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Advances in Pharmacology and Pharmacy

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A Review Article: Formulation of Topical Gel by QbD Approach

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ABSTRACT

Application of drug by topical route is an alternate route for the treatment of skin diseases for systemic route. The skin diseases can be treated by administration of drug by local application and may avoid first pass metabolism. It minimizes systemic side effects and when applied locally can be removed easily if any side effects occur like, irritation, skin rash, redness at the application site. The topical drug delivery has been beneficial for longer period of time because of availability of large surface area of skin which exposed to circulatory routes. Because of this route, one can be directly applied to any external body surface and it is only for local application. Amongst many types of topical dosage form delivery, gel is most likely to be used and is a patient-friendly dosage form. Due to the lack of insoluble excipients and oily bases, the gel represents better release of drug as compared to other topical drug delivery system. Nowadays, many industries follow QbD (Quality by Design) approach for the formulation of Gel to prepare a quality medicine delivery to patients. The QbD approach describes the CQA, CMA and CPP of the formulation which ensures the quality of dosage form. This review article focuses on the different dosage forms, types of gel, evaluation by taking parameters such as drug content, pH, spreadability, extrudability, viscosity, swelling index and in-vitro drug diffusion and application of QbD approach to gel formulation.

Keywords - Gel, QbD Approach, Topical Drug Delivery

1. Introduction

1.1. Drug Delivery System (DDS) by Topical Route

The administrations of topically applied drugs are considered as local drug delivery system anywhere on the body such as skin, vaginal, rectal and ophthalmic topical routes. Skin is the major way of drug delivery system for topical administration because skin is one of the largest and most easily available organs on the human body. Skin plays a major obstruction for access of many substances keen on the body and this is mostly due to stratum corneum which is outer layer of the skin, it allows only small molecules to penetrate over a period of time into a systemic circulation. Avoidance of the risk and inconveniences of injectable delivery and varied physiological condition like gastric emptying time, pH change, absorption, presence of enzyme are advantages of drug delivery by topical route.The topical drug delivery systems generally used where the other systems of drug administration fail or it is mainly used in pain management, contraception and acne. Topical drug delivery system is well-defined as an application of drug comprising preparation onto the skin which directly delight cutaneous maladies (e.g. acne) or the cutaneous appearances of common disease (e.g. psoriasis) with the intention of restricting the pharmacological or further consequence of the drug to the apparent surface of skin or inside the skin

[1-3].

Active moiety penetrates the skin through tortuous and continueous intercellular path. By addition of permeation enhancer and solvents in the topical formulation, the drug can transport by hair follicles, a transcellular route and sweat ducts [4].Previously it was considered that, the skin is an impervious shielding barrier but then investigations prove that the skin will be used as route of systemic administration, as per figure 1 [5]. Absorption of drug may occur through following three major routes:

1. Transcellular Route: The drug may transport through keratin crowded corneocytes by way of partitioning into and away from the cell membrane.
2. Intercellular Route: The transportation around the corneocytes in the lipid rich extracellular regions.
3. Transappendageal Route: The shunt transport by sebaceous gland, sweat glands and hair follicles.

1.2. Transport Mechanism

Drug molecule absorbs through the skin by passive diffusion, which is major process of absorption of drug by way of topical preparations. The transport of drugs through skin follows Fick's law of diffusion. The drug diffusion process stops when concentration gradient reaches to zero. Following is the equation which describes the drug flux: [6]

$$J = DPA\Delta C/h$$

Where,

J – Steady state flux across the stratum corneum D – Diffusion coefficient or diffusivity of drug molecule (cm2/sec)

P – Partition coefficient of drug between skin and formulationA – Surface area of stratum corneum (cm2)ΔC – Concentration gradient of drug across the stratum corneum (g/cm3)h – Stratum corneum thickness

According to the equation, drug passage depends upon its aqueous solubility, directly proportional to partition coefficient, skin surface area and concentration of drug in the formulation and inversely proportional to skin thickness.

1.3. Different Types of Topical Preparations: [2]

1.3.1. Gel

The pharmaceutical sciences developed many advanced dosage forms to treat different ailments with some advantages and disadvantages. The carriers are developed to immobilize or encapsulate the drug into it allows to deliver at required site. Considering biocompatibility, integrity and flexibility drugs are used in this carrier material with three dimensional matrices. The materials of this class are identified as gels. To give nomenclature to some semisolid materials as per their physiological characteristic the term 'Gel' was introduced in the late 1800. Gels are rich in liquid but does not flow in the steady-state and comprise of substantially dilute cross-linked, and two component semi-solid system. Gels become more popular drug delivery system because of its molecular stability of the drug, network structure and biocompatibility. The USP (United State Pharmacopoeia) defines gel is semisolid system comprising of dispersion fabricated of either large organic molecule or small inorganic molecule interpenetrated and enclosed by liquid. The inorganic particle forms a 3-D house of card structure. Gels consists of two phase system in which inorganic particles are not dissolved but simply discrete all over the continuous phase, arbitrarily twisting in the flexible chains.The use of gels has been increased in the pharmaceutical and cosmetics preparations. A gel is 99% weight liquid and colloid in nature immobilized by its surface tension and gelating agent forms a macromolecular arrangement of fibers [1,2].

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1.3.2. Structure of Gels

As depicted in figure 2, a gel comprised of synthetic or natural polymer which forms three dimensional matrixesall over a hydrophilic liquid or a dispersion medium. The rigidity of gel is achieved by the gelling agent because particles interlocked and formed a network. The type of form and mature of particles which are responsible for the formation of linkages determine the composition of network and property of gel. When gel is applied on to the skin, the liquid evaporates and entrapped drug leaves in form of thin-film of gel-forming matrix which covers the skin [1].

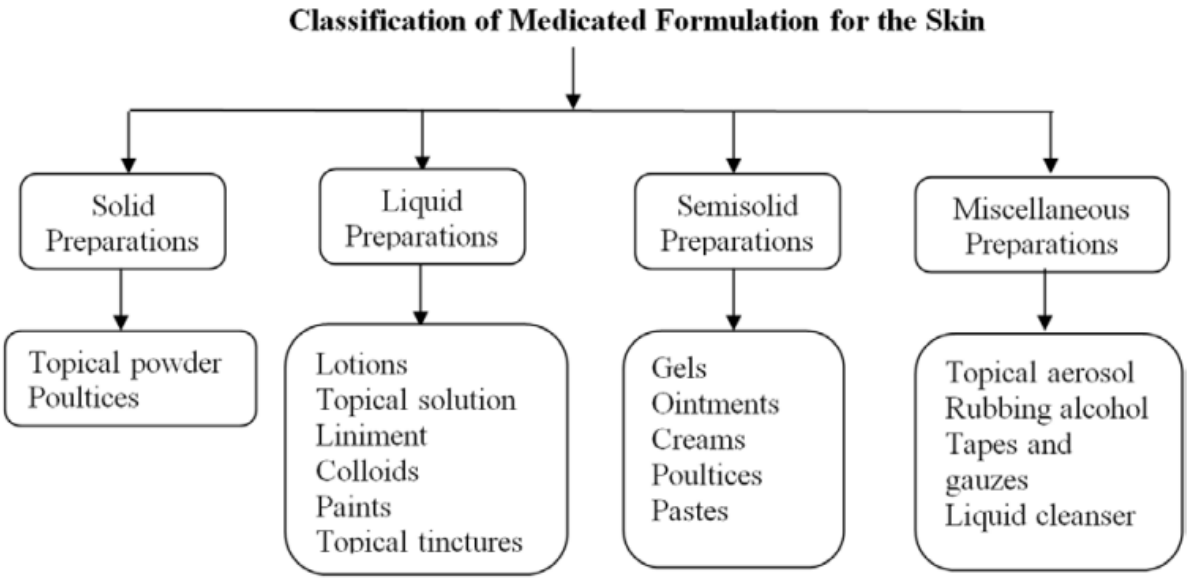

Figure 1. Classification of Medicated Formulation for the skin

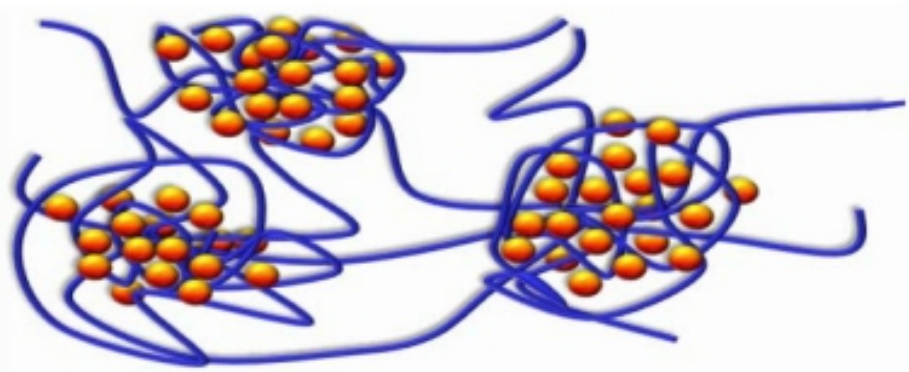

Figure 2. Structure of Gel

1.3.3. Properties of Gel [7]

1. Gel should be convenient in usage and its appliance.
2. Should be inert, non-toxic and compatible with other additives.
3. Should not alter biological character of drug.
4. Should be steady during storage condition.

5. Should have properties like thixotropic, non-staining, emollient, and greaseless.
6. The gelling agents incorporated in the formulation should bring into being a realistic solid like character throughout the storage which can be effortlessly broken by squeezing of tube, shear force generate by trembling the bottle or during application by topical route.
7. It should be non-tacky.
8. It should possess an appropriate anti-microbial agent which prevents commencing of microbial attack.

1.3.4. Qualities of Gels [1,8]

A. Swelling

When gelling agent comes up to contact with the liquid, it absorbs water and the volume increases, this process is known as swelling. Gel-gel links are supplanted by association of gel-solvent. The level of swelling relies on the quantity of linkages between individual particles of gelling operator and on the quality of these linkages.

B. Syneresis

Syneresis means oozing of liquid medium from gels on standing because it contracts spontaneously. The phenomenon linked with the use of gelling agent as the concentration decreases, the occurrences of syneresis increases. It indicates the gel is thermodynamically unstable. During the preparation of gel elastic stress is developed and it may relax on standing and cause contraction. As this stress relieved the interstitial space gets reduced for solvent and forcing out the liquid.

C. Ageing

Colloidal systems generally demonstrate deliberate unstructured aggregation and this progression is known as ageing. Gelling agent gradually form denser network which results into ageing of gels.

D. Structure

The particles of gelling agent form a network by interlinking which gives rigidity to a gel. The property and structure of gel depend on the character of the particle and the type of forces which are responsible for the linkages. The hydrophilic colloid particles may perhaps consist of single macromolecules or have equal aggregate of small molecules.

E. Rheology

Dispersion of agglomerated solid and solutions of gelling agents are pseudo plastic which exhibits non-newtonian flow behavior and which is characterized by decreasing viscosity with increasing shear rate.

1.3.5. Classification of Gels [7]

Gels are classified on the basis of character of solvents used, colloidal phases, rheological properties and physical nature.

1. Nature of Solvent Used:

a. Hydrogel:

Here water is used as continuous liquid phase.

Eg. Derivatives of Cellulose, Bentonite magma, Gelatin,
and poloxamer containing gels

b. Organic Gel (which contains non-aqueous solvent):

A non-aqueous solvents are used as continuous phase.

Eg. Plastibase (low molecular weight polyethylene dissolve in Mineral oil and little cooled), dispersion of metallic stearate in oils and Olag gel.

c. Xerogels:

Xerogels are solid gel with low solvent concentration and produced by evaporation of solvent or freeze drying.Eg. Tragacanth ribbons, acacia tear β-cyclodextrin, dry cellulose and polystyrene.

2. Based on Colloidal System

a. Single Phase System (Organic):

This system consists of big organic molecule presented on warped stands dissolved in continueous phase. Eg. Carbopol, Tragacanth, etc

b. Two Phase System (Inorganic):

If dispersed phase particle size is relatively large and forms 3 dimensional structures all over the gel such as an arrangement which consistsfloccules of relatively smallerparticles than the layer molecule and hence the gel structure in such systems is not stable.Eg. Aluminium Hydroxide Gel

3. Based on rheological properties

Usually gels exhibit non-newtonian flow properties.

They are classified into:

a. Plastic Gels:

Eg. Flocculated suspension of aluminium hydroxide which shows plastic flow, Bingham bodies.

b. Pseudo Plastic Gels:

Eg. Sodium CMC (Carboxy Methyl Cellulose) exhibits pseudo plastic flow, Sodium alginate and tragacanth in liquid disoersion. The viscosity of such types of gels decreases with increase in the shear rate, without yield value.

c. Thixotropic Gels:

In these gels the bonds are very weak between the particles and can be easily broken by normal shaking. The consequential solution will slip back to gel due to again linking and collision of particles.

Eg. Bentonite, Kaolin, Agar.

4. Based on Physical Nature:

a. Rigid Gels:

In this type of gels the framework of gel will be linked by primary valance bond which is formed from macromolecule.

Eg. Silica gel, where silic acid molecules apprehended by Si-O-Si-O bond to build a polymer structure which possess a network of pores.

b. Elastic Gel:

Alginates display an elastic behavior, pectin, agar gels, guar gum.

1.3.6. Advantages of Topical Gel Preparation [1,9]

The non-invasive drug delivery hence better patient compliance.
Easy and convenient for application.
Medication can be easily terminated if found any side effects.
First pass metabolism is avoided.
This is the substitute for other routes of drug administration like oral and intravenous.
Gastro-intestinal incompatibility avoided.
Single application can provide prolonged therapy and may improve patient agreement.

Self-medication can be suitable.
Site precise drug delivery can be achieved.
Narrow therapeutic window and short biological half-life drugs can be formulated.
Intra and inter patient variability is avoided.
More drug can be administered due to availability of large surface area.
Less greasy and easily remove from the skin.

1.3.7. Disadvantages of Topical Preparation [9]

Possibility of allergic response on the application area.
Drug or excipients may cause dermatitis or irritation to the skin.
Large particle size of drugs cannot be absorbed easily through the skin.
The drugs which cause irritation or sensitize skin are not suitable.
Drugs which have poor permeability are not suitable.

1.3.8. Factors Affecting Skin Penetration [6,9]

Drug penetration through exterior of the skin depends on following factors:
1. Skin Condition: Drug penetrate through abraded or injured skin surface more than normal skin. Chemicals increases penetration of drug because of causing injury to the skin.
2. Content of Fat: Epidermis contains fat and does not affect drug penetration.
3. Age: The skin of newborn and children's is very soft and hence penetration is more than adult.
4. Hyperemia: Drug penetration through skin increases due to vasodilatation of the blood vessels though it is because of local or generalized stimulation.
5. Hydration of Skin: Hydrated skin shows more drug penetration than dry skin. Water is operated as penetration enhancer in hydrated skin and increases permeability through stratum corneum.
6. Types of Vehicles: Vehicles play an important role in the drug absorption and penetration through the skin surface. It depends on the type of skin and its condition like, dry or hydrated.
7. Lipophilic substances facilitate more penetration through skin.
8. Physiological factors such as pH of the skin, density of the hair follicles, blood flow to the site of application, inflammation of skin, density of sweat glands, and content of lipid.
9. Physiochemical factors: Partition coefficient, molecular weight of the drug.

1.3.9. Preparation Methods of Gels [7]

1. Dispersion Method

In dispersion method the polymer is added in water for 2 hours or kept in water till the polymer is soaked completely in the water, then add remaining ingredients with stirring until a homogeneous mass is formed.

2. Cold Method

In the cold method the entire ingredients should mix together which forms a homogeneous mass, under low temperature at about 50°C. The penetration enhancer and polymer were mixed together which forms solution A, then solution B prepared by mixing drug in the solvent. Then solution B poured into solution A with continueous stirring.

3. Chemical Reaction

In this chemical reaction method, gel is formulated by chemical interaction of solvent and solute.Eg. Preparation of silica gel and aluminium hydroxide gel.

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4. Flocculation

In this flocculation method, gelatin is produced by addition of appropriate quantity of salt to bring into being age state but inadequate to entire precipitation.

5. Temperature Effect

The solubility of lipophilic colloid agar is decreased by decreasing the temperature.

1.3.10. Ideal Drug Substance for Formulation of Gel [6]

Selection of active pharmaceutical ingredient is very important for the winning preparation of topical gel. The most important properties which have been taken into consideration are as follows:
a) Physicochemical Properties:
I). Drug candidate should have lipophilic property.
ii). The molecular weight should be less than 500 dalton.
iii). pH of saturated solution should be between 5 to 9.
iv). Highly alkaline or acidic drug candidate are not suitable.

b) Biological Properties:

I). Drugs shows first pass effects are suitable candidate.
ii). Degraded in GI (Gastro-intestinal) tract are suitable.

iii). Drugs which are non-irritated to dermis.
iv). Drugs which do not stimulate immunologic reaction to the skin.

1.3.11. Novel Approaches for Gel Formulations [7]

Preparation of a gel is traditional approach of mixing the soluble ingredients into each other and produces a formulation to use topically. Now-a-days novel approaches are invented to prepare a gel with poorly soluble drug by using following methods such as hydrogel, emulgel, in-situ gel, microemulsion based gel, solid lipid nano particles based gels, ethosomes based gels, liposomes based gels, solid dispersion based gels, microsphere based gels, niosomes based gels, microsponged based gels. The method, advantages and disadvantages of each approach are described as below:

1.3.11.1. Hydrogel [3,6,10]

A Hydrophillic gel is called as hydrogels which are cross-linked materials and without dissolving absorbs ample quantity of water. Storing capacity of water makes hydrogels unique nature and is soft. Hydrophilic functional group is attached to backbone of polymer which gives ability to absorb water and the cross-linking in connecting network chain gives resistance to dissolution. A free diffusion of solute molecule occurs because of inside water of hydrogel, whereas the polymer holds the water and serves as a matrix. The hydrogel is a well-known as solo polymer molecule, it means, the group of chains in the gel are coupled with each other and forms a giant molecule on the macroscopic scale. It's likely to look ahead to the conformational changeover of the elastically dynamic arrangement of chains suit able to be seen onto the macroscopic range of hydrogel samples. Gel is a condition which is neither totally liquid nor fully solid. These half-liquid like and half-solid like properties cause many interesting relaxation behaviours that are not found either a pure solid or a pure liquid. Some examples of hydrogels include contact lenses, wound dressing.

