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THE FUTURE OF NON-INVASIVE WAYS TO TREAT CANCER

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ABSTRACT: The non-invasive technique in which a variety of nanoparticles are used to deliver the chemotherapy drug to the location of metastatic cancer has fewer side effects, high bio-availability, and high bio-stability of a drug. The anti-proliferative activity of EGCG is very efficient against a variety of cancer, but the problem is that it can't withstand the environment of an intestine. One of the nano-system is Spanlastic (type of niosome system) which can carry both lipophilic and hydrophilic drugs. Spanlastic is capable of holding a large amount of drugs; also, the penetration potential of spanlastic is very efficient due to the addition of cholesterol and ethanol in it. Non-ionic surfactant made up of Span-60 (mono-stearate), and the edge activator made of Tween-80 increases the flexibility and deformability of the system. Ethanol injection will be used to prepare the EGCG loaded spanlastic system. After preparing, the Z-average diameter, polydispersity index, and spanlastic will be determined by dynamic light scattering (DLS) and, with the help TEM imaging, will check the shape and size of the system. Further, the system will be tested against SIF and SGF incubators to calculate the cellular antioxidant activities and FRAP. Detection of apoptosis will be checked by Annexin V–FLUOS staining kit. The EGCG loaded in spanlastic will treat cancer and many other diseases as EGCG has many other healing properties.

INTRODUCTION: Cancer is the second cardinal cause of death globally, and it is responsible for an estimated 10 million deaths in 2020. Out of every six deaths, one is due to cancer. Approximately 70% of deaths from cancer occur in low and middle-income countries [1]. Chemotherapy is still considered the main treatment for different types of cancer (advanced levels). But chemotherapy causes a lot of severe side effects such as iron-poor blood (anaemia), headache, even migraine in major cases, nausea, severe hair loss, vomiting, drastic weight loss, and loss of appetite [2].

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The non-invasive techniques are preferred over traditional techniques as they are more convenient, have fewer side effects and are economical [3]. There are several non-invasive techniques used in the treatment of cancer. Still, there is a lot of problems depicted in the various studies, such as low solubility, less permeability and also the release of some cytotoxins in the body.

Green, oolong and black there are three different groups of tea [4]. Green tea is extracted from *Camellia sinensis* L. of the Theaceae family. Green tea contains polyphenolic compounds known as catechins and caffeine (1, 3, 7-trimethylxanthine). The major component of Green tea is (−)-Epigallocatechin gallate (EGCG), which is a water-soluble flavonoid. There are also some other catechins present in green tea, which are (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin (EC), *etc*.

Among all these catechins, EGCG has a better chemopreventive property [5, 6]. However, due to the instability and low bioavailability, developing treatments based on EGCG is challenging. In humans, the maximum plasma EGCG concentrations of 0.15 µM are achieved after consuming two cups of green tea. The oral bioavailability of EGCG is only 0.2-2% [7]. The main reasons for low bioavailability are instability and biodegradation in the gastrointestinal tract. Lipinski's rule of five (which states that compounds with a molecular weight greater than 500 g/mol and 5 or more hydrogen bond donors do not easily pass through the plasma membrane) explains the low absorption of EGCG in the intestine [8, 9]. As EGCG is like a perfect anti-cancerous agent, but because of low bioavailability, instability and biodegradation in the gastro-intestinal tract, we need a better approach to deliver EGCG at the target cell [10].

The use of nanotechnology to deliver the EGCG to the target cell can be seen as the potential player among all other techniques. The success rate depends upon the nanoparticle used. Several Lipid-based nanocarriers such as Niosomes containing cholesterol, Layered double hydroxide (LDH) nanoparticles can be used as a good carrier in transferring EGCG to the target cell. Niosomes and LDH both increase the bio-permeability of the EGCG and also the stability of the compound when inside the body. EGCG cannot survive in a slightly alkaline pH, but when loaded in niosomes or LDH, it can easily survive at the pH of 7.4, also it is stable at high temperature, and the effect of EGCG on the tumour cell is also increased significantly as the more and more anti-proliferative potential of EGCG is reaching the cells. There is no doubt that the LDH and niosomes are increasing the anti-proliferative property of EGCG, but still, the upgrade is needed. The up-gradation is loading of EGCG inside the Spanlastic, and then Spanlastic will provide a better and safe route to EGCG. Spanlastic is used in this technique due to its excellent bio-permeability, highly elastic and deformable nature, and can hold a larger amount of drug inside. The combination of EGCG and Spanlastic may change the traditional way of treating cancer because this combination may have a few or zero side effects and may show better results.

