken gently. A reddish brown color in the chloroform layer and green fluorescence in the acid layer confirm the presence of the steroidal ring.
3	Flavonoids	**Alkaline reagent test**	Test solution mixed with 20% sodium hydroxide solution. Then added dilute HCl, a yellow color forms and disappears on the addition of HCL which indicate the presence of flavonoids.
4	Saponin	**Foam test**	The test solution was mixed with 5mL of distilled water in a test tube and shaken vigorously. The formation of stable foam indicates the presence of Saponin.
5	Glycosides	**Baljet Test**	The test solution was mixed with picric acid. The formation of an orange color indicates the presence of glycosides.
		Keller-Kiliani test	One ml of plant extract was treated with 2mL glacial acetic acid containing a drop of $FeCl_3$. A reddish brown color indicates the confirmation of Keller-Kiliani test.
		Legal test	One mL test solution was dissolved in Pyridine and sodium nitroprusside solution added and made alkaline. A pink to red color is produced.
6	Tannins	**Ferric Chloride test**	The test solution was mixed with 2mL of ferric chloride. Formation of a dark brown color indicates the presence of tannins.
7	Proteins	**Xanthoproteic test**	The test solution was mixed with 1mL of concentrated nitric acid and boiled. A yellow color precipitate was formed. After cooling, sodium hydroxide solution was added. Formation of orange color conflicts proteins.
8	Phenolic compounds	**Ferric chloride test**	The test solution was mixed with 2mL of ferric chloride. Formation of blue or green color indicates the presence of phenolic compounds.
9	Fixed Oils	**Spot test**	The test solution was pressed between filter papers. Oil stains on the filter paper confirm the presence of fixed oils.
10	Carbohydrates	**Molish Test**	The test sample was mixed with α-napthol and conc.

			H_2SO_4. Ring formation occurs which indicate the presence of carbohydrates.
11	Terpenoids	The test solution was mixed with chloroform and conc. H_2SO_4. Formation of red-brown color indicates the presence of Terpenoids.	

Formulation and evaluations of silver nanoparticles

The silver nanoparticles of peel extract of *Citrus lemon* were formulated by chemical and microwave method. On the basis of various parameters like time, concentration of $AgNO_3$ microwave method was conducted. Further, optimized on the basis of particle size, entrapment efficiency, and *in-vitro* release of silver nanoparticles.

Formulation and evaluations of silver nanogel

Optimized batch of silver nanoparticles containing *Citrus lemon* peel extract were incorporated into gel separately. The optimization of topical gel was carried out using different gelling agents with varying concentrations, like Carbopol 940, CMC, and HPMC. The pH of the final formulations was adjusted using triethanolamine.

Research Application

In the current study, novel herbal formulations to deliver the silver nanoparticles of *Citrus lemon* peel extract for inflammation and arthritis were developed. These formulations provide more efficient therapy with no side effects, higher bioavailability, and cost effective compared to synthetic drugs. As silver itself showed anti-microbial activity, it reduced the growth of micro-organisms during inflammation. The ethanolic extract of *Citrus lemon* peel based silver nanoparticles showed best result for particle size, polydispersity index, zeta potential, entrapment efficiency, and *in- vitro* release studies. On the basis of these evaluation parameters best batch of silver nanoparticles was incorporated in to topical gel for anti-inflammation and anti-arthritic activity. The efficiency of silver nanogel containing *Citrus lemon* peel extract was confirmed by *in-vitro* and *in-vivo* activity.

Future Prospects

Citrus lemon s peel extract based silver nanogel was successfully formulated and this could be a promising approach for treatment of inflammation and arthritis.

CONCLUSION

The present study was based on the novel, a fast, accurate, feasible and convenient technique was utilized for the formulation of silver nanogel containing *Citrus lemon* peel extract for

high yield, good drug loading, and increased drug efficiency, etc. Silver nanogel provides faster and prolonged action, increases product efficiency. *In-vitro* release studies of silver nanogel containing *Citrus lemon* peel extract showed that silver nanogel provide faster action and prolonged activity as compared to normal topical gel.

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