Advantages

Easy to modify.

Biodegradable, biocompatible.

Entrapment in the polyurethane hydrogel of microbial cells leads to low toxicity Environmentally susceptible hydrogels have the capability to intellect changes of temperature, the concentration of metabolite or pH and discharge their load because of such type of changes.

Natural hydrogel resources should be investigated for the purpose of tissue engineering, which consist of methylcellulose, agarose and supplementary naturally resultant polymers.

Decreased administration of dose.

1.3.11.2. In-Situ Gel [11,12]

In recent years, In-Situ gel has become prominent in new preparation delivery system because of its advantages such as prolonged and sustained drug action, reduced regularity of drug direction and improved patient compliance. This is a polymeric formulation available in sole form previous to administration and forms gel by gelation when making in contact with the body fluids. Various water soluble polymers are used which are biocompatible, natural and biodegradable.E.g. Gellan Gum, Pluronic F127, Poly-Caprolactone, Chitosan, Carbopol, Pectin, Glycoginic acid, Poly-D, L-lactide-co-glycolide, and xyloglucan.

In-Situ Gelling System Importance

In-Situ gel reduces drug administration frequency, and increases patient compliance and ease of administration.

The conventional ophthalmic solution exhibits poor bioavailability because of quick precorneal elimination of drug and this overcome by use of gel system which instilled as drop into eye and undergoes a sol-gel transition.

1.3.11.3. Microemulsion Based Gels [13,14]

Microemulsions are thermodynamically stable and isotropic multicomponent fluids which composed of oil, water and surfactants whose diameter will be in the range of 10 – 140 nm. The study proves that the stability of microemulsion based gels ismore stable than conventional hydrogels. The microemulsion based gels were prepared with w/o microemulsion which is thermodynamically steady and organic solvent as an external phase offers greater confrontation to microbial contamination as compared to the aqueous phase. The microemulsion based gels are used as sustained release drug delivery sytem by incorporating gelation into w/o microemulsion which increases viscosity of the system. Electrical conductivity can be applied to microemulsion based gels in iontophoretic drug delivery system.

Advantages of Microemulsion Based Gels

It helps in solubilization of lipophilic drug
Drug moiety penetrates rapidly and efficiently.
Increased rate of absorption.
Less amount of energy required.
Variability in absorption eliminates.
Increased bioavailability.

1.3.11.4. Solid Lipid Nanoparticles Based Gels [15-17]

Nanoparticles are colloidal particles having range size between 10 and 1000 nm. Nanoparticles are manufactured by using natural or synthetic polymers which are ideally suited to reduce toxicity and to optimize drug delivery. The successful drug delivery of nanoparticles depends on the capability to enter through numerous anatomical barriers, steadiness in nanometer size and sustained discharge of their contents. To conquer such limitations lipids introduced as an alternative carrier. These lipid nanoparticles are identified as solid lipid nanoparticles.

Advantages of Solid Lipid Nanoparticles

It enhances the bioavailability and chemical manufacture of labile integrated compound.
Enhance penetration of drug into skin by topical application.
It possesses better stability than liposomes.
Chemically labile drugs can be protected from the degradation in gut and from outer environment.
Exhibits improved and greater bioavailability of lipophilic drugs.
More concentration of functional drugs can be achived.
Can be easily scaled up.

1.3.11.5. Ethosomes Based Gels [18-21]

Ethosomes are soft lipid vesicle contains water, phospholipids and alcohol which is relatively in higher concentration. The size of ethosomes varies from 10's of nanometers to microns and hence permeate through skin quickly and have power over significantly elevated transdermal flux.

Advantages of Ethosomes

The components and composition of ethosomes are safe and have various applications in veterinary,

pharmaceutical and cosmetic field.

Permeation of active ingredient enhances through skin and ultimately into the systemic circulation.

Comparatively smaller in size than conventional vesicles.

Better patient compliance.

It improves delivery of active ingredient through the skin in occlusive as well as non-occlusive conditions.

As compared to conventional vesicles it shows better solubility and stability of most of the drugs.

Limitations of Ethosomes

If shell locking is unsuccessful, the Ethosomes may coalesce and collapse apart on transfer into water.

Loss of product may occur during the relocation from organic medium to water medium.

Yield is poor.

1.3.11.6. Liposomes Based Gels [22-24]

Liposomes are made up with lipid bilayer and an aqueous volume entirely enclosed by a membrane. Liposomes are used for controlled drug delivery as it carries hydrophilic and hydrophobic active ingredients and used for different therapeutic purposes. It acts as encapsulating agent and prevents damages from external environment. The liposomes can be acted as solubilizing matrix for inadequately water soluble actives when applied on the skin. This dosage form provides controlled and prolonged release of drug substance, which may lead to better patient compliance and improved efficiency.

Advantages of Liposomes Based Gel

Phospholipids can be well tolerated by intravenous route.

Precipitation of drug can be prevented in blood circulation and at the location of injection.

Encapsulation of drug increases stability.

Selective passive targeting of tumour tissues can be possible.

Encapsuletion reduces toxicity.

Therapeutic index and safety increase.

1.3.11.7. Solid Dispersion Based Gels [25-27]

Solid dispersion prepared with minimum of two components and mostly with hydrophobic actives and hydrophilic matrix. The hydrophobic drug matrix can amorphous or crystalline. The drug may disperse in crystalline particles or in amorphous particles. Now-a-days, many poorly soluble drug candidates are increased and difficult for formulation as conventional oral dosage forms hence to enhance solubility many techniques have been developed which increases dissolution of drug candidates such as salt formation, inclusion complexation, solvent deposition, etc. Amongst many techniques solid dispersion method is effective to increase the dissolution of poorly water soluble active ingredients which ultimately increases the bioavailability.

Advantages of Solid Dispersion

This technique increases porosity of particles which enhances drug release profile.

Increases the rate and extent of drug absorption through rapid dissolution.

The surface area is improved by reduced particle size which increases dissolution rate and ultimately bioavailability.

The drug present in solid dispersion as a supersaturated solution which is considered as metastable polymorphic form and drug in amorphous form increases solubility.

The carrier plays an important role to increase wettability and results in increased solubility.

1.3.11.8. Microsphere Based Gels [27-29]

Microspheres are also known as microparticles which are very small spherical particles within range of 1 μm to 1000 μm. The microspheres consist of biodegradable synthetic polymers or protein and free flowing powder.

The microspheres are in two types as below:

Micromatrices

Microcapsules

In micromatrices the entrapped active ingredient is dispersing all through matrix of microspheres and in microcapsules the entrapped substance is enclosed by distinctive capsule wall. The poorly soluble drug substance dissolved or dispersed through particle matrix incorporated in solid biodegradable microspheres having potential for controlled release of active. Biodegradable microspheres are made up from polymeric, waxy or other protecting material i.e. Modified natural products and biodegradable synthetic polymers.

Advantages

Better drug utilization enhances bioavailability and minimizes intensity of incidence of adverse effects.

Reduction in frequency of dosing improves patient compliance.

The morphology of microspheres allows controllable predictability in degradation and drug release.

It offers constant and extended therapeutic effect.

The smaller size and spherical shape add an additional advantage for injectable drug delivery.

1.3.11.9. Niosomes Based Gels [30-32]

Niosomes are non-ionic surfactant based liposomes and mostly formed by cholesterol. Neosomes are microscopic in size and range from 10 nm to 100 nm. Niosomes are lipophilic in nature and hence possess additional penetrating ability than other emulsion based dosage forms. Niosomes are more stable than liposomes and hence offers many advantages than liposome based delivery system.

Advantages of Niosomes

The release of drug can be delivered in controllable manner.

Patient compliance is better than oily-based dosage forms.

Permeation through skin is enhanced.

Wide range of soluble drugs can be easily accommodated.

No special conditions require for handling and storage.

It increases stability of entrap active molecule and isosmotically energetic and stable.

1.3.11.10. Microsponge Based Gels [33-37]

Microsponge are polymer based microspheres which are microscopic and can be suspended or entrapped variety of drug substances. The size of microsponge ranges from 5 to 300 μm and a typical 25 μm microsponge sphere can have upto 250000 pores which provides greater volume about 1 mL/g for extensive drug retention. It is a novel technique to release a drug in controlled manner and target precise drug release system. Microsponges when applied to the skin releases drug slowly and act as a prolonged release drug delivery.

Advantages

Possesses good chemical, physical and thermal stability.
Provides extended release drug release
It absorbs skin secretions and hence reduces feeling of oiliness.

1.3.11.11. Emulgel [14,27,37-40]

The name itself indicates that it is a combination of emulsion and gel. It is a promising drug delivery system for poorly water soluble drugs. Polymers perform dual behavior as thickner and emulsifier for the reason that the gelling capability of such polymers forms stable emulsion and cream with decreasing interfacial and surface tension and increases the aqueous phase viscosity. The gelling agents available in the aqueous phase alter a classical emulsion into emulgel. These emulgels have the most important advantages on conventional and novel vesicular system. Both w/o and o/w emulsions are used as vehicles for delivery of various drugs to the skin. Emulgel for topical use have severably favourable properties such as greaseless, thixotropic, emollient, easily spreadable, nonstaining, easily removable, transparent and pleasing appearance, biofriendly, long shelf-life.

Advantages

Low preparation cost
Use to make longer drug effect having short half-life
Better drug loading capacity and better stability
Rigorous sonication is not needed
Self-medication possible
Site precise active drug delivery
Avoiding first pass metabolism
Improve patient compliance

1.3.12. Evaluation Parameters for Gel [2,5,9,14,37,39]

1.3.12.1. Physical Appearance

The emulgel have to be inspected visually for colour and phase separation.

1.3.12.2. pH Measurement

The 1% aqueous solution of formulation should be prepared and kept for two hours then measure for pH value by pH meter.

1.3.12.3. Rheological Study

The viscosity of preparation should be determined at 25 °C by using a viscometer.

1.3.12.4. Spreadability

It expresses in the term of time taken by two slides in seconds to slip off from formulations which place between the two slides under the application of a certain load, the lesser time for taking apart of two slides indicates better spreadability.

Procedure

The apparatus typically comprise of a wooden block with pulley at one side. Take two glass slides of size 6 cm x 2 cm. Place the formulation on one slide which is being to be tested. Place second slide on it in such a technique that the preparation will be sandwiched between both the slides. Place 100 g weight on

upper slide about approximate 5 min to expel the air, the excess gel will come out from the slide and have to be removed after removal of weight. Fix the lower slide on apparatus and tie the upper slide with a string and apply 20 g of load with facilitated by pulley. The time taken to separate the upper slide from ground slide should be noted. The procedure should be repeated for 3 times and average can be considered.

$$\text{Spreadability} = M/T * L$$

Where,
M = Weight which is attached to upper slide (e.g. 20 g)
L = Glass slide's length (e.g. 6 cm)
T = Separation time of two slides (seconds)

1.3.12.5. Swelling Index

Swelling index of formulation can be determined by taking 1 g weighed quantity of gel formulation on a porous aluminium foil which is placed in beaker in such a method that it should completely deep into 10 mL of 0.1N sodium hydroxide. The small quantity of gel removed at predecided time intervals and kept on dry place for some time and reweighed.
The swelling index can be calculated as below:

$$SW\ \% = [(Wt - Wo) / Wo] \times 100$$

Where:
SW % - Percent Swelling Index
Wo – Initial weight of Gel
Wt – Weight of swollen gel formulation after time t

1.3.12.6. Extrudability Study

This test is performed by filling the gel into tube and applied a force to expel it and measures the required force to extrude the gel from tube. The filled aluminum tubes pressed by help of finger and the gel will extrude. The weight applied on the gel filled aluminum collapsible tube to extrude the gel at least 0.5 cm ribbon in 10 seconds. The applied weight is measured in grams. Repeat the same experiment 3 times and calculate the average of this determination. Calculate the extrudability by using subsequent formula:

$$\text{Extrudability} = \text{Applied weight to extrude emulgel from tube (g)/Area (cm2)}$$

1.3.12.7. Viscosity

Brookfield viscometer is used to measure viscosity of emulgel at 37°C. Sample to be tested filled in sample holder and appropriate spindle immersed in sample. Allow the spindle to revolve at a particular speed and after 2 min measure the viscosity.

1.3.12.8. Globule Size and Distribution

Generally size of globule and its distribution will be resolute by using Malvern Zeta Sizer. To get homogeneous dispersion, one gram of gel dissolved with sufficient agitation in purified water. The distribution and mean of globule size are measured by injecting the sample in photocell of zetasizer.

1.3.12.9. Content of Drug and Uniformity

Dissolve known quantity of gel formulation in suitable solvent by sonication and filter it to get clear solution. Absorbance of solution measures by using UV visible spectrophotometer. Prepare the calibration curve by using aliquots of different concentration solutions and determine the content of drug

by using equation, which is obtained by linear regression analysis of calibration curve.

1.3.12.10. In-vitro Drug Diffusion Study

In-vitro drug release from gel can be evaluated by using egg membrane which exhibits stratum corneum of human and mainly consists of keratin. Put a fresh egg into concentrated HCl for 15 min and then in fresh water for 5 min and then remove the egg membrane. The resultant egg membrane should be carefully clamped between receptor and donor chamber of Franz diffusion cell. Apply known quantity of gel on to the egg membrane. Prepare fresh pH 5.5 phosphate buffer solution and fill into receptor chamber to solubilize the drug. The solution of receptor chamber should be stirred continuously with magnetic stirrer and 5 mL of aliquots were withdrawn at suitable time intervals. Analyze the samples for drug content by using UV visible spectrophotometer with required dilutions. Calculate the cumulative corrections at each interval to obtain the totality of drug release.

1.3.13. Gel by QbD [41-43]

The subsequent elements are taken into consideration for gel formulation and process development:

Quality target product profile (QTPP)

Critical Quality Attributes of the drug substance, excipients and drug product.

Product blueprint and understanding together with recognition of CMA (Critical Material Attributes) of excipients, drug substance.

Process design and consideration including identification of CPP's (Critical Process Parameters) and in-process material attributes of a drug product.

Control strategy and justification to ensure the product reliability.

1.3.13.1. Target Design Implementation

The following elements are taken into consideration for Gel product and process development:

The QTPP describes the use, safety and efficacy of gel.

Defining QTPP will be a quantitative surrogate for aspects of chemical safety and efficacy during gel formulation development.

To collect appropriate aforementioned knowledge about the drug substance, excipients and process into knowledge space.

Use of risk assessment for further investigations.

Designing a formulation and identifying the CMA (Critical Material Attributes) of gel formulation must be restricted to meet QTPP.

Designing a formulation process to manufacture an ultimate product having above mentioned CMA.

Identifying the CPP (Critical Process Parameters) and input raw materials attributes must be controlled to attain CMA of the finished product.

1.3.13.2. QbD Approach for Gel Formulation [42,43]

Product Development Outline:

Defining Quality Target Product Profile.

Identification of CQAs (Critical Quality Attributes) for the drug substance, excipients and drug product.

Identification and prioritization of potential risks for each unit operation (Risk assessment)

Screening as well as optimization of the formulation.

Development of robust manufacturing process.

Establishment of control strategies.

Raw material specification
Process controls and monitoring, design spaces about individual or various unit operations.
Finished product specifications

The quality target product profile was defined in table 1.Table 2 summarizes critical quality attributes of Gel preparation and indicates which attributes were classified as drug product CQAs. For this product physical attributes, uniformity of content, assay, and preservative content are recognized as subset of critical quality attributes which have potential to impact by the finished formulation or/ and process variables, therefore, will have to investigate and discuss in aspect in succeeding formulation and process improvement studies.

Table 1. Quality Target Product Profile for proposed Gel Formulation

QTPP Elements	Target	Justification
Dosage Form	Gel	The proposed dosage form is for topical application
Dosage Design	Immediate Release	Equivalent type of immediate release
Route of administration	Topical	Taken as generally applied
Dosage Strength	Dose	Pharmaceutical Equivalent
Pharmacokinetics	Clinically equivalent	Clinical study requirement as per USFDA guidance for Acne Vulgaris
Drug Product Quality Attributes	Assay	Meets the requirement of compendia and other quality standards with safety and efficacy of drug product
	Drug Permeation	
	pH	
	Colour and appearance	
	Viscosity	
	Extrudability	
	Spreadability	
	Gel Strength	
Container Closure System	Tube or Wide mouth container	Acceptable to patient

Table 2. Critical Quality Attributes of Gel Preparation

CQA	Specification	Justification
Assay	100% w/w of label claim	Assay affects safety and efficacy. Process and formulation variables can affect assay
Drug Permeation	Flux	Important for the therapeutic point of view
pH	Between 5 and 8	pH to compatible with skin
Colour and appearance	White to off-white	Colour and appearance are not critical attributes although they are long term stability indicator of physical incompatibilities and should be evaluated
Viscosity	Medium	Viscosity may impact on drug release hence it should be medium
Extrudability	Medium	Extruding from the tube during application
Spreadability	Medium	Spreading uniformity on the skin
Gel Strength	Medium	Polymer concentration that determines gel strength

On the other hand, Critical Quality Attributes including identity, microbial limits and preservative content which are expected to be impact the process and formulation variables were not discussed in details. However the targetelements of QTPP are ensured through the product and process design and the control strategy as per table 2.

2. Conclusions

There are different dosage forms existing to treat skin disease locally. Gel is patient friendly dosage forms, which can deliver hydrophobic drug. It helps in permeation of drug through stratum corneum and exerts its action locally. It is non-greasy and does not require rubbing. Gel is the most stable dosage form and easy to handle. QbD approach to the gel preparation gives safe, effective, and quality medicated dosage form for treatment of locally. QbD approach minimizes the cost on research and ultimately dosage form available at affordable cost.