(−)-Epigallocatechin gallate (EGCG): Green tea is produced from leaves that have not undergone fermentation. Flavonoids (polyphenols) are generally found in plant foods, fruits, herbs and tea; they have low molecular weight. Flavan-3-ol (also called catechin) accounts for 30-40% of the total weight of green tea, making it the most abundant compound in green tea [11].

Basic catechins contain two or more aromatic rings. Hydroxyl group on carbon three positions and/or the higher degree of hydroxylation of the B ring would be primarily responsible for the potent antioxidant activities of catechins [12, 13]. Tea polyphenols possess chemopreventive effects and anti-proliferative effects on many different types of cancer, such as prostate cancer [14], breast cancer [15], lung cancer [16], and colorectal cancer [17]. The anti-proliferative effect of EGCG is even more powerful than 5-fluorouracil, which is a chemotherapy drug for colorectal cancer and skin cancer. EGCG can inhibit leukocyte chemotaxis, quench free radicals, chelate transition metals, and interrupt lipid peroxidation chain reaction [18]. It is also used as an antioxidant, anti-inflammatory, anti-atherosclerotic, cardio-protective, anti-viral, neuroprotective, anti-diabetic, anti-bacterial, and anti-obesity [19, 20]. The antioxidant effects of EGCG are exhibited in foraging the free radicals in the body and inhibiting the formation of ROS (Reactive oxygen species). Also, the EGCG shows significant prooxidant effects under high-dose conditions [13]. The half-life of EGCG is between 3-4 h hence rarely accumulates in the body.

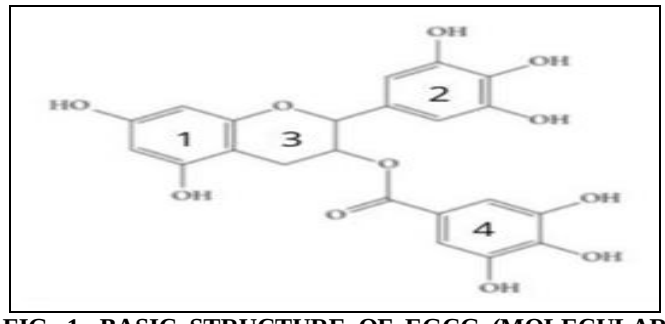

FIG. 1: BASIC STRUCTURE OF EGCG (MOLECULAR WEIGHT: 458.375, MOLECULAR FORMULA $C_{22}H_{18}O_{11}$)

There is research [21] in which the different types of tea phenols have been tested on different categories. 10 major green tea polyphenols are chosen and they are tested against two human colorectal cancer cell lines, SW-480 and HCT-116.

Tea polyphenols are catechin (C), caffeic acid (CA), gallic acid (GA), epicatechin (EC), gallocatechin (GC), catechin gallate (CG), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG).

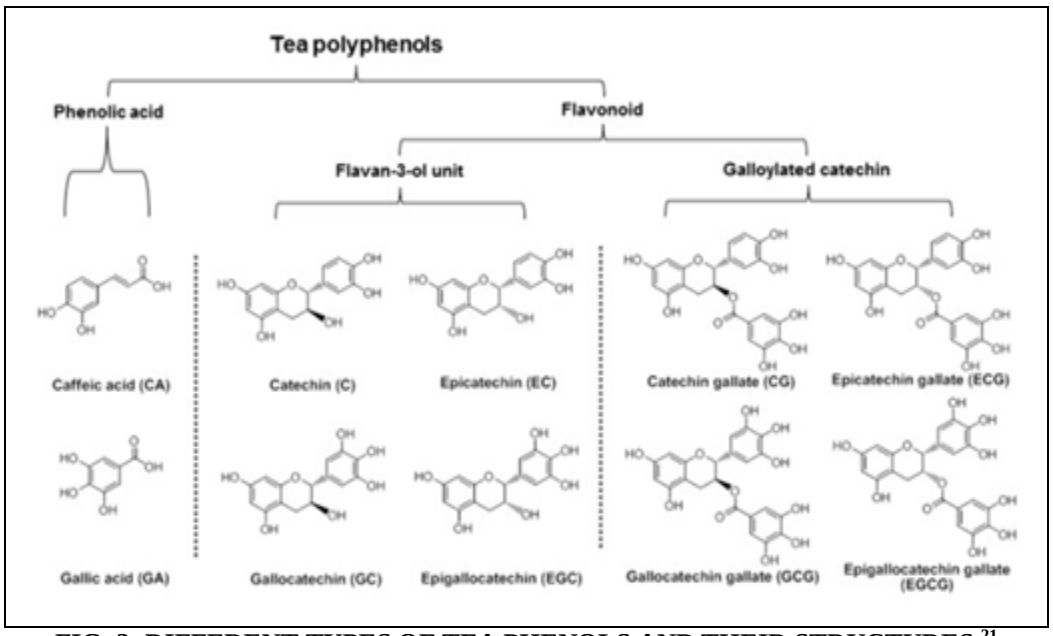

FIG. 2: DIFFERENT TYPES OF TEA PHENOLS AND THEIR STRUCTURES [21]

The Result from the Research:

Phenolic Acids: CA and GA inhibited HCT-116 cell growth, and also there is a dose-dependent effect observed in the anti-proliferative activities of CA and GA.

Flavan-3-ol: C, EC, GC, and EGC inhibited HCT-116 cell growth but there are very low effects observed in C and EC. Similar to phenolic acid, there are dose-dependent effects observed in the anti-proliferative activities of GC and EGC but no dose-dependent effects are observed in C and EC.

Galloylatedcatechin: CG, ECG, GCG and EGCG inhibited HCT-116 cell growth, and also in all four compounds CG, ECG, GCG, and EGCG dose-dependent effects were observed.

The anti-proliferative effects of the different types of polyphenols compounds on SW-480 cells are very similar to HCT-116 cells.

EGCG showed the strongest anti-proliferative effects on HCT-116 cells and SW-480 cells in comparison to the other 9 tea polyphenols.

Also, there are some other points observed during the research while observing the effects of C and EC (flavan-3-ol) they showed low or no anti-proliferative effects but after the addition of a phenolic hydroxyl group to the C and EC compound to compose GC and EGC, it increases the anti-proliferative effect of both the compounds.

Also, the anti-proliferative effects of C and EC are increased significantly when GA is esterified with them to produce CG and ECG. These similar effects were also observed in the GC and EGC when they are esterified with GA to produce GCG and EGCG.

All-inclusive, EGCG shows the highest amount of anti-proliferative potential in comparison to the others [21]. Also, the research tells that the EGCG shows an anti-proliferative activity in HeLa cells with an IC_{50} value of 54 μM[22].

How EGCG Works: Apoptosis (also known as programmed cell death) is a type of cell death in which a series of molecular reactions occurs in a cell that leads to its death. The body uses this method to get rid of unneeded or abnormal cells. The process of apoptosis may be blocked in cancer cells, but simultaneously, it is the best therapeutic strategy for cancer control. Researches [23] concluded that EGCG could inhibit cell proliferation and induce apoptosis through cellular DNA breakage in different cancer cell lines. There are 2 pathways for inducing apoptosis, either by intrinsic

mitochondrial pathway or external death receptor pathway [24, 25]. Intracellular stresses like oxidative stress induce the mitochondrial pathway. It is found that the decrease in the number of viable cancer cells was mainly due to apoptosis which was induced by the EGCG-induced intracellular ROS (reactive oxygen species). In the research [26] there is confirmation of the presence of Thioredoxin (Trx) and thioredoxin reductase (TrxR) in the process of apoptosis. Thioredoxin and thioredoxin reductase are vitally important regulators of cellular redox homeostasis. The decrease in the activity of Trx/TrxR might contribute to the increased level of ROS in the body. Cancer cell deaths were observed in the HeLa cells and the death is due to the elevation of ROS level and this rise of the ROS level is because of the high concentration of EGCG (high concentration because EGCG activities are dose dependant) inactivated Trx/TrxR *via* the formation of EGCG-Trx and EGCG-TrxR conjugates.

Molecular Mechanism of Intrinsic Apoptosis: The major step in the mechanism is the release of mitochondrial cytochrome C. When the cytochrome C is released in the cytoplasm, the apoptosome formation starts, which initiates the activation of the caspase cascade [24] (Caspases are a family of cysteine proteases). If the anti-proliferative potential of the EGCG reaches the matrix of the cytoplasm (cytosol) it will cure cancer more efficiently, which the EGCG-mediated mitochondrial ROS could do.

IC_{50} value suggests that the cancer cells are more sensitive to EGCG than normal body cells, which might predict that the ROS produced by EGCG is selectively toxic to cancer cells only, not to normal cells [27].