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Applications of Carbon Dots (CDs) in Drug Delivery

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ABSTRACT

The star of the carbon nanomaterials industry is now carbon dots (C-Dots). C-Dots are a potential toolbecause of their applications in imaging, environmental, catalytic, biological, and energy fields thanks to their numerous distinctive physicochemical and photochemical characteristics. C-Dots have received plenty of research attention and have shown remarkable application growth to date. Green biomass or sustainable raw materials may be used to create C-Dots since they are economical, cost-effective, and most significantly, they encourage waste reduction. However, there is still a problem that must be solved regarding the production of high-quality C-Dots from biomass waste. Chemotherapy is currently recognized as the most successful way of cancer treatment, although it is known to induce severe side effects in patients due to its non-discriminatory harmful impact on both normal and tumor cells. The fundamental issue in cancer and other complicated disorders of chemotherapy is understanding drug distribution throughout organs and designing a site-specific drug delivery method that targets cancer cells. Therefore, it is of crucial importance to create advanced routes for the targeted and traceable administration of anti-cancer drugs. C-Dots can be produced using top-down or bottom-up approaches, with the latter method being more frequently employed for high-volume and low-cost syntheses. In this comprehensive review article, we mainly discuss the structure of CDs, classification of CDs, their properties, limitations, source of CDs, fabrication techniques, and characterization techniques. More significantly, we bring readers update on the most recent trends of Cds development in health care.

Keywords Carbon Dots, Drug Delivery, FabricationStrategies, Targeted Drug Delivery, Applications of Carbon Dots

1. Introduction

Modern medicine's ultimate aims include targeted medication delivery, localized disease therapy, and tailored treatments. Such progress is dependent on exact basic biochemical understanding as well as visible biochemical activity markers [1]. Research and application in nanoscience and nanotechnology have grown at an unprecedented rate in recent years. Growing expectations exist that the use of nanotechnology in medicine will result in significant improvements in the diagnosis and management of illnesses. Drug delivery, in vitro and in vivo diagnostics, nutraceuticals, and the improvement of biocompatible materials are a few of the anticipated medical uses [2]. Different carbon dots (CDs) and nanoparticles (NPs) such as gold NPs, iron oxide Nps, semiconductor quantum dots (Qds), polymer

NPs, carbon NPs, and graphene have been investigated as prospective candidates for the integration of various functions [3–5].

New nanostructures called carbon dots (C-dots) are very flexible. Scrivens and collaborators identified it in 2004 [6]. Polymer dots, carbon nanodots, carbon nitride dots (CNDs) and graphene quantum dots (GQDs)are all members of the wide family of carbon-based nanomaterials known as Cds. Carbon dots (Cds), a type of fluorescent carbon nanomaterial with an emission spectrum that goes from blue to near-infrared (NIR), are becoming more and more popular in the biomedical field around the world. C-dots are another name for these dots made of carbon. These are new zero-dimensional nanocarbons that light up and are smaller than 20 nm. CQDs are spherical nanoparticles made of a carbon core with several functional groups bonded to the carbon surface [7]. These materials are made from organic molecules and are water stable, which is important for biological applications. The chemical and photophysical properties of CDs vary substantially depending on their form and size, as well as heteroatom doping with oxygen, nitrogen, phosphorus, sulphur, and boron. Laser ablation, Microwave irradiation, ultrasonic irradiation, hydrothermal treatments, electrochemistry, arc discharge, and pyrolysis are among the synthetic processes used in this field [8]. The three main determining factors in fluorescent CD design are surface state, quantum confinement effects, and molecule state. Fortunately, these characteristics were easily adjustable by altering the synthesis approach, i.e. by employing various precursors or synthetic processes. When compared to traditional fluorescent dyes, this novel form of carbon-based fluorescent material offers several benefits, including simple production, brilliant emission, minimal toxicity, and high biocompatibility. Significant research effort has been devoted in recent years on the original materials, production techniques, and prospective uses of Cds, resulting in a diverse range of CDs [9].

2. Structure of C-Dots

C-dots are typically composed of carbon skeletonsmixed with two additional essential elements, hydrogen,and oxygen, in variable proportions. Most of the time, they are amorphous spheres made of sp2 and sp3 hybridised carbon atoms that are less than 10 nm in size [10]. Different types of carbon-based materials are generated during the synthesis of C-dots, depending on the precursor and synthesis techniques used. Polar groups like carboxyl and carbonyl can be employed to significantly change the surface of C-Dots. Figure 1 shows a schematic representation of basic graphene, graphene oxide, and C-dots [11]. 3. Classification of Carbon Dots Although all carbon dots have identical photoluminescent (PL) characteristics, their intrinsic structures and surface functional groups differ [12]. They are classified into three groups based on this: 3.1. Graphene Quantum Dots (GQD) It has a single or many layers of graphene linked at the edges by chemical groups. They have anisotropic properties. Their length is usually bigger than their height [13].

3.2. Carbon Nanodots (CND)

It is usually spherical. Particles without crystal lattice carbon nanoparticles and particles with crystal lattice carbon quantum dots are included [14].

3.3. Polymeric Dots (PD)

They are polymers that have been aggregated and crosslinked from linear polymers/monomers. Polymer dots are formed by joining a carbon core and linked polymer chains [15].

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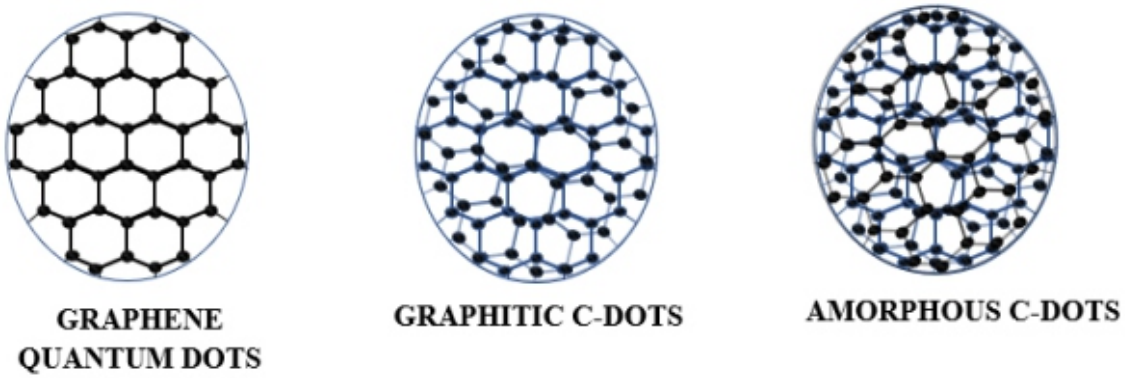

Figure 1. Structure of Carbon dots (C-Dots)

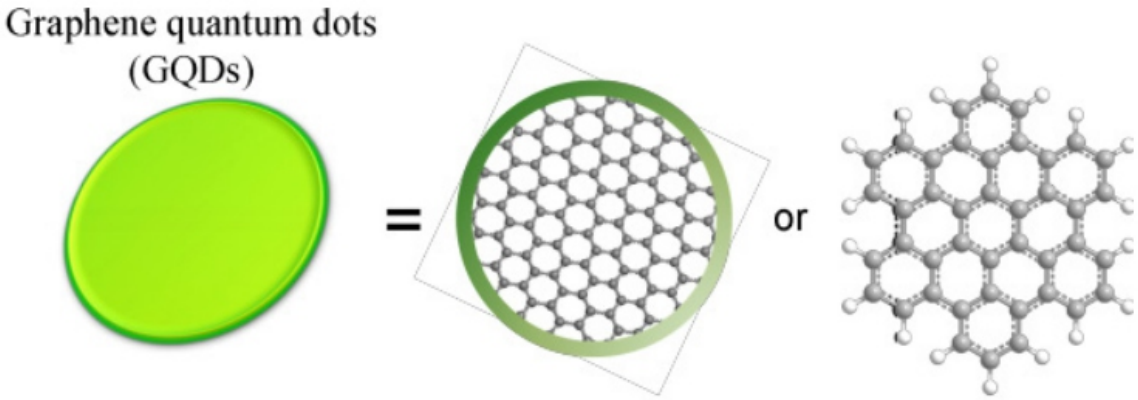

Figure 2. Graphene carbon dots structure

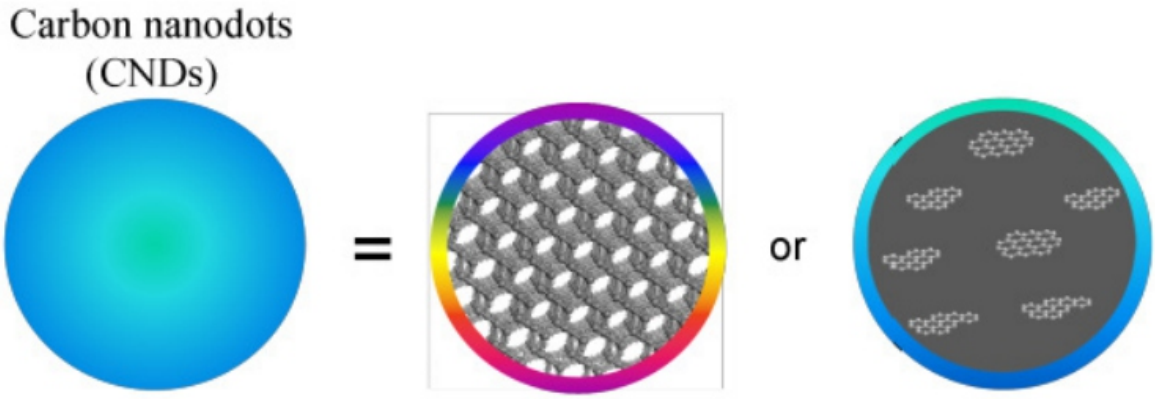

Figure 3. Carbon Nanodots

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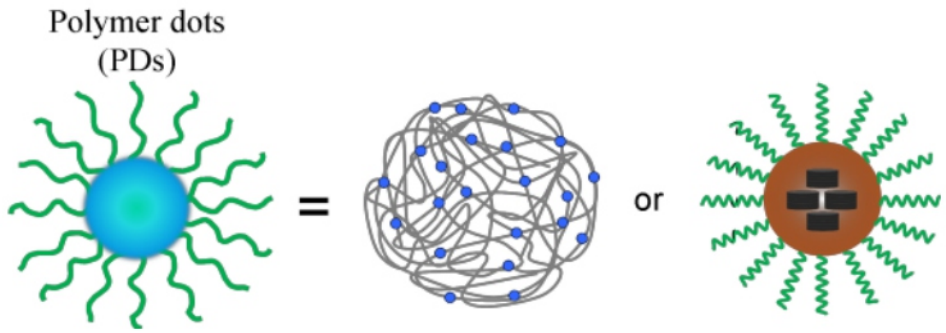

Figure 4. Polymeric Dots

4. Properties of Carbon Dots

CDs are small in size and regulate excellent photoluminescence (PL) [16]. It has great waterdistribution with better drug targeting and targeting. Itlessens toxicity while preserving therapeutic advantages.These are more biocompatible and safer. Faster development of new, safer drugs, simple and inexpensivemanufacturing technologies based on renewable rawmaterials, fluorescence properties with high quantum yield(QY), high thermal and optical photostability, adjustable excitation and emission, as well as simple surface functionalization environmentally friendly, are advantages.

5. Limitations

Difficulties in large-scale synthesis and purification
Inadequate purification techniques
Low fluorescence without functionalization

6. Source of C-Dots

For the synthesis of C-Dots, carbon sources from recyclable trash and renewable raw materials are the most cost effective and environmentally friendly options.

Various wastes, such as sago waste, mint leaves, orange peel trash, banana plant stalks, and frying oil waste, have been found throughout time as sustainable green predecessors for gathering C-dots [17]. Figure 5 depicts more C-dots precursors produced from biomass. Making C-dots from high-carbon trash from recycling and reuse will help to minimize waste, which will help to create an ecological community.

7. Fabrication Methods of Carbon Dots

Fluorescent CDs production procedures have been enhanced in recent decades via the employment of a wide range of manufacturing techniques. CDs can be synthesised from the top down or the bottom up [18]. They work by cutting the graphite matrix into nanoscale particles and chemically fusing small aromatic molecules.

7.1. Top-down Methods

Physical processes like laser ablation technology, electrochemical oxidation and arc discharge are used to reduce the large carbon materials like graphene, graphene oxide sheets, carbon nanotubes, carbon fibres and graphite to nanosizes.

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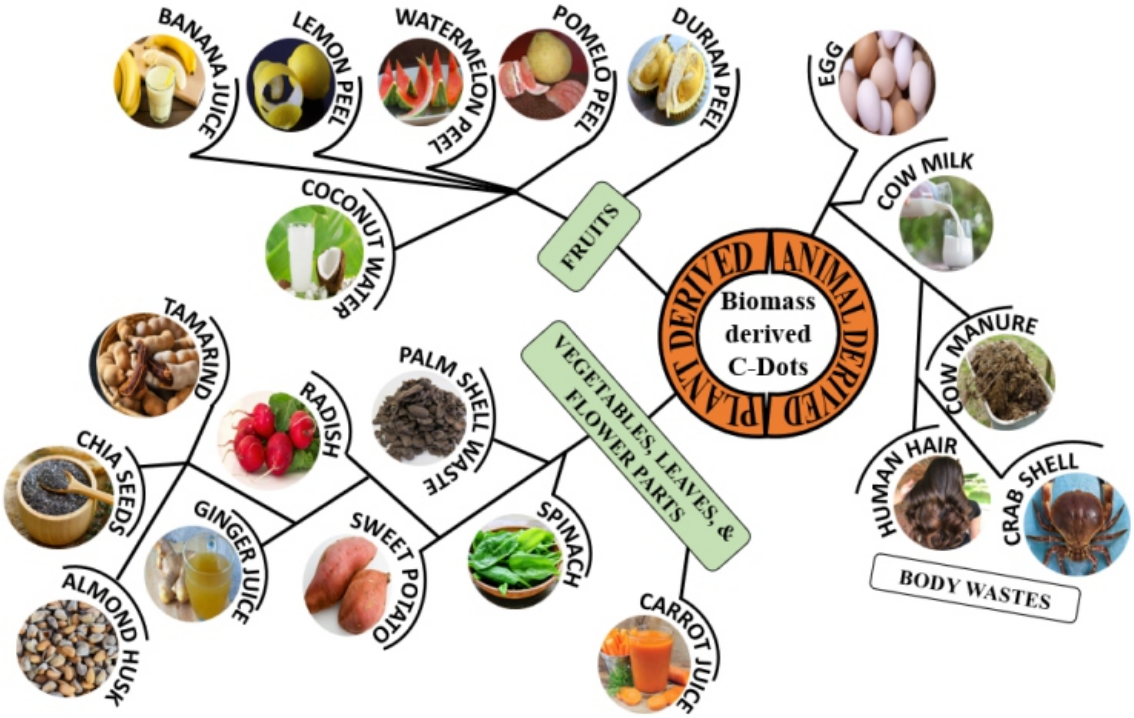

Figure 5. Biomass derived C-Dots from plant and animal source

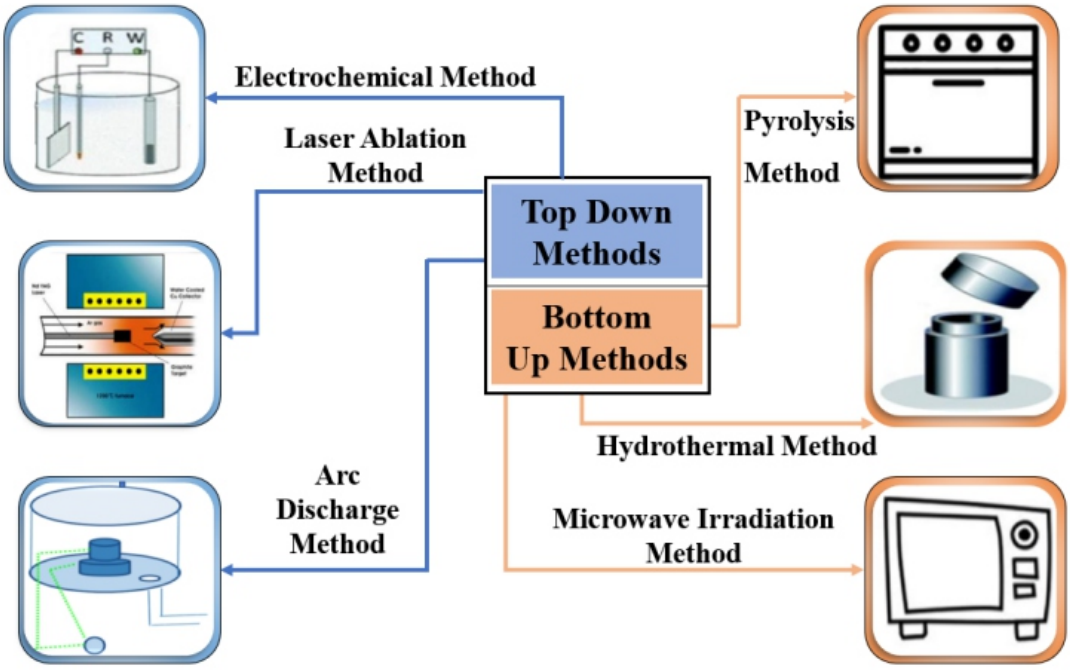

Figure 6. Fabrication strategies of Carbon Dots (C-Dots)

7.1.1. Electrochemical Method

An electrolytic process that uses larger organic molecules as electrodes in the presence of suitableelectrolytes is utilised in the electrochemical method forthe production of high-purity CDs from larger such as carbon nanotubes, graphite, and carbon fibres. This method involves the utilisation of an electrolytic process.In the presence of the electrolyte tetrabutylammonium perchlorate, Zhou et al. were the first to report the synthesis of CDs by starting with multiwalled carbon nanotubes as the starting material. Zheng et al. [19] developed water-soluble clean CDs by employing an electrochemical method that utilised graphite as an electrode and a phosphate buffer at a pH that was considered to be neutral. The CDs made in this method have been successfully utilised as potential biosensors in research and development [20].