At pH of 7.4 and temperature of 37°C, EGCG is auto-oxidized and converted to o-quinone through nonenzymatic dehydrogenation of phenolic hydroxyl groups at ring no 2 and as o-quinone is used in the production of H_2O_2, which is efficient in stopping cancer growths [28] which means; at small intestine due to auto-oxidation of EGCG cancer cells present in the intestinal areas can be easily cured with the help of o-quinone [29].

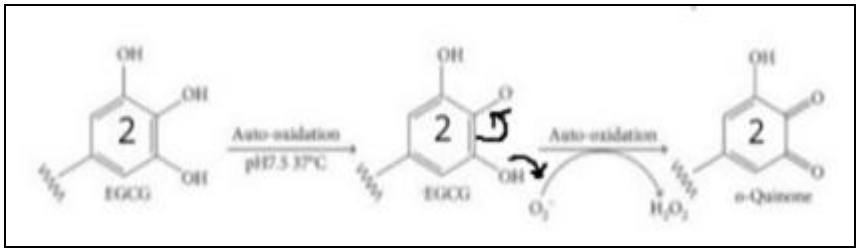

FIG. 3: THE PROCESS OF AUTO-OXIDATION OF RING NO 2 OF EGCG COMPOUND [13]

Also, in the presence of transition metals (such as Cu(II) and Fe(III)) the H_2O_2 converts into a potent oxidant hydroxyl radical which is very efficient in cell deaths by oxidative DNA damage [30]. Hydroxyl ions also play a vital role in DNA breakdown.

These results suggest that the EGCG could impart significant damage to the cell's DNA through a metal ion-dependent pathway.

Other Activities of EGCG that can be used against Cancer: H_2O_2 production by EGCG increases the potency of Cisplatin in ovarian cancer by many folds [31]. By the above results, it is clear that the EGCG mediated environment can reduce the Cancer cells *in-vitro* conditions. EGCG might be the solution to many cancers, but their stability inside the body is very low, so we need to search for a better carrier.

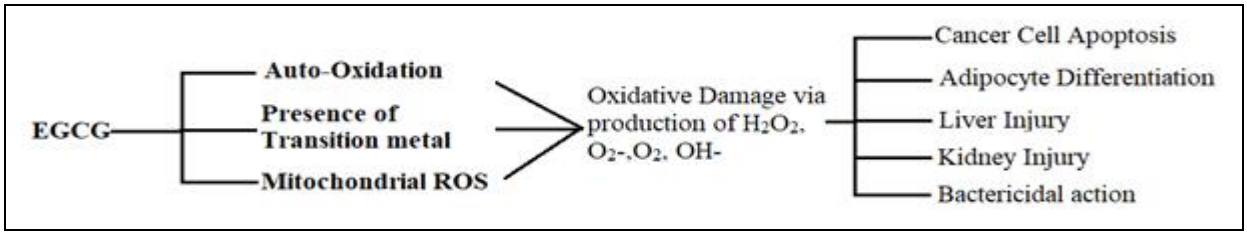

FIG. 4: THE PROOXIDANT EFFECTS OF EGCG

Use of Nanotechnology-Particles: The use of nanoparticles in the field of cancer treatment is quite recent. For cancer diagnosis, nanoparticles are being applied to capture cancer biomarkers, such as cancer-associated proteins, circulating tumor DNA, circulating tumor cells, and exosomes [32]. The traditional or conventional methods achieve a good clearance rate, but they have a lot of side effects. Conventional topical medications compromise patient compliance, safety and efficacy of the therapy. So, for better treatment and a good clearance rate, one should develop techniques that have higher flexibility and permeability.

Major Advantages:

- Nanotechnology provides high sensitivity, highly specific and multi-fold evaluation capacity.
- Expert in the detection of extracellular cancer biomarkers and cancer cells, as well as for in vivo imaging.
- Using nanoparticles for cancer detection lies in their large surface area to volume ratio relative to bulk materials. Due to this property, nanoparticle surfaces can be densely covered with antibodies, small molecules, peptides, aptamers, and other moieties. These moieties can bind and recognize specific cancer molecules [33].
- Nano-particle also plays a crucial role in the penetration of drugs in the deeper layers of the stratum corneum easily.

There are several nanoparticles used in the treatment of cancer. Three of them will be discussed here: Niosomes tween-60 containing cholesterol, Ca/Al-NO3 Layered double hydroxide (LDH) nanoparticles, and Spanlastics. Firstly, Niosomes tween-60 containing cholesterol, the research [9] showed that the EGCG loaded with Niosomes tween-60 containing cholesterol show any significant increase in the anti-proliferative activities to the tumor cells.