7.1.2. Laser Ablation Method

In recent years, the production of CDs of varying sizes has increasingly resorted to the use of laser ablation. During the laser ablation process, massive organic macromolecules are exposed to pulsed laser light. This causes the formation of nanoscale carbon particles, which are then isolated from the larger molecular structures. It was reported by Sun et al. (2006) that a laser ablation technology was used for the first time to make CDs out of graphite powder. Carbon was obtained from a discarded cement mixture as well as graphite powder [21]. After the ablating process, the sample was subjected to a 12-hour long HNO3 reflux. The resulting solution was subsequently chilled and centrifuged to produce C-dots that were smaller than 5 nm.

7.1.3. Arc Discharge Method

Arc discharge accidentally made CDs. In the synthesis of SWCNTs, Xu et al. devised this approach. An electrical discharge across two graphite electrodes generates microscopic carbon fragments or CDs. Bottini et al. discovered CDs with vivid PL in purple, blue, and blue, green sections generated by an arc discharge technique on bare and SWCNTs [22]. Recently, boron and nitrogen-doped graphite QDs were created using the arc discharge method. Boron was doped with B2H6, while nitrogen was doped with Nh3.

7.2. Bottom-up Approach

CDs are produced in this small molecule using a varietyof processes including microwave irradiation,hydrothermal, and pyrolysis.

7.2.1. Microwave Irradiation Method

The synthesis of N-doped carbon dots (N-CDs) usingp-phenylenedia mine as the carbon precursor was accomplished by Ding et al. using the microwave approach that they invented. The NCDs that were developed exhibit excellent water solubility, the potential to be biocompatible, a sensitive responsiveness to pH, and robust optical stability. Through the use of microwave synthesis and phenylenediamine as the carbon source were able to produce carbon dots (CDs) that emit a bright red colour [21]. The CDs that are produced have the following characteristics: (a) a quantum yield that is up to 15%, (b) long-wave radiation (excitation below 470 nm with a maximum of 620 nm), (c) high photostability, and (d) a normal diameter that is 3.8 nanometers. These CDs have a pH-dependent response range that goes from 5 to 10, and the fluorescence that they emit can be inhibited by ferric ion.

7.2.2. Hydrothermal Method

Wu et al. developed a simple quantitative pH sensor that has potential applications both within cells and outside of cells, such as in the environment. Citric acid (CA) was used in the hydrothermal synthesis of nitrogen-doped (N-doped) graphene quantum dots (GQDs) with good quantum yields. This was accomplished by employing citric acid as the carbon source (QY) [23]. The N-doped GQDs that were manufactured show remarkable photoluminescence (PL) properties and a comparatively high quantum yield (QY) of about 36.5 percent. In order to produce two distinct kinds of N-CDs, Yang et al. utilised a solvothermal method. As precursors, they used citric acid combined with either monoethanolamine or L-serine. They used N, N-dimethylformamide (DMF) for the monoethanolamine, and they used deionized water for the L-serine [27–29].
Both of these were used as solvents. Under 365 nm UV light, N-CDs dissolved in DMF (DMF-CDS) released a blue-green fluorescence and a dark green colour, whereas N-CDs dissolved in deionized water (DW-CDS) emitted a blue fluorescence and a yellow in colour visible light.

7.2.3. Pyrolysis Method

Pyrolysis is a straightforward approach for creating Cds from organic molecules that employ fundamental chemicalreactions at extremely high temperatures in the presence of a strong acid or base. Martindale et al. [27] used sun radiation to pyrolyze citric acid at 180°C to make CDs with an average diameter of 6 nm. Guo et al. used a one-step pyrolysis procedure at 200°C for 24 hours to make durable CDs from hair (keratin). They obtained CDs and used them to detect Hg2+ more accurately and selectively. Rong et al. [28] created extremely photoluminescent nitrogen-doped CDs (N-CDs) by pyrolysis and fluorescence quenching in the presence of Fe3+ from guanidinium chloride and citric acid. N-CDs produced from their synthesis have found widespread application in metal ion detection and bioimaging.

8. Evaluation of C-Dots

To better understand the unique features of C-dots, extensive research on C-dot characterization has recently been presented.

8.1. Transmission Electron Microscopy Analysis

C-dots, which have an average size of less than 10 nm,may be measured in terms of their size and form using TEM.

8.2. Fourier Transform Infrared Spectroscopy and X-ray Photoelectron Spectroscopy

FTIR and XPS are used to analyse the C-dots' surfacecharacteristics. The FTIR spectrum captures the functionalgroups in C-dots, while XPS recognizes the identity of theelements [29].

8.3. Raman Spectroscopy (RS)

It is widely used for material identification and spectralcharacteristics of molecules.

8.4. Ultraviolet-visible (UV-vis) and Photoluminescence (PL) Spectroscopy

These are spectroscopic techniques, commonly used tostudy the linear optical absorption behavior of C-dots. Ingeneral, the optical absorption spectrum of C-dots peakedat about 230 - 340 nm with a tail extending into the visibleregion.

8.5. Electron Paramagnetic Resonance (EPR)

It is a spectroscopic method based on electron spin's physical characteristics. C-Dots feature several paramagnetic defect centres that are detectable with EPR. EPR spectroscopy may provide a wealth of information, such as structural and dynamical features of paramagnetic species and their interactions with the surrounding environment, to help increase understanding of electronic coupling effects in nanocarbons [30].

8.6. Quantum Yield Measurements

The quantum yield was determined using the referencesample quinine sulphate in 0.1 M H2SO4 as the standard,and the absorbance of both the reference and the CQD was kept in a fluorescence cuvette to avoid reabsorption effects [31]. Using the equation below, the quantum efficiency of the CQD sample was computed.

$$\emptyset_{CQD} = \emptyset_R X \frac{I_{CQD}}{I_R} X \frac{A_R}{A_{CDS}} X \frac{\eta^2_{CQD}}{\eta^2_R}$$

Where,
R and CQD = reference and sample,
I = Intensity of integrated fluorescence emission
A = Value of absorbance at the existing wavelength
"Ƞ" = Refractive index of the solvent medium

8.7. Fluorescence Detection

Stock solutions of numerous metal salts were made at the 100 µM concentration using deionized water. The highest fluorescence intensity was measured as F0 using 2 ml of diluted CQD in a cuvette (blank). Following that, each metal ion was injected at 100 µM and incubated for 5 minutes before the fluorescence spectra at the excitation wavelength were measured. The very selective metal ions were identified using the recorded fluorescence spectra. Finally, the limit of detection experiment for highly detected metal ions was repeated three times at lower concentrations and the findings of CQD quenching efficiency and CQD fluorescence enhancement are fitted to the Stern Volmer equation.

$$\frac{F_0}{F} = K_{sv}(X) + 1 \;\&\; \frac{F}{F_0} = K_{sv}(X) + 1$$

Where,
F0 = Fluorescence intensity in the absence of metal ions,
F = Fluorescence intensity in the presence of metal ions,
Ksv = The linear fit's slope
[X] = The concentration of metal ions.

8.8. Toxicological Profile (Biosafety and Biodistribution)

Cytotoxicity is a severe issue since it can have serious consequences on both diseased and healthy tissues. As a result, medications and treatments are being developed tom combat cytotoxicity. Although quantum dots are thought to have severe cytotoxicity issues, CDs have superior biocompatibility and are less dangerous. CDs are biocompatible on a variety of cell lines at various concentrations, with/without surface doping. In a similar vein, to get further knowledge on the cytotoxicity of NCDs, which are biocompatible with the majority of cellular organisms. Establishing safe doses or concentration ranges for bioapplications such as diagnostics, bio-imaging, treatments, and drug delivery at the molecular and

cellular levels across the animal system, organs, or integratedorgans is the primary goal of a biosafety assessment. This can be done at any level: molecular, cellular, organ, orintegrated [32]. As a result, creating their bio security profiles is critical. There have been some in vivo and invitro assessments, but there haven't been many systemiccomparisons (in vitro and in vivo) that are useful for futurebiological applications, nor are there particular responsevariances, such as B. Sensitivity has been identified inspecies (rats). Furthermore, an understanding of biodistribution is required for a thorough in vivo toxicity evaluation, both qualitative and quantitative.

9. Application of C-Dots Drug Delivery

Because of their small particle size, ease of synthesis and purification, prolonged drug release, biocompatibility, low cytotoxicity, and other features, C-Dots are excellent drug delivery methods [33]. C-dots address the issue of traditional drug carriers' low observability and traceability. 9.1. CDs as Sensing and Tracing Probes in Drug Delivery Because of the similarities in the fluorescence processes that NCDs and CDs share, they both offer real-time tracking and detecting properties that make it possible to administer drugs. Real-time tracking capabilities include the visualisation of drug translocation through microtubules, membrane-bound receptor diffusion,receptor-mediated signalling, and endocytic uptake.

Real-time tracking capabilities also include the monitoringof CD/NCDs between cells and the visualisation of virus behaviour in target cells. These capabilities contribute to an understanding of the in vitro and in vivo interactions of these nanocarriers with target cells [34]. In addition to this, CDs and NCDs have cognitive properties such as cell attachment, uptake, and the release of drugs within cells. The brilliant feature also helps when analysing how efficiently drugs are loaded and monitoring how quickly drugs are released.

9.1.1. Antimicrobial Drug Delivery

In compared to the currently available nanostructures, which have a low photosensitivity, high cost, and high level of toxicity, these NCDs may be easily produced, may coat pharmaceuticals with a variety of antimicrobial agents, and may combat the increasing antibiotic resistance of bacterial pathogens [35]. In order to stop the spread of these diseases, there is a wide variety of treatment options available, one of which is photodynamic inactivation. NCDs that have photosensitizing properties make visible light more brilliant by causing microbiological reactive oxygen species to be produced from molecular oxygen (ROS). These reactive oxygen species have an unspecific reaction to viral or cellular components, which causes severe damage and kills a wide variety of microorganisms including bacteria, fungi, viruses, and parasites. As a consequence of this, antibiotic-resistant bacteria are eventually rendered inactive, much like their drug-sensitive counterparts [36]. Furthermore, these bacteria produce non-specific damage to ROS, which suggests that resistance to NCDs is unlikely to develop. Extracts of aloe vera, for instance, have been shown to be effective against the bacteria Staphylococcus aureus and Escherichia coli (E.coli). The aloe vera extract NCDs emitted a brilliantly blue luminescence when examined with ultraviolet light and had a quantum yield of 12.3 percent. Antiviral, antibacterial, and antifungal actions are subcategories that fall under the umbrella term "antimicrobial".

9.1.2. Antiviral Drug Delivery

The pandemic problem caused by certain viruses, most notably the coronavirus, is exacerbated by the fact that NCDs offer a promising opportunity to cure infectious viral disorders with antiviral drugs. For example, Cur-NCDs have been shown to be effective antiviral agents against the Porcine Epidemic Diarrhea Virus (PEDV), which is widely acknowledged as a model for the coronavirus model. These

tests have been carried out and passed with flying colours. The researchers came to theconclusion that the hydrothermal method was successful after using it to produce uniform cationic CDs, which were then tested for their ability to inhibit viral replication by inducing the release of pro-inflammatory cytokines and interferon-stimulating genes. Based on the results of these tests, the researchers determined that the hydrothermal method was effective (ISGs). Experiments conducted in the field of virology demonstrated that Cur-NCDs have the potential to alter the surface protein structure of the virus. If this were to take place, it would result in an inhibition of virus entrance as well as a reduction in the production ofvirus negative-strand RNA, a suppression of ROS buildup, and an inhibition of virus buddin. All of these effects would be caused by an inhibition of virus buddin. The antiviral capability of Cur-NCDs against enterovirus 71 (EV71) demonstrated a good level of protection against a lethal dosage of EV71, lowered mortality, and great biocompatibility. This was shown by the fact that them ortality rate was reduced [37].

9.1.3. Anti-bacterial

Certain critical problems, such as the rapid spread of pathogens from contaminated surfaces to hosts and the development of antibiotic resistance, pose an increasing threat to humanity. Photodynamic treatment to limit microbial growth is promising to overcome such a situation [38–41]. Green CDs are said to be low-cost because they are abundant, do not require chromatographic purification, are inexpensive, compact, biocompatible, and have scalable photosensitization and multicolor emission capabilities. According to Bhamore et al. findings, the color-emitting NCDs from Manilkara Zapota fruits are appropriate imaging agents for bacterial cells like E. coli, Fomitopsis sp., and Aspergillus aculeatus because of their relatively small size and biocompatibility [42]. Green Cds are considered inexpensive because they are plentiful and require no special equipment. The nanosize enhanced intracellular diffusion and its non-toxic nature revealed its biocompatibility, allowing its use in imaging and antibacterial drug delivery. 9.1.4. Anti-cancer Activity Any nano-vehicle that may localise to diseased tissue via conjugation should have abundance, high affinity, chemical modification flexibility, and receptor binding selectivity. Interactions between ligands and receptors, aptamer targeting, and antigens and antibodies aid sick cell detection.[43] With their ability to conjugate and submicron size range, CDs have the ability to pass through physiological barriers and reach a wide variety of tissues, hence enhancing the process of intracellular internalisation and cellular absorption. The use of CDs enables maximum absorption and enables the administration of the appropriate quantity of medicine via conjugation.

9.1.5. Neurodegenerative Disease

Because of the electrostatic interactions that they have with positively charged CDs and negatively charged nucleic acids, cationic CDs have the potential to serve as gene carriers and delivery agents. Che et al. made positively charged CDs with a QY of 56.3% using porphyria polysaccharide and ethylenediamine precursors. This was done to stimulate neuronal differentiation of adult stem cells using non-viral gene transfer.[44]

9.2. Gene and Drug Delivery

Caionic CDs have demonstrated a great deal of potential for usage as gene carriers and delivery applications due to their ability to electrostatically interact with positively charged functionalized CDs and negatively charged nucleic acids. Che et al. [45] generated positively charged CDs with a high QY of 56.3 percent using porphyria polysaccharide and ethylenediamine precursors in order to stimulate neuronal differentiation of adult stem cells by non-viral gene delivery. In order to do this, the researchers

used porphyria polysaccharide and ethylenediamine precursors.

9.3. Catalysis and Energy

CDs have recently found applications in energy conversion and storage, as well as electrocatalytic and photocatalytic devices. This is due to the exceptional properties that CDs possess, which include low cost, broad optical absorption, high photo- and chemical stability, environmental friendliness and non-toxicity, and scalable synthetic methods. In their study, Xu et al. described ZnO nanorod-functionalized CDs (ZnO@CDs) as a medium for energy conversion and storage in the context of photoelectrochemical (PEC) wate splitting from solar energy conversion to hydrogen energy. ZnO@CDs as the photoanode boosted PEC activity for solar water splitting when compared to bare ZnO nanorods. This was possible because of the broader spectrum response region, which improved photoconversion efficiency.

10. Conclusion and Future Perspectives

CDs have become one of the most important presents in nanotechnology due to their magical characteristics and applications. CD synthesis strategies have been developed using both top-down and bottom-up approaches. Top-down CD production employs acid oxidation, arc discharge, laser ablation, and ultrasonic techniques to fragment bulk carbon materials. Bottom-up methods, on the other hand, carbonise molecular precursor materials through hydrothermal, microwave, and thermal pyrolysis. Microwave irradiation is the most extensively utilised technology for making CDs due to its quick response time and consistent heating [46]. According to in vitro and in vivo studies, C-dots are also non-toxic compared to other nanomaterials.

Although C-dots are expected to have more efficient nanoarchitectures for drug delivery and biological imaging, they have several disadvantages such as control over their size and surface properties that limit their usefulness [56– 58]. C-dots have been studied extensively, but the cause and mechanism of their photo luminescence remain a mystery, as different fabrication methods produce C-dots with non-uniform photo luminescence and different sizes. The application of C-dots is limited due to their complicated structure and additional cleaning problems. However, more research should be done to explain the activities of C-dots. It would also be beneficial to research the origin and mechanism of photo luminescence, as well as revolutionary high-quality Y fabrication technologies. C-dots could be used for drug targeting and diagnostic imaging in the future, which would be beneficial for human healthcare.

Conflicts of Interest

There is no conflict of interest.

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Evaluation of Phytochemicals, Anti-inflammatory and Antioxidant Potential of Aegle marmelos L. Leaves

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ABSTRACT

Aegle marmelos is a deciduous tree with trifoliate leave commonly found in the Indian subcontinent. The present study is to diagnose the phytochemicals physiochemical, qualitative and quantitative estimation. Total phenolic content was recorded at 53.52±3.25 µg Gallic acid equivalent (GAE) /mg of plant extract, total tannin content was observed at 6.4±1.02 µg GAE/mg and total flavonoid content was recorded at 37.4±2.65 µg Quercetin equivalent/mg. The study also comprised the Anti-inflammatory and anti-oxidant activity of plant leaves. Anti-inflammatory activity was examined through the HRBC method and compared with the standard drug diclofenac sodium. Ethanolic extract of leaves shows significant anti-inflammatory activity and provides 61.04±2.89 % of protection against hemolysis at 2000µg/ml of leave extract whereas at the same concentration diclofenac sodium provides only 54.55±2.35 % protection against hemolysis. The antioxidant potential of plant extract examines through DPPH free radical scavenging method. Leave extract showed better antioxidant activity in comparison to ascorbic acid. IC50 of plant extract is 48.99 ±1.96 % which is also better than the value of IC50 of ascorbic acid which was recorded at 50.39±2.05. The study showed that leaves extract of the plant has significant potential for the treatment of oxidative stress and inflammation.

Keywords Antioxidant Activity, Anti-inflammatory Activity, Natural Product, Herbal Drugs, Medicinal Plants

1. Introduction

From the time of the evolution of human beings,diseases and various health problems have been associated with them. To get rid of these health disorders, we need specific types of compounds that provide relief to the body from the illness [1]. These compounds can be considered drugs. Normally the drug molecules are a type of secondary metabolite synthesized in the plant body. Alkaloids, Flavonoids, Tannins, Glycosides, and Phenolic compounds are the most important types of secondary metabolites [2]. The therapeutic activity of plants has been subjected to evaluation since human civilization. To date, various drug molecules had been obtained from plant sources. The use of plants for medicinal purposes is increasing. According to the WHO report, 80 % of the world's population is still dependent on plants for their primary healthcare needs [3]. In India, since a long time ago back plants are used for medicinal purposes. Around two to five centuries ago, knowledge about the medicinal uses of plants came into existence. There were many researchers and physicians at that time. Sushruta who is considered the father of surgery and Charak who knows medicinal plants got popular because of their vast utilization of knowledge. Both the ancient scientist scripted their knowledge in the form of books i.e. Charak Samhita and Sushruta Samhita [4,5]. These books provide the basis of Ayurveda. From that period, the use of plants as medicine has increased. Various plants are subjected to analysis for their medicinal property. Plant-based medicine has various advantages. It is much more economical, easily available, no or fewer side effects. The major advantage is that it can be used in those conditions where traditional allopathic medicine does not show any improvement in patient condition. These advantages enlarge the market of herbal medicine in many countries. Not only India but the US, UK, Australia, and many other European

countries are investing their resources in the promotion and development of plant-based medicine. Inflammation and oxidative stress are associated with each other. Whenever the body's defense system may collapse due to the massive production of ROS and free radicals, the body or related organs suffer from the problem of oxidative stress. This oxidative stress leads to many pathophysiological problems in the body. Inflammation is the major sign or symptom of oxidative stress. So it is necessary to keep out or eliminate the problem of oxidative stress by taking antioxidants from outside to counter the problem. Several known antioxidants provide relief from inflammation and oxidative stress. Recently researchers aim to prepare plant-based drugs with several active biomolecules. Mainly these drugs contain many therapeutic agents having a wide range to treat many pathological conditions without any remarkable adverse or side effects.

Aegle marmelos has widely used in Indian society, its fruit is edible, and its leaves were donated to please lord Shiva in Indian methodology. Instead of this plant possess many pharmacological activities. Previously researchers reported that plant root has anti-inflammatory activity [6]. Later anti-microbial and anti-cancer activity of leaf extract was examined by Seemaisamy et al 2018 who found that acetone and methanolic extract has significant potential to kill or inhibit the growth of various bacterial strain and also potential to inhibit the growth of various cell lines including MDA-MB-231, HEp-2 and Vero cell [7].

2. Material and Method

Collection & Preparation of Plant Material

Fresh leaves of Aegle marmelos were collected from the Kalinga University campus in February 2022. The plant material was first washed with tap water and then with distilled water to remove all physical impurities, and then the plant material was dried for seven days in an air dryer at 35 °C. The coarse powder has been prepared for soxhlet extraction. A total of 47.4996 grams of plant sample was loaded into the extraction chamber for 48h. Ethanol is used as a solvent. The solvent has been evaporated and a semi-solid extract has been obtained.

Chemicals and Reagents

All the chemicals were of laboratory grade and purchased from Loba Chemicals Pvt. Ltd.

Microscopic Analysis

Microscopic identification with the help of a microscope, of the botanical ingredients is a standard for identification purposes in several solid and semi-solid compound formulations as well as plant raw material.

Determination of Moisture Content (Loss on drying,LOD)

Any drug must have a certain amount of moisture. High humidity increases the risk of microbial contamination and low humidity affects the solubility of the drug. To find out the moisture content in any plant powder or drug, weigh it by weighing a certain amount of it, then keep it in a hot air oven for half an hour, then cool and weigh it. Repeat until the weight of the drug or plant powder is stable. After that, with the help of the given formula, we find the percentage of moisture [8].

$$\text{Percentage of LOD} = \frac{\text{Average weight differnce of plant powder}}{\text{Weight of plant powder}} \text{ X } 100$$

Determination of Alcohol Soluble and Water Soluble Extractive

To determine the alcohol and aqueous solubility, dissolve 1 g of air dry plant powder or drug with 100 ml of solvent in a 250 ml conical flask and place it in a rotator for 6 h. After 6 hours, turn off the rotator and keep the flask overnight. The next day the filtrate is separated with the help of a Whatman filter paper. With the help of a water bath, the solvent is drained, the remainder is weighed and the solubility is determined with the help of the formula given below [8].

$$\text{Percentage of Solubility} = \frac{\text{Average weight difference of plant powder}}{\text{Weight of plant powder}} \text{ X } 100$$

Determination of Total Ash

Incinerated about 2g of accurately weighed drug in a crucible at a temperature up to 450 C, until free from carbon, then it was cooled in a desiccator and weighed. Then the percentage of total ash was calculated concerning the air-dried drug.

The total ash and acid insoluble ash concentrations of herbal medicines are essential criteria for determining quality and purity. The total ash values of leaves were obtained by gradually burning the powdered sample between 500°C to 600°C until it turned white, then desiccating and weighing the resulting powder [8].

Phytochemical Screening

The ethanolic extracts of leaves were tested for the presence of alkaloids, steroids, tannins, saponins, and glycosides. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Test for Alkaloids

About 15 mg of leaves extract was taken with 6 ml of 1% HCL stirred in a water bath for 5 to 6 min. This solution is equally divided into 3 parts.

1. Dragendroff's test: added 1 ml of Dragendroff's reagent to one portion of the above solution (2 ml); if an orange-coloured precipitate appeared that shows the presence of alkaloids [8,9].
2. Mayer's test: added 1ml of Mayer's reagent to one portion of the above solution (2ml); Alkaloids produce a cream-coloured precipitate [9,10].
3. Wagner's test: A few drops of Wagner's reagent were added to one portion above the solution. The presence of alkaloids is indicated by a brown precipitate [9,10].

Test for Steroids and Terpenoids

Using 100 mg of extract, 2 ml of chloroform and 2 ml of concentrated H2SO4 were added along the side of the test tube; a reddish-brown colour indicates terpenoids are present [11].

Test for Tannins

We mixed 0.5 g of leaf extract with 10 ml of distilled water. We added a few drops of 5% ferric chloride. Tannins cause black or blue-green precipitation [11].

Test for Saponins

0.5 g of leaf extract was shaken with 10 ml of distilled water in a test tube. The formation of foaming,

prevailing on warming in a water bath for 5 min, indicates the presence of saponins [11].

Test for Glycoside

1. Anthraquinone glycoside (Born Trager's test)

The mixture of 1 ml of extract solution and 1 ml of 5% H2SO4 was boiled in a water bath and filtrated. The filtrate was added with an equal volume of chloroform and stood for 5 min and later added half of its volume of dilute ammonia. The changes in the rose-pink to red colour of the ammonium layer indicate anthraquinone glycoside [9].

2. Cardiac glycoside (Keller-Killiani test)

5 ml of distilled water and 0.5 g of extract were shaken together. A solution of 2 ml acetic acid, some ferric chloride, and 1 ml H2SO4 was added along the side of the test tube. During the synthesis of cardiac glycoside, a brown ring appears at the interface, followed by a violet ring [12,13].

Determination of Phenolic Content

Estimation of phenolic contents in plant extract was estimated by UV- spectrophotometric method using a folin-ciocalteu reagent. The assay mixture contains 1 ml of an extract with 9 ml of water in 25 ml of a volumetric flask. One ml of folin-ciocalteu reagent was added to the flask and shaken well. After 5 minutes 10ml of 7% sodium carbonate was added and the mixture volume was adjustedn to 25ml. Standard solutions of Gallic acid (20, 40, 40, 60, 80, and 100 µg/ml) were prepared in the same manner, and absorbance was taken at 550nm with the help of a UV-spectrophotometer after 90 minutes of incubation. Total phenol content was expressed as µg of GAE/mg of the extract [14-16].

Determination of Tannin Content

The tannins were estimated by the Folin-Ciocalteu method. 0.1 ml of sample extracts was added to 7.5 ml distilled water followed by 0.5 ml of Folin-Ciocalteu phenol reagent to a volumetric flask of 10 ml and shaken well then kept at room temperature. Standard solutions of Gallic acid of 20, 40, 60, 80 and 100 µg/ml were prepared. Using a UV/VIS spectrophotometer, the absorbance of test and standard solutions was measured against prepared blank solutions at 725 nm [16-20].

Determination of Total Flavonoid Content

The determination of total flavonoid content in leaf extract has been measured by aluminium chloride colorimetric assay. To perform this assay 1 ml of plant extract has been taken. Add 4 ml of distilled water then add 0.3 ml of 5% NaNO2. After 5 minutes we added 0.3 ml of 10% of AlCl3. After adding aluminium chloride 2ml of 1M NaOH has been added to 10ml of the volumetric flask. Make the final volume 10ml. A set of standard drugs Quercetin (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml) has been prepared as discussed procedure. After 30 minutes of incubation period absorbance has been taken for standard and test solution at 510 nm with the help of Labtronics spectrophotometer model LT-2201 [21-24].

Preparation of Human Red Blood Cell (HRBC) Suspension

Mix fresh human blood with sterilized Alsever solution. Centrifuged at 300 rpm for 10 minutes, packed cells were washed three times with isosaline. A 10% v/v suspension of isosaline was prepared from the measured volume of blood.

Heat-induced Hemolysis

The theory states that hypo-tonicity stabilized the membrane of human blood cells, causing membrane lysis. 1 ml phosphate buffer (PH 7.4, 0.15M), 2 ml hyposaline (0.36 percent), 0.5 ml HRBC suspension (10 % v/v) with 0.5 ml plant extracts and various concentrations of standard drug diclofenac sodium (50, 100, 250, 500, 1000, 2000 µg/ml) and distilled water were incubated at 37 degrees Celsius for 30 minutes before centrifugation. A spectrophotometer set at 560 nm was used to calculate the protein content [25-29].

The formula below can be used to calculate the percentage of hemolysis.

$$\text{Percentage of Hemolysis} = \frac{\text{Absorbance of the test sample}}{\text{Absorbance of Control}} \text{ X } 100$$

Protection or membrane stabilization can be extracted by the following equation,

$$\textbf{Percentage of Protection} = \frac{\text{Absorbance of the test sample}}{\text{Absorbance of Control}} \text{ X } 100$$

Free Radical Scavenging Activity (DPPH assay)

To determine the free radical scavenging capacity of the ethanolic extract of Aegle marmelos leaves, the DPPH method was used. In this case, the sample caused a decrease in absorbance at 510 nm of a solution of coloured DPPH in methanol. A DPPH stock solution (1.3 mg/ml in methanol) was made. 10 mg of plant extract has been dissolved in 10 ml of distilled water 1 ml of this solution was taken in test tubes and diluted with the same solvent for up to 10 ml. This is a generic solution. In various test tubes, 0.10, 0.15, 0.25, 0.50, and 0.60 ml of stock solution were taken whose concentrations were successively 10, 15, 25, 50, and 60µg/ml. DPPH solution that has been freshly produced. Each of these test tubes containing ethanolic extracts (5µg /ml, 10 µg /ml 15 µg /ml, 20 µg/ml 25 µg ml, 50 µg /ml, 75 µg/ml) received 75 µl of DPPH (1.3 mg/ml) and was kept in the dark for 30 minutes before the absorbance was measured at 517 nm using a Labtronics UV-visible spectrophotometer model no.-2201. A control sample was made with the same volume of 95% methanol and DPPH, and a set of ascorbic acids with the same concentration. The percentage of radical scavenging activity of test and standard samples had been calculated using the below equation [30-34]. All experiments were performed in triplicate.

$$\%\text{ of inhibition} = \frac{\text{Optical density of Control} - \text{Optical density of Test sample}}{\text{optical density of Control}} \text{ X } 100$$

Control= Absorbance of DPPH alone

Test Sample= Absorbance of DPPH along with the different concentrations of plant extract/standard

An equation of the line obtained from plotting concentration versus % inhibition was used to calculate the Ic50.

3. Result and Discussion

Morphological Features of Plant

Aegle marmelos is a deciduous tree with trifoliate scented leaves that grows to 6–8 meters in height. Long straight spines can be found on the branches. The bark is corky and shallowly wrinkled. The hermaphrodite flowers are almost 2 cm wide, clustered, sweetly perfumed, and greenish-white in

colour. The small calyx is glabrous on the outside and features five short sepals. Five petals are rectangular ovoid, blunt, thick, pale greenish-white, and dotted with oil glands. The stamens are abundant and occasionally grouped in bundles. Usually oblong, oval, and slightly tapering, the ovary has many tiny cells (8-20), arranged in a circle, and each cell contains multiple ovules. Fruits are globose, oblong pyriform, 5–7.5 cm in diameter, rind grey or yellow, and fresh pleasant, thick yellow, orange to brown in hue. Seeds are abundant and are organized in cells surrounded by slimy, translucent mucilage. Woody hairs cover the seeds.

There is a dimorphism in the twigs of the trees. Twigs have a leaf at each node, a spine at each internode, and 3–5 cm long internodes. Foliage spurs are another form of twig that grows on the principal branches. There is a lot of little internodes on the foliage spurs, which are shorter than normal twigs (1–3 cm).

There is a leaf at each node in foliage spurs, but no spine. New twigs produced on second and third-year twigs have striated surfaces, while new twigs produced on first-year twigs have glabrous surfaces. The prickles on the stem are usually absent, or if they are present, they are not persistent. When the suckers reach a sufficient height and crown width, they branch off from the primary Bael trees. To protect the suckers against herbivores, the immature suckers have the strongest and sharpest spines. In most cases, gum-like sap is secreted from the wounds. The sap discharges first appear as long, thick threads, then dry out into lengthy solid crystals. Figure 1 shows the trifoliate compound of the leaf of the plant (Aegle marmelos) and Figure 2 shows the whole plant.

Figure 1. Trifoliate compound of Aegle marmelos leaf

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The leaves are alternating, solitary, or compound, with one or two pairs of opposite leaflets with short stalks.There are no wings on the petioles of the leaves, which are glabrous and long. When cut, the leaves are trifoliate and fragrant. The leaves are deciduous, alternating, and solitary or complex in appearance. Leaflets come in 2–5 oval-ovate or ovate-shaped, pointy, and frivolously toothed leaflets in compound leaves. A leaflet measures 4–10 cm in length and 2–5 cm in width. The leaflets are narrow, with conspicuous midribs on the underside. The petiole of the terminal leaflet is longer. After a dormant or reproductive phase, fresh foliage emerges that is lustrous and pink or burgundy in colour.

Figure 2. Aegle marmelos whole plant

Anatomical Study of Plant

Anatomy is a branch of biology that studies the interior structure of plants and is used to correctly identify taxa. The spatial arrangement of the dermal, ground and vascular tissue systems is fundamental to anatomy. Similarly, foliar epidermal microscopic traits such as epidermal cell shape**,** stomata type, pubescence presence or absence, and cell wall thickness are regarded as important aids for taxon identification and evolutionary relationships with other taxa.

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Anatomy of Young Leaf and Stem

The leaflets have a dorsiventral orientation. The epidermis of the adaxial surface has isodiametric cells that are larger than those of the abaxial surface in the transverse section. Stomata can be seen on the abaxial surface, at the same level as the surrounding cells. Palisade tissue is unilayered, contains 50% of the mesophyll, and is continuous throughout the midrib area. The secretory cavities are only found at the leaflet edges. The vascular bundles are tiny, collateral, and proximal to one another, with bundle sheaths. The midrib is encased with parenchyma and stands out on the abaxial surface. The epidermis of the wings and leaflets are comparable, but there is a variation in the percentage of palisade tissue, which accounts for less than half of the mesophyll. Secondary growth is shown in the center zone of the rachis, which has a pith surrounded by the xylem and phloem. The secondary phloem makes touch with chlorenchyma on the abaxial surface, which also contains a layer of collenchyma as shown in Figure 3.

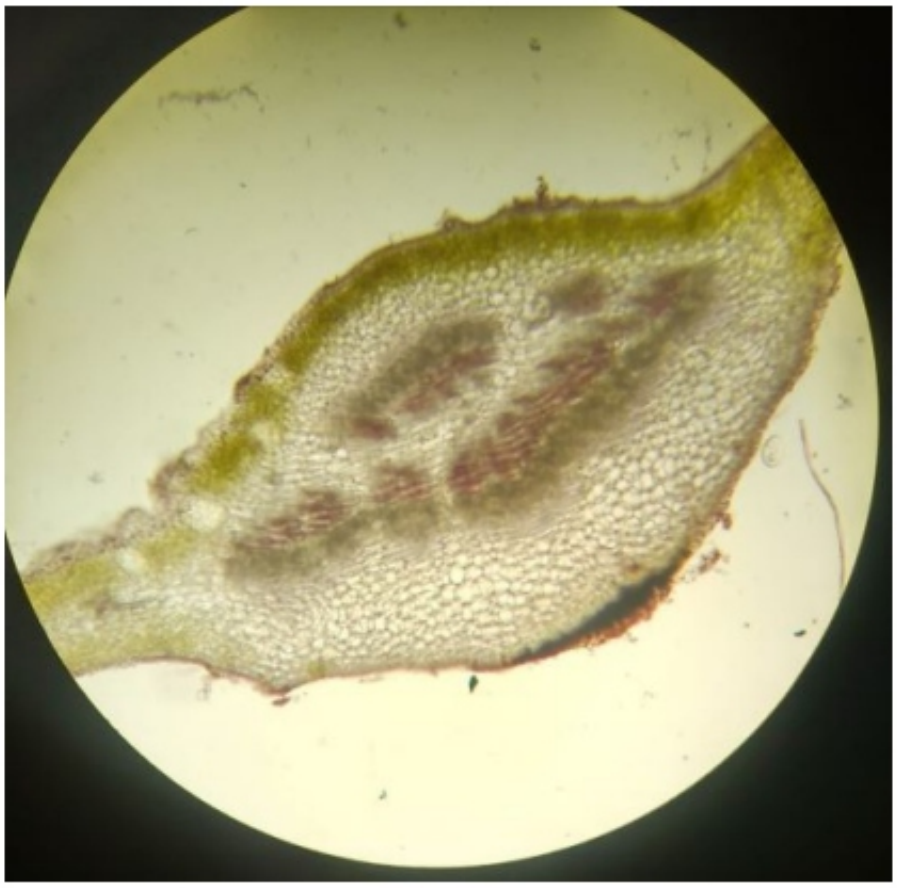

Figure 3. Leaf anatomy of Aegle marmelos

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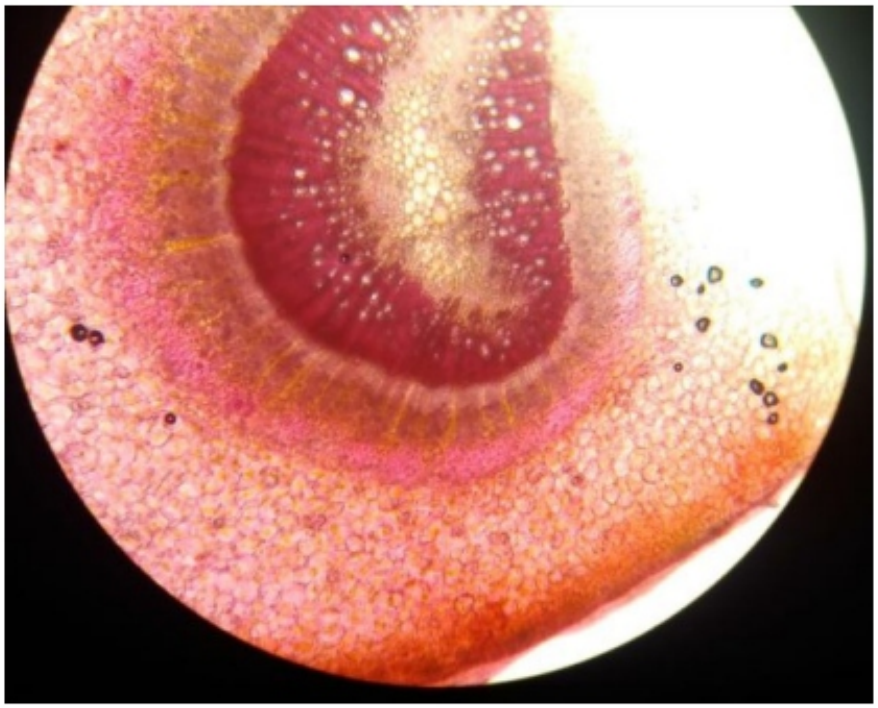

Figure 4. T.S. of Petiole of Aegle marmelos

In the epidermis, the trichomes are sunken. Both the adaxial and abaxial surfaces of leaves have a single-layeredepidermis with a striated cuticle. The epidermal cells on the adaxial surface have straight walls and are pentagonal or quadrangular in shape, but those on the abaxial surface have sinuous anticlinal walls and are irregular. Both epidermal faces have multicellular peltate hairs. A multicellular peduncle and a head with four core cells with thick walls and numerous peripheric cells characterise these trichrome. The leaves are hypostomatic, the stomata are anomocytic, and they come in a variety of sizes and distributional patterns, with some of them being so closed that the occlusive cells of adjoining stomata come into touch. A dermal tissue consists of a unilayered epidermis and a cortex composed of storage parenchyma subepidermal strata with numerous starch granules in young stems. A parenchymatous pith surrounds the parenchyma with numerous starch granules and secondary xylem, forming a continuous cylinder with rays in the middle zone of the stem. Figure 4 is demonstrating the anatomy of the petiole of Aegle marmelos. A roundish and squarish epidermal layer is present which is followed by a hypodermal layer. Hypodermal layers can be differentiated into adaxial, abaxial and lateral layers. In the case of Aegle marmelos, petiole 3-4 adaxial 4-5 abaxial and 2-3 lateral layers are present. Vascular bundles are completely closed and medullary rays are present.

Solubility or the Extractive Value of Plant The extractive value of the plant part will be calculated by using the following formula

$$\text{Solubility} = \frac{\text{Weight of extract} - \text{weight of empty petridish}}{\text{Weight of Plant Powder}} \times 100$$

Methanolic Soluble Extractive Value [MSEV] extractive value was recorded at 13.78%, whereas ethanolic soluble extractive value was noted at 12.66% and the highest extractive value was recorded with water which is 15.47%.

Physiochemical Study of Plant

The powdered drug was evaluated for its Physico-chemical parameters like a loss of drying, total ash values, acid insoluble ash and water soluble ash, and the results were tabulated (table 1).

Table 1. Physico-chemical analysis of plant

Physiochemical Analysis of plant powder	Outcome
Loss on drying (LOD)	7.68±1.42%
Total Ash	11.8±1.67%
Acid Ash Insolubility	2.17±0.61%

Phytochemical Screening

Table 2. Phytochemical screening of the leave extract

Test	Presence
Alkaloids	
(a) Dragendroff's test	+
(b) Mayer's Test	-
(c) Wagner's Test	+
Terpenoids	
Salkowski Test	-
Tannin	+
Saponins	+
Glycoside	
(a) Anthraquinone Glycoside (Borntrager's Test)	-
(b) Cardiac Glycoside (Killer-Killiani Test)	+

Estimation of Phenolic Compound

Using the folin-ciocalteu reagent method, the phenolic compound was estimated. The plant extract contained a significant amount of phenolic compound. Total phenolic compounds were expressed in terms of Gallic acid equivalent. The value obtained for the concentration of total phenolic compounds is expressed as µg GAE/mg of plant extract. The total phenolic content examined in the plant extract is 53.52 ±3.25 µg GAE/mg of plant extract.

Phenolic compounds were calculated by using the formula ($y = 0.0025x - 0.0008$, $R^2 = 0.9999$) (Shown in Figure 5).

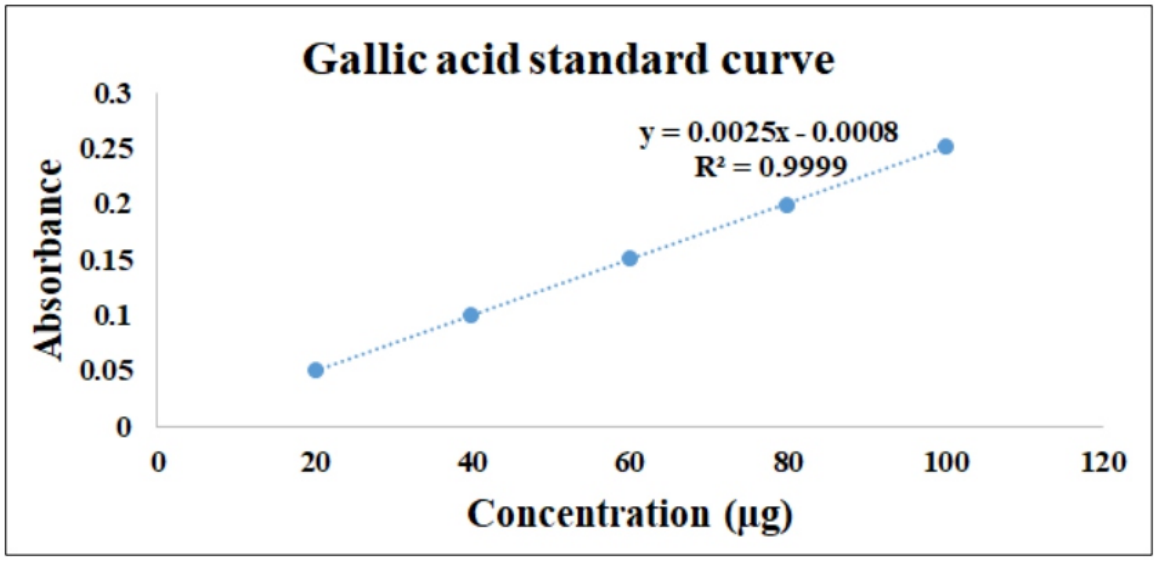

Figure 5. Gallic acid standard curve for phenolic content

Estimation of Tannin Compound

The estimation of the tannin compound was determined by the folin-ciocalteu reagent. The plant extract contains a large amount of tannin content. The total tannin compounds were measured in Gallic acid equivalents. The concentration of the total tannin component obtained is defined as µg GAE/mg of plant extract. The total tannin content of the plant extract was measured as 6.4±1.02 µgGAE/mg of plant extract. Tannin compounds were calculated by using the formula (y=0.0103x-0.0119, R2=0.9997) (shown in Figure 6).

Estimation of Flavonoid Compound

The estimation of Flavonoid compounds was determined by aluminum chloride colorimetric assay. A high level of flavonoid component was found in the plant extract. The total flavonoid compounds were measured in Quercetin. The concentration of the total flavonoid component obtained is defined as µg quercetin/mg of plant extract. The total flavonoid content of the plant extract was measured as 37.4±2.65 µg quercetin/mg of plant extract.

Flavonoid compounds were calculated by using the formula (y=0.0009x-0.0097, R2=0.9999) (shown in Figure7).

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Anti-inflammatory Activity of Plant Extract

In comparison to the standard drug diclofenac sodium and Aegle marmelos show quite significant anti-inflammatory activity. At higher concentrations i.e. 2000µg/ml of plant extract prevents hemolysis which was reduced up to 38.96±2.89 % while it would be reduced by diclofenac sodium 45.45±4.62% with the same concentration. Protection from hemolysis was also observed higher with plant extract which was recorded at 61.04±2.89 % while at the same concentration Diclofenacsodium showed 54.55±2.35% prevention of hemolysis. The comparative anti-inflammatory activity of plant extract and the standard drug has been demonstrated in Figure 8 and Figure 9. The present study showed that the anti-inflammatory activity of plant extract is dependent on concentration. As the concentration increases, plant extract stabilizes the erythrocytes membrane with more efficiency and protects against haemolysis.

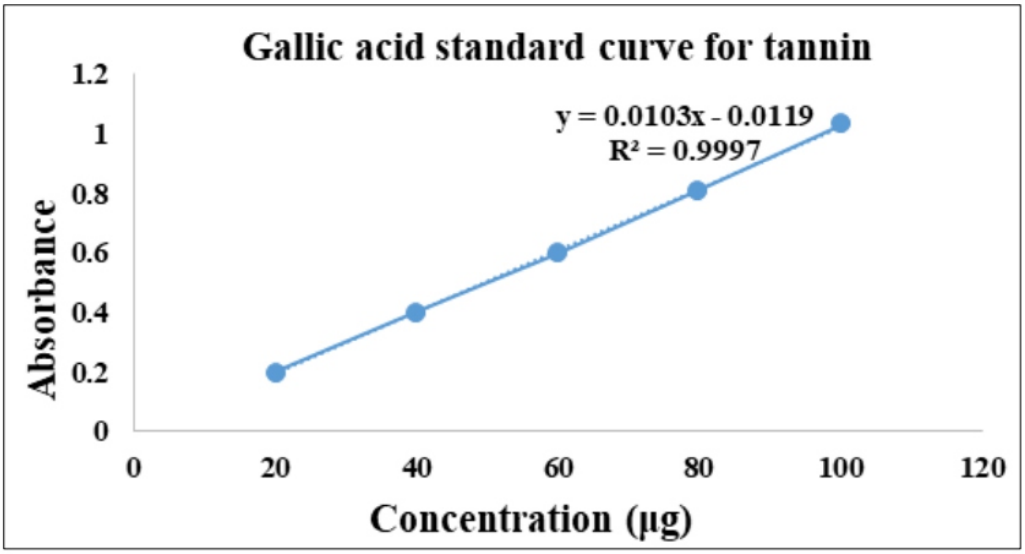

Figure 6. Gallic acid standard curve for tannin content

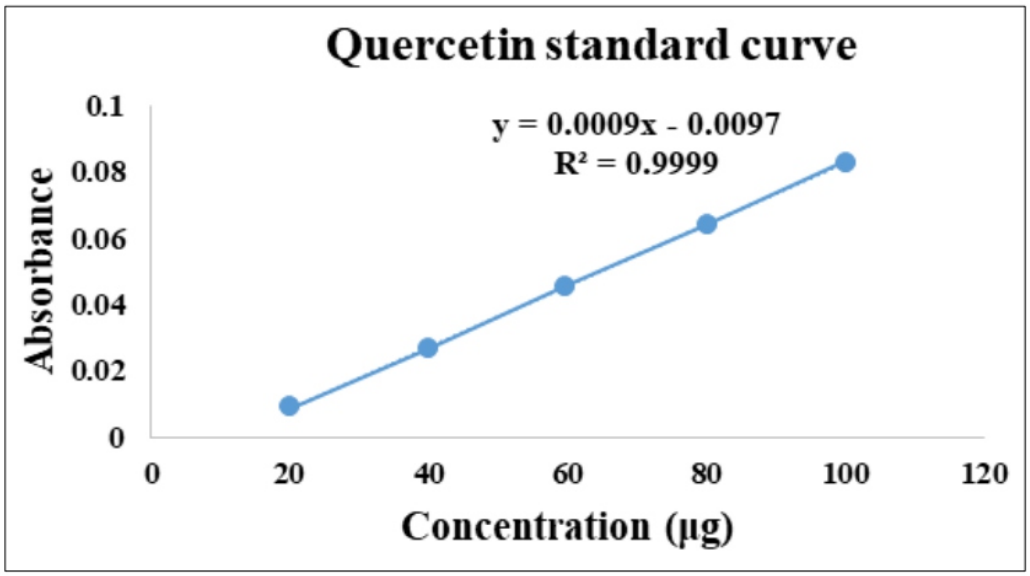

Figure 7. Quercetin standard curve for flavonoid content

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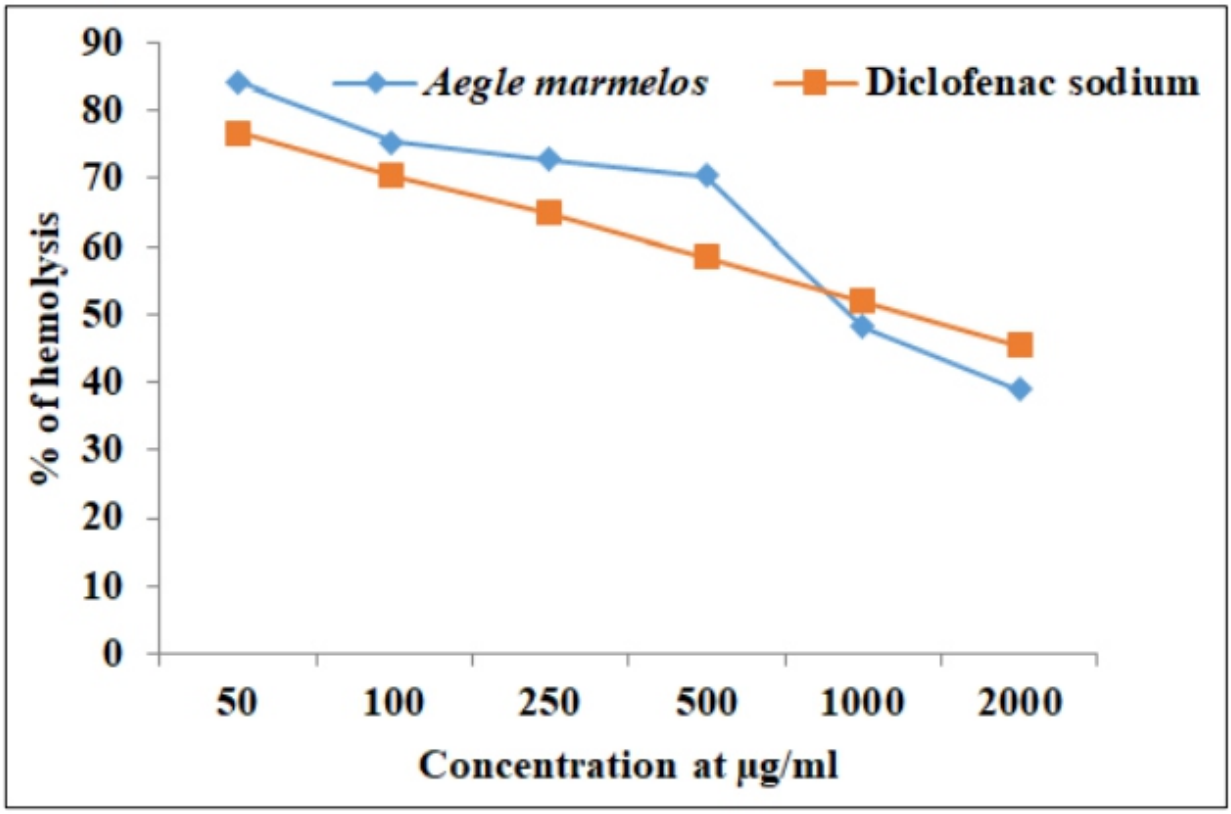

Figure 8. Comparative analysis of hemolysis inhibition of Diclofenac Sodium and Aegle marmelos

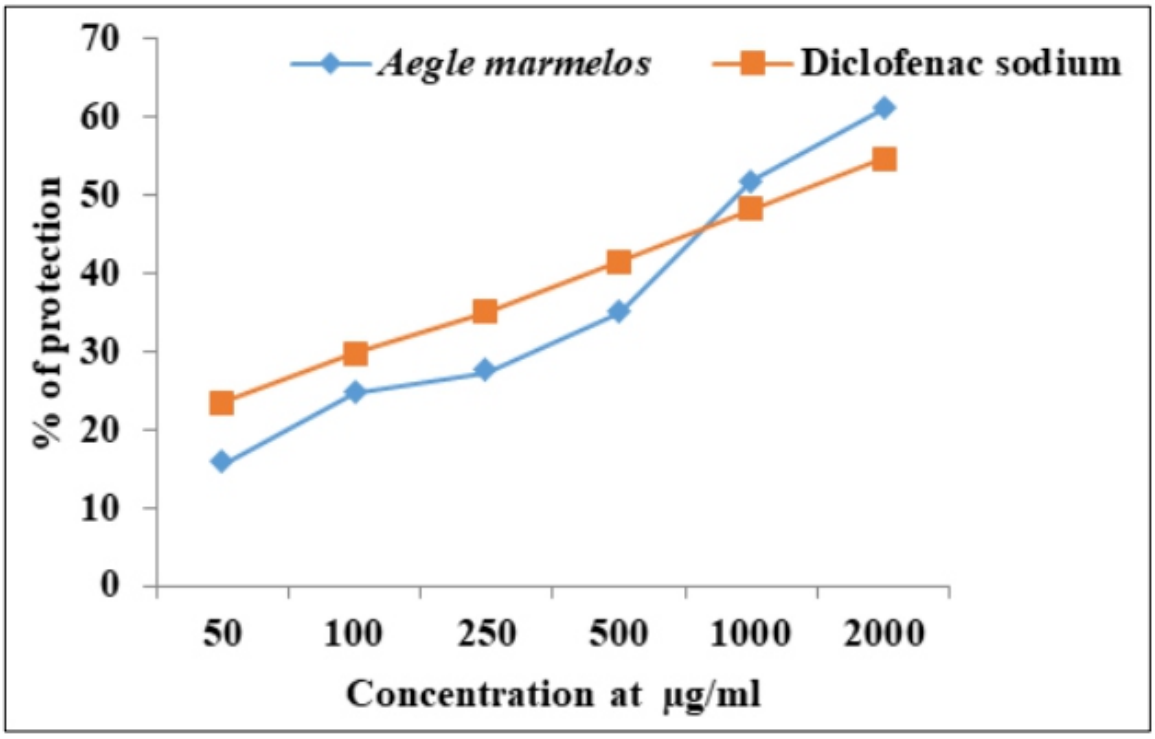

Figure 9. Comparative analysis of protection from hemolysis after treatment with Diclofenac Sodium and Aegle marmelos

Antioxidant Activity

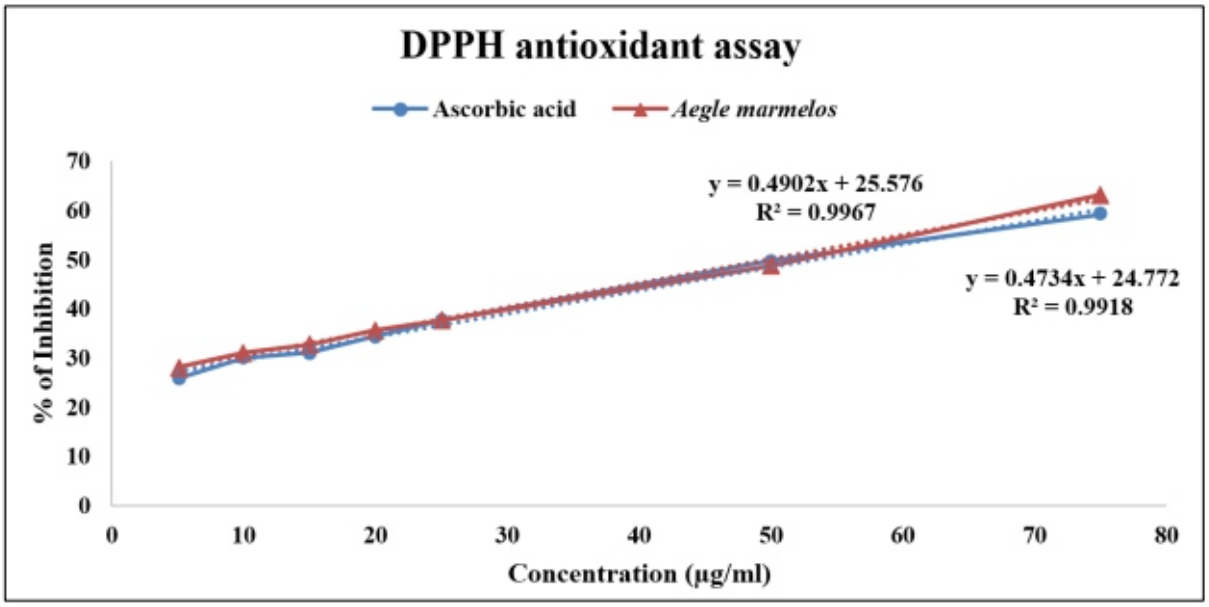

Figure 10. DPPH antioxidant Activity of Ascorbic acid and Aegle marmelos

The antioxidant potential of the ethanolic extract of Aegle marmelos (EEAM) is quite similar to the standard drug Ascorbic acid. Although the higher concentration of plant extract showed a little bit higher antioxidant properties than ascorbic acid. It scavenges 63.2±1.20 % of the DPPH while ascorbic acid scavenges 59.13±2.34 of the DPPH at 75ug/ml. The IC50 value of EEAM was recorded as 48.99 ±1.96 slightly better than the IC50 of ascorbic acid i.e. 50.39±2.05. The calibration curve of plant extract and ascorbic acid is described in Figure 10. It has been seen in many studies that the antioxidant activity of plant extract is dependent on the phenolic content of the plant, as this plant is also showing a good amount of phenolic compounds. Other secondary metabolites such as flavonoids and tannin may also add some more efficiency to its antioxidant potential.This in vitro method of anti-inflammatory activity wasmore convenient, flexible, and time-saving in comparisonto other methods. The main principle behind this model is that during inflammation, the lysosomal membrane burst and release lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases which cause further tissue inflammation. HRBC and lysosomal membrane are physiologically the same. If any drug can stabilize the RBC membrane, it will also stabilize the lysosomal membrane [35]. The result of the study showed that the ethanolic extract of the leaf of Aegle marmelos showed remarkable antioxidant and anti-inflammatory activity. It was just because of the presence of various polyphenols and flavonoid compounds. It has been previously seen that these compounds are effective in reducing acute inflammation. Various polyphenolic, flavonoids, and tannin compounds possess potent inhibitory activity against a variety of enzymes including protein kinase C,protein tyrosine kinases, phospholipase A2, and phosphodiesterases [36].

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In vitro Anti-inflammatory, Anti-diabetic and Anticancer Properties of Copper Nanoparticles Synthesized by Medicinal Plant Leucas aspera (Willd)

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ABSTRACT

Introduction: *The present study focuses on the use of Leucas aspera aqueous extract as a reduction agent for the synthesis of copper nanoparticles (CuNPs). The entire plant is employed in conventional medicine and as an insecticide. Scientific research has been done on the plant's potential as an anti-inflammatory, analgesic, and wound-healing agent. Intoxication by cobra venom also makes use of it. This study was intended to explore the in vitro anti-inflammatory, antidiabetic and anticancer activities of copper nanoparticles. Methods: Protein denaturation and membrane stabilization of human red blood cells (HRBC) were used to assess the in vitro anti-inflammatory activity. The antidiabetic activity of the CuNPs was evaluated by inhibiting both amylase and glucosidase in vitro, whereas anticancer activity against vero and MCF-7 cell line. Results: The Leucas aspera copper nanoparticles (LACuNPs) showed a dose dependent -inflammatory activity. The highest inhibition percentage in protein denaturation is 70.30±1.72% and 67.68±1.41% in HRBC membrane stabilization at 1000µg/ml concentration. In vitro antidiabetic activity exhibited 76.03 ± 1.14 of α-amylase and 71.54 ± 1.55 at α-glucosidase inhibition assay. The in vitro anti-cancer study revealed 50% apoptosis (IC50) of LACuNPs treated vero and MCF7 are displayed as 248.84±0.01 µg/ml and 137.39±1.51, respectively. Conclusion: The results clearly demonstrated the application of biosynthesized LACuNPs as potential anti-inflammatory, anti-diabetic, and anticancer agents in addition to their environmental friendliness.*

Keywords In vitro Anti-inflammatory, Copper Nanoparticles, HRBC, MCF-7, α-glucosidase

1. Introduction

Nanotechnology has recently attracted a lot of attention due to its adaptable qualities and possible uses in contemporary material science. Owing to the special characteristics of nanomaterials compared to bulk materials, there is a broad spectrum of investigation in many scientific disciplines. Because of its unique benefits, such as their straightforward, scalable, non-toxic method, and better biomedical applications, the synthesis of nanomaterials using biological channels has received a lot of interest in contemporary nanotechnology [1]. Copper is one of the most often found elements incorporated into crucial biological pathways, a transition metal. Many biologically significant compounds exhibit catalytic activity or transfer activities, such as the transfer of oxygen, and incorporate transition metals into their active sites [2]. As a result of being more biocompatible than other metal nanoparticles with medical value, copper nanoparticles (CuNPs) decrease the danger for toxicity. As a result, recently, biologically produced metal nanoparticles with a variety of therapeutic possibilities have become more significant [3]. The pathological condition known as diabetes mellitus (DM) is characterized by significant physiological abnormalities. It's a predominantly endocrine and metabolic condition defined by chronic hyperglycemia, which causes oxidative stress and a number of biochemical abnormalities [4]. Post-prandial hyperglycemia is a hallmark of type 2 diabetes mellitus (T2DM), often referred to as not

insulin-dependent type 2 diabetes (NIDDM), a chronic metabolic condition (PPHG). Although several different synthetic medicines have been employed recently to treat T2DM, most of them have significant long-term side effects, including drug resistance, hepatotoxicity, stomach pain, flatulence, and diarrhoea [5]. Finding an alternate drug with a hypoglycemic effect on T2DM is therefore necessary. Metal ions with antidiabetic activity both in-vitro and in vivo, including vanadium, zinc, manganese, copper, chromium, and tungsten, have been the subject of attempts to find alternative antidiabetic compounds [6]. α-glucosidase and α-amylase are interesting therapeutic options for the treatment and prevention of T2DM because of their involvement in sugar absorption. [7].

Anti-inflammatory medicines which are non-steroidal (NSAID) are typically used for relieving inflammation; however, long-term use has severe adverse effects, including gastrointestinal problems, hepatitis, nephritis, and more [8]. Opioids are a strong NSAID substitute, but they worsen psychiatric disorders like depression, addiction, and withdrawal symptoms [9]. Therefore, a powerful medicine that s ccessfully heals inflammation and decreases pain without causing any side effects is needed today. Scientific reports on cancer-related illnesses and fatalities have been steadily rising recently, necessitating efficient remedies to prevent and manage it. To address the core cause of cancer's destructive results on natural cells and lessen the extreme side issues due to their failure, conventional methods used for cancer detection and therapy have been ineffective [10,11]. To combat this, a number of researchers and scientists are striving to utilise different types of innovative therapeutic materials that are nano structured for focusing on a variety of cancer cells [12]. With fewer side effects, these nanomaterials are the leading contender for directly delivering drugs to the specific location of the targeted cancer cells [13].
India's wastelands and roadside vegetation include Leucas aspera. A common medicinal plant in tropical Asia and Africa is the Leucas aspera species, which belongs to the Leucas genus and the Lamiaceae family [14]. It has a pleasant odour and is frequently used as an antipyretic in crude form in south India. Its leaves' juice is applied externally to relieve uncomfortable swellings. Snake bites are treated locally with bruised leaves. [15]. The aim of this work was to investigate the in vitro anti-inflammatory,anti-diabetic and anticancer properties of Copper Nanoparticles from Leucas aspera aqueous extract.

2. Materials and Methods

Preparation of Plant Extract

Plants were collected from Sathyamangalam region, Erode, Tamilnadu. The Leucas aspera plants were shade-dried at ambient temperature and pulverized into powder. A hot air oven was used to extract 100 g of plant powder using the cold maceration method and dual-distilled water for twenty- four hours. The concentrate was kept at 4°C for use in subsequent experiments after filtering by Whatmann No. 1 to eliminate any debris, like cellular components and other substances.

Biosynthesis of Copper Nanoparticles

Leucas aspera copper nanoparticles (LACuNPs) were synthesized by mixing a 50 mL (5 mM) copper sulphate solution with a 5 mL aqueous plant elicit. NaOH (1N) solution was included in the mixture to bring pH level down to 7.0. The blend of green colours was also produced. The mixture was centrifuged, pellets were gathered, and they were dried in a hot air oven at 60°C overnight. The brunswick green colour powder was kept at ambient temperature for later usage.

Anti-inflammatory Activity

Albumin Denaturation Assay [16]

The albumin denaturation technique is used in the anti-inflammatory assay of LACuNPs. 2.8 mL of PBS, 2 mL of egg albumin, and the sample were combined, and the amalgamation was then incubated for 15 min. at 37°C. Bovine serum albumin (0.45 ml) and 0.05 ml of distilled water made up the reaction mixture, which had a pH of 6.3. Samples of varying concentrations are added to the reaction mixture, which is then heated for five minutes at 57o C and incubated at 37o C for 20 minutes. Later on 2.5 ml of phosphate buffer saline is added after the samples have of phosphate buffer saline is added after the samples have cooled. At 600 nm, the ELISA reader measures inhibition percentage. HRBC Membrane Stabilization Method [17] The LACuNPs at the concentration of 15.65, 31.25, 62.5, 125, 250, 500 and 1000At 600 nm, the ELISA reader measures inhibition percentage.

HRBC Membrane Stabilization Method [17]

The LACuNPs at the concentration of 15.65, 31.25, 62.5, 125, 250, 500 and 1000 *ug/* ml are nourished with HRBC solution for membrane stabilization assay separately. From a healthy volunteer, blood (2 ml) is collected, mixed with an equivalent volume of sterilized Alsevers solution (2% dextrose, 8% sodium citrate, 5% citric acid, and 0.42% sodium chloride), and centrifuged at 3000 rpm. The packed cells were washed in a saline solution. Aspirin serves as a standard and a control when different extract concentrations are prepared in normal saline. The haemoglobin content of each assay mixture is calculated by spectrophotometer at 560 nm after 30 minutes of incubation at 37o C and 20 minutes of centrifugation at 3000 rpm.

Antidiabetic Activity

α-amylase Inhibition Assay [18]

The test solution (LACuNPs) mixture is prepared by different concentrations with 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) which contains porcine pancreatic α-amylase enzyme (EC 3.2.1.1) (0.5 mg/ml) and were nurture at 25◦ C for 10 min. Later on when the reaction mixture had been incubated, five hundered micro litre of one percentage starch solution in a 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) were supplemented. Then the reaction mixture was incubated at 25°C for 10 minutes, after which 1.0 ml of dinitrosalicylic acid was added (DNSA). After 5 minutes of incubation in boiling water, the reaction was finally stopped and permitted to cool. A spectrophotometer was used to detect the absorbance of reaction mixture with 10 ml of distilled water at 540 nm after the dilution. As a control, the mixture of the enzyme and all other reagents was employed.

α-glucosidase Inhibition Analysis [19]

Divergent concentrations of LACuNPs were combined with 100 µl of α-glucosidase (0.5 mg/ml) in a 0.1 M phosphate buffer solution (pH 6.9) for a 10-minute incubation period. Fifty micro liters of a 5M p-nitrophenyl-D-glucopyranoside solution in a 0.1M phosphate buffer (pH 6.9) solution were then added. After 5 minutes of incubation at 25°C, reaction mixtures were measured using a spectrophotometer for absorbance at 405 nm. Results of α-glucosidase inhibitory activity were expressed in terms of inhibition percentage using the mixture of all other reagents and the enzyme excluding the control.

In-vitro Cytotoxicity [20]

The monolayer cell culture was trypsinized, and growth media was used to increase the cell density to 1.0x105 cells/ml. The diluted cell suspension 0.1ml (about 10,000 cells/well) was added to each well of a 96-well microtitre plate. As soon as a partial monolayer had developed after 24 hours, the supernatant was removed, the monolayer was washed once, and 100 µl of a drug intensity made in maintenance media were applied at each well on plates of microtitre. Later than, the plates were incubated for three days at 37°C in a 5% CO2 environment, with microscopic examinations and observations being made

every 24 hours. The medication solutions were removed after 72 hours from the wells and 50 µl of MTT in HBSS-PR were appended to all the wells. Incubation was carried out at 37°C in 5% CO2 with the plates gently shaken for 3 hours, the supernatant was removed, 50ml of propanol is added, and the plates are gently shaken again to solubilize the formation of formazan in the propanol solution. Microplate readers measuring absorbance were used at 540nm wavelengths.

3. Results

Anti-inflammatory Analysis

Inhibition of Albumin Denaturation Assay

Table 1 summarizes the in vitro bioassay results of LACuNPs impact evaluated against albumin denaturation. The denaturation of egg albumin was considerably (P<00.05) reduced by all doses that were tested. At 1000 µg/ml concentration, the highest percentage of inhibition was attained, which was 70.30±1.72%. At the same concentration, aspirin displayed an inhibition of 83.62±1.71% (Figure 1).

HRBC Membrane Stabilization Method

The results of anti-inflammatory activity which was indomitable by the human red blood cell membrane stabilization (HRBC) test were depicted in figure 1. LACuNPs showed anti-inflammatory activity depending on concentration of the samples. At 1000µg/ml of LACuNPS concentration produced 67.68±1.41% inhibition of HRBC hemolysis, respectively as compared with 83.40±0.70% produced by standard drug aspirin (Table 1)

Table 1. Anti-inflammatory activity of LACuNPs sample

Sl.No.	Sample Concentration µg/ml	Protein denaturation		HRBC	
		CULA	Aspirin	CULA	Aspirin
1	1000	70.30 ± 1.72^{b}	83.62 ± 1.71^{a}	67.68 ± 1.41^{b}	83.40 ± 0.70^{a}
2	500	62.83 ± 1.13^{c}	72.36 ± 0.77^{b}	58.70 ± 1.39^{d}	75.29 ± 1.11^{a}
3	250	51.31 ± 2.17^{c}	60.65 ± 1.59^{b}	47.78 ± 1.65^{d}	66.31 ± 0.71^{a}
4	125	40.29 ± 2.11^{c}	48.95 ± 1.23^{b}	37.74 ± 1.27^{c}	55.82 ± 1.37^{a}
5	62.5	30.34 ± 1.16^{c}	38.66 ± 1.45^{b}	31.66 ± 1.45^{c}	47.51 ± 1.23^{a}
6	31.25	18.49 ± 0.80^{d}	29.79 ± 1.41^{b}	20.88 ± 0.61^{c}	39.13 ± 1.52^{a}
7	15.62	11.59 ± 0.92^{d}	18.50 ± 0.88^{b}	14.15 ± 0.76^{c}	28.99 ± 1.42^{a}

Each value in the table is represented as Mean ± SD

Values in the same column followed by different letters are significantly different (P<0.05)

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2.3. ModernAnthelmintics

2.3.1. Phenothiazinesandpiperazines

The breakthrough in chemotherapy of helminthoses occurred in the 1940/1950's, with introduction of synthetic organic chemicals – phenothiazine (1940; the first phenothiazine, methylene blue, was applied as fabric dye) [27] and piperazine (1953) [28] – in nematodosis treatment:

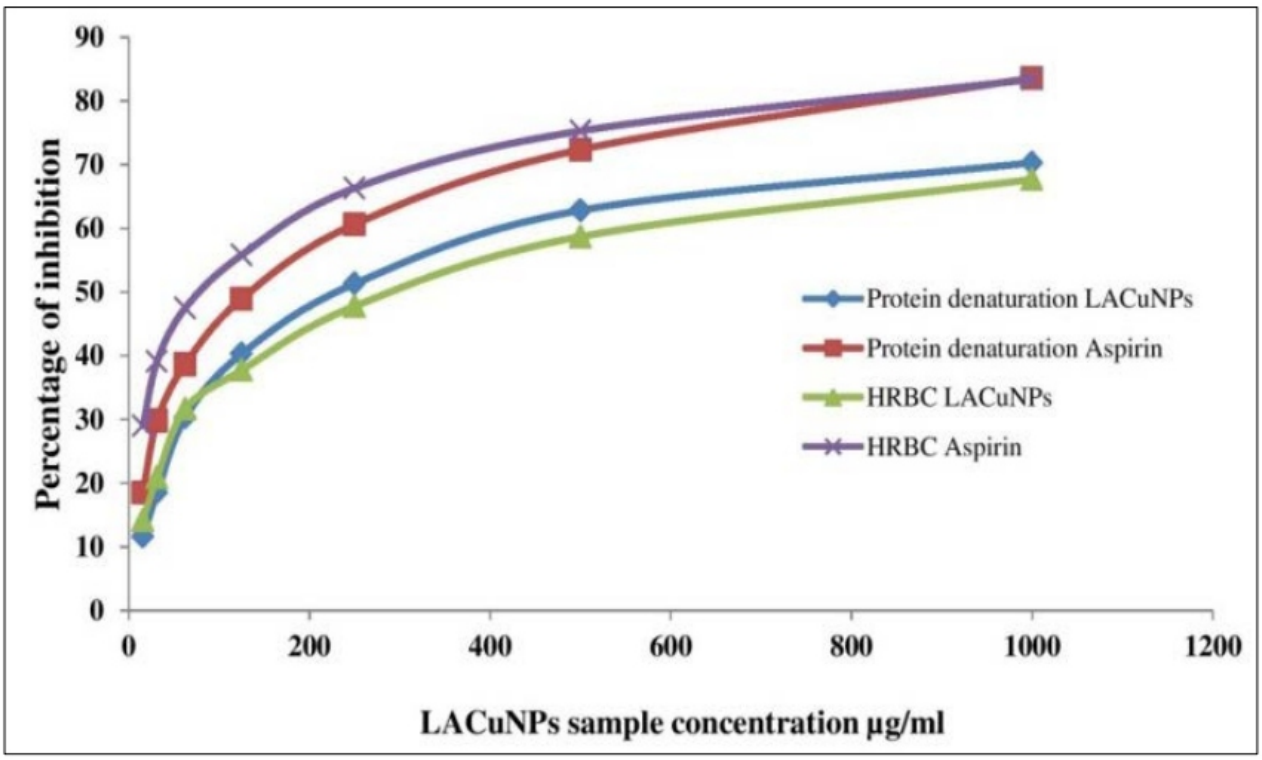

Figure 1. Anti-inflammatory activity of LACuNPs sample

Antidiabetic Activity

α-amylase Inhibition Assay

Table 2 illustrates LACuNPs ability to inhibit alpha-amylase. Since the concentration of the sample significantly affects the amount of enzyme inhibited, it indicates that the inhibition of enzyme is dose dependent. At 1000 µg/ml, the inhibition percentage was 76.03±1.14 with an IC50 of 176.47±1.85 µg/ml for LACuNPs and 94.22±1.48 µg/ml for acarbose. It was determined that LACuNPs have greater inhibitory potential than acarbose. α-glucosidase Inhibition Assay The LACuNPs ability to inhibit α-glucosidase is illustrated in figure 2. The LACuNPs concentration is related to the inhibition impact since the greatest concentrations showed the strongest inhibition action. At 1000 µg/ml, LACuNPs exhibit an inhibition percentage of 71.54±1.55. According to the estimated IC50, LACuNPs have a higher inhibitory potential IC50 197.23±1.28 than acarbose 119.17±0.48 µg/ml. Even with its modest efficacy, LACuNPs inhibition of α-glucosidase is however one of their suggested probable modes of action.

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Table 2. Anti-diabetic activity of LACuNPs

S. No	Concentration (µg/ml)	Percentage of inhibition value* ± SD µg/ml		
		α-amylase	α-glucosidase	Acarbose
1.	1000	**76.03±1.14^{b}**	**71.54±1.55^{c}**	83.05±1.36^{a}
2.	500	65.38±1.70^{b}	61.15±0.82^{c}	71.49±0.64^{a}
3.	250	55.39±1.71^{b}	52.91±1.59^{b}	63.04±1.79^{a}
4.	125	46.16±1.64^{b}	44.69±1.48^{b}	51.04±1.46^{a}
5.	62.5	35.55±0.58^{b}	35.62±1.34^{b}	41.27±1.21^{a}
6.	31.25	25.57±1.43^{b}	26.98±1.53^{b}	53.96±1.59^{a}
7.	15.65	18.72±1.61^{b}	17.96±1.13^{b}	26.09±1.11^{a}

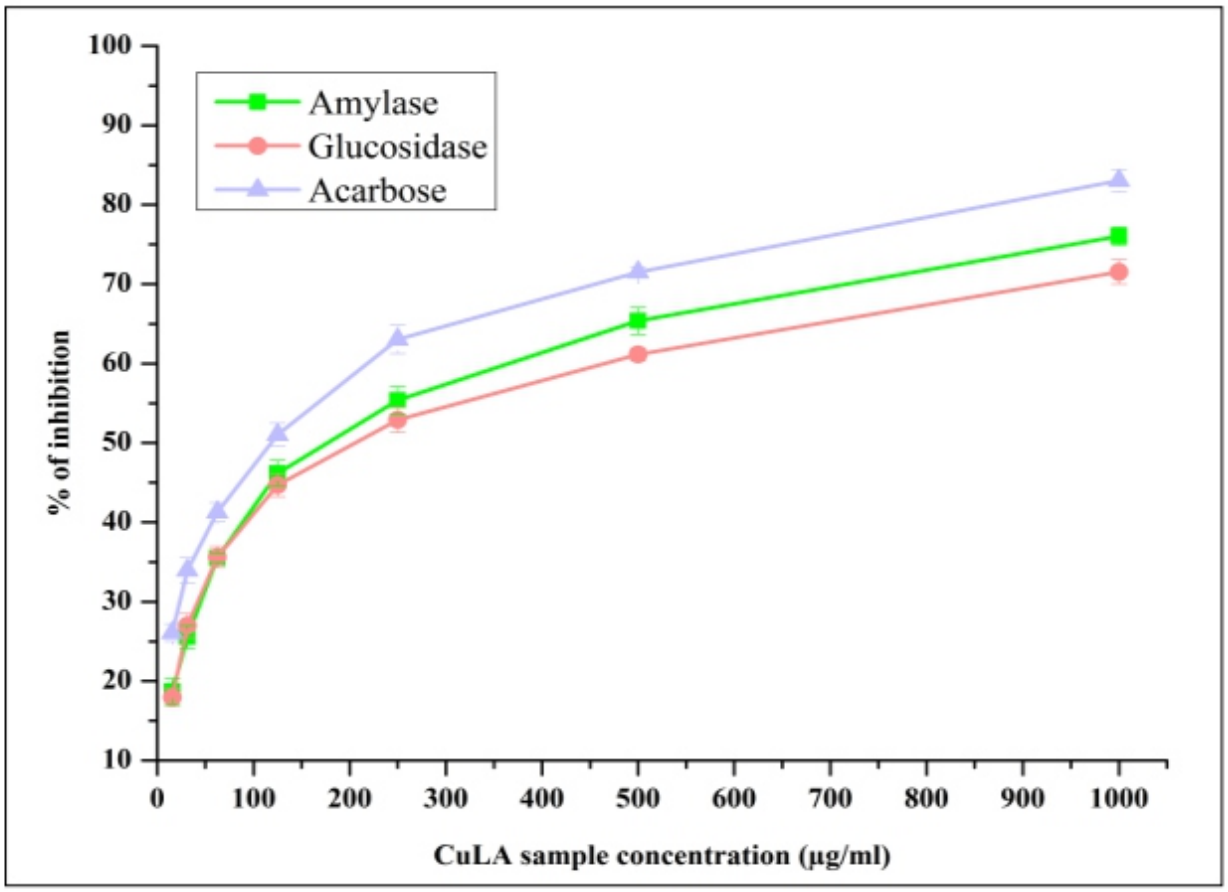

Figure 2. Anti-diabetic activity of LACuNPs

In-vitro Cytotoxicity

Figure 3 illustrates the cytotoxicity of biosynthesized LACuNPs against Vero and MCF-7 cells. The anti-cancer activity of LACuNPs against Vero and MCF-7 was observed according to the dose. After incubation, 38.98±1.60a % and 32.94±1.55% of cell viability was observed against Vero and MCF-7 cell lines at 500 µg/ml (Table 3). The inhibitory concentration 50% (IC50) was observed against Vero and MCF-7 cell lines is 248.84±1.05 µg/ml and 137.39±1.51µg/ml, respectively (Table 4).

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Table 3. Anti-cancer activity of LACuNPs

Sl.No.	Sample Concentration (µg/ml)	Percentage of cell viability in various cancer carcinoma	
		LACuNPs sample	
		Vero	MCF-7
1	500	38.98±1.60[a]	32.94±1.55[b]
2	250	49.51±1.67[a]	41.65±1.54[c]
3	125	60.51±1.25[a]	50.59±0.85[c]
4	62.5	69.61±1.55[a]	58.45±0.57[c]
5	31.25	78.53±1.22[a]	68.66±1.32[b]
6	15.62	87.68±1.58[a]	75.29±1.05[c]

Each value in the table is represented as Mean ± SD

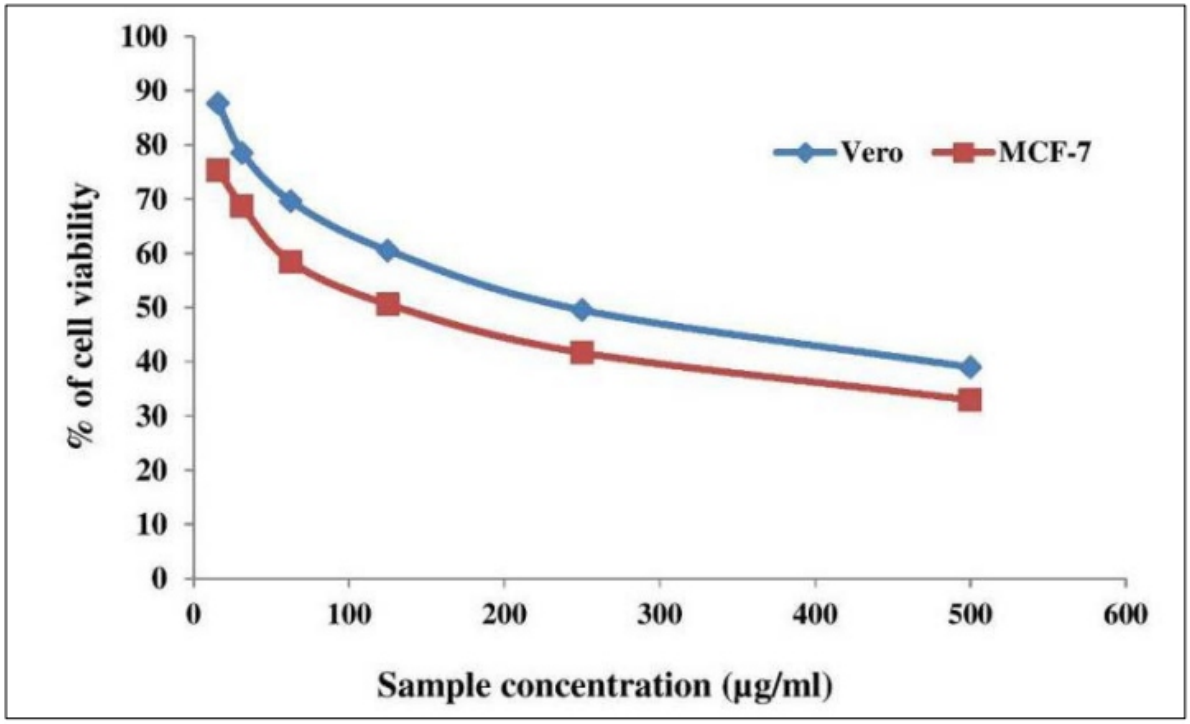

Figure 3. Anti-cancer activity of LACuNPs

Table 4. IC50 value of LACuNPs

S.No.	Samples	IC50 values of cell lines (µg/ml)	
		Vero cell	MCF-7 carcinoma
1	LACuNPs	248.84±1.05	137.39±1.51

4. Discussion

Biological, optoelectronic, semiconductor, and sensor applications are common uses for nanoparticles. Both plants' extensive phytochemistry may have contributed to the CuNPs' synthesis and stability. One of the reasons for autoimmune illness is macromolecular denaturation. In denaturation, the hydrogen, hydrophobic, and disulfide bonds may be modified electrostatically [21]. Inflammation may be a complex process that frequently results in pain and involves changes to membranes, increased protein denaturation, and increased vascular permeability, among other things. Proteins lose their tertiary and secondary structures when exposed to external stressors or substances such as strong acids and bases, concentrated salts, organic solvents, or heat. The breakdown of biological proteins causes them to cease functioning biologically. The most common cause of inflammation is thought to be protein denaturation [22]. The anti-inflammatory activity of the produced CuNPs was examined in vitro. When tested for in vitro anti-inflammatory action using the method for denaturing proteins, these LACuNPs showed the greatest level of inhibition. The inclusion of flavonoids, phenols, tannins, and glycosides in the aqueous fractions accounts for the high activity they displayed. Our findings support those of Malarvizh [23].

By inhibiting the release of lysomal components of active neutrophils including proteases and bactericidal enzymes, which induce further inflammation in tissues and damages the extra-cellular release, the regulation of inflammation depends on lysosome stabilization. The processes that could increase or accelerate the intracellular components may be inhibited by the extract [24]. Based on in vitro testing outcomes, LACuNPs show appreciable anti-inflammatory activity in comparison to the control.

Both flavanoids and tannins have anti-inflammatory properties. It is well known that flavonoids like quercetin can effectively reduce acute inflammation., Protein tyrosine kinases, protein kinase C, phosphodiesterase and phospholipase A2, are just a few of the enzymes that certain flavonoids have strong inhibitory effect against. The presence of flavonoids, tannins, etc. in the fraction, moreover alone or in combination, may be the cause of the fraction's anti-inflammatory activity [25]. To assess the ability of the produced particles to inhibit enzymes, the L. aspera extract-mediated CuNPs were subjected to an experiment for α-amylase inhibition. The results showed that synthetic CuNPs had a modest level of inhibition for the -amylase enzymes, which is comparable to the sample's percent inhibition. At 1000 µg/ml concentration, CuNPs inhibited the -amylase enzyme by 76.03±1.14 %, which is roughly comparable to the other report of Silybum marianum copper nanoparticles [26]. Our research suggested that the LACuNPs have a significant capacity for enzyme inhibition. The ability of medicinal plants to inhibit the activity of the enzyme -amylase can be attributed to a variety of factors [27]. The essential enzymes for glucose metabolism, α-glucosidase, could be effectively inhibited by the phytogenic CuNPs. The change in size and form may be the cause of the variation in the level of inhibition. This is supported by the observation that synthesised LACuNPs inhibited glucosidase the most, (71.54±1.55) at 1000 µg /ml. This result was comparable with the previous report of Dioscorea bulbifera copper nanoparticle [2]. Postprandial hyperglycemia is a complication of Type II diabetes mellitus (T2DM), which can be efficiently managed with -amylase and α-glucosidase inhibitors.

Over the past 20 years, copper, among the crucial biological trace elements, has had a significant biological impact and is a crucial component of anticancer medications [28]. Nano ionisation is a routine contemporary technique of getting beyond this restriction and is probably a targeted therapy for the growth of cancer cells. CuNPs damage to cancer cells is primarily caused by the release of copper ions from the nanoparticles, which bind to the cell's DNA. Consequently, it leads to cell death and damages DNA [29]. The current study evaluated the cytotoxicity of LACuNPs against MCF-7 cell lines, and it also looked at the cytotoxicity of healthy (Vero) cell lines. A dose-dependent cytotoxic response to LACuNPs was seen in breast cancer cells. LACuNPs were discovered to have IC50 values of

137.39±1.51 µg/ml and 248.84±1.05 µg/ml against Vero Cells (non-tumor cell). This result was comparable to that of the earlier Wrightia tinctoria copper nanoparticle investigation [30].

5. Conclusions

In conclusion, a simple, easy, and environmentally friendly method for creating well-dispersed Cu nanoparticles was disclosed. Without the employment of any additional reducing and capping agents, L. aspera leaf aqueous extract served as both a stabilizing and reducing agent during the process. Although L. aspera's anti-inflammatory, anti-diabetic, and anticancer activity is less potent than standard, it can be more potent when its concentration is raised to tolerable levels. It is recommended that clinical trials be conducted in order to validate these findings in humans due to the considerable outcomes obtained in the in vitro studies.

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