The Results of the Research are:

EGCG with Niosomes tween-60 containing Cholesterol (Dissolved in Ethanol):

Loading of EGCG with Niosomes Tween-60 containing Cholesterol: The encapsulation efficiency of EGCG loaded niosomes was good enough. The galloyl group of EGCG facilitates its absorption by the lipophilic segments of surfactants, while the hydrophilic group of EGCG facilitates partition to the inner water phase [34, 35]. Bio stability of Niosomes tween-60 containing cholesterol loaded with EGCG

The addition of Niosomes tween-60 containing cholesterol in ECGC increases the bio-stability of EGCG in the gastrointestinal tract. The percentage of leakage of EGCG from niosomes was not very big but still enough to impart a result but still better than many other nanoparticles. The retention rate of EGCG loaded in niosome in SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) mediums was very good.

Chemical Antioxidant Activity (CAA): EGCG is taken orally; hence the CAA after digestion needs to be determined. The CAA of EGCG was increased by about 1.5 fold by encapsulation. There is a significant decrease in the CAA value of free EGCG after digestion; the value of EGCG loaded in niosomes did not change notably. The CAA assay also proves that the niosomes bilayer appears to protect EGCG against degradation.

FRAP (Ferric-Reducing/Antioxidant Power) values of EGCG loaded niosomes showed a continuous decrease which is very good as the lower FRAP value means a better retention rate. This is due to the shielding effect of the niosomal bilayer membrane. There is a research [5] paper on Ca/Al-NO_3 Layered double hydroxide (LDH) nanoparticles; we also check the stability and other major properties of this nanoparticle when loaded with EGCG.

The results of this nanoparticle are as follows:

EGCG with Ca/Al-NO_3 Layered double hydroxide (LDH):

Loading of EGCG with Ca/Al-NO_3 Layered double hydroxide (LDH): Through TEM imaging, we can say that the loading was pretty good and the zeta potential calculated is positive, which is good because the negatively charged cell membranes can easily interact with the LDH because of their high surface charge difference.

***In-vitro* Antiproliferative Activity of EGCG, EGCG-LDH, and LDH:** Cytotoxic effects were

observed (the effects were observed on Annexin V–FLUOS staining assay) in a dose-dependent manner when the PC3 line is treated against EGCG-LDH (even it shows a higher tumour subduing efficiency compared to EGCG approximately 5 times). LDH alone cannot impart an effect on the PC3 cell line. Leakage is very low.

Release of EGCG: A burst release was observed at the initial stage, followed by a stable release of drug from the nano-vesicular system. The acidic attack is why there is a higher amount of release of drug at pH 4.25 compared to pH 7.45.

The first part of the paper suggests that EGCG has excellent anti-proliferative properties. The EGCG can easily be taken up by the cells of the tumour, and through the process of apoptosis, it destroys the tumour cells [36]. There is no doubt that if the EGCG can be used efficiently, it will change the entire syllabus of Non-invasive techniques to cure cancer.

In the second part, we studied that the EGCG is not very stable in the gastrointestinal tract, and there are several techniques to tackle this problem. There is a technique in which EGCG is loaded inside a very stable compound that helps it to deliver its anti-proliferative ability to the tumour site [37]. Out of certain techniques, Niosomes tween-60 containing cholesterol and Ca/Al-NO3 LDH nanoparticles were considered a very good binding agent for EGCG. They are very good and effective, but an upgrade is needed.

In this third part, the process of up-gradation will be discussed. This upgrade is purely a hypothesis but can be an important invention in the chapter on non-invasive techniques. The technique is based on the binding and stability of EGCG with Spanlastic. In this hypothesis, preparation of EGCG loaded Spanlastic particles will be discussed.

A Hypothesis: In this hypothesis, EGCG is loaded inside the Spanlastic. To better understand this technique, first, the properties of Spanlastic will be discussed and analyze why it is better for up-gradation.

Spanlastics: Spanlastics (Span + Elastic) was introduced in 2011 [38]. They are named Spanlastic because of its vesicles are primarily composed of Spans (Surfactants) [39]. Spanlastic entraps the drug inside its core cavity in the form of a bilayer. They are highly elastic and highly deformable and also amphiphilic [40]. The elastic nature of these vesicles is because of the presence of edge activators on the surface of spanlastic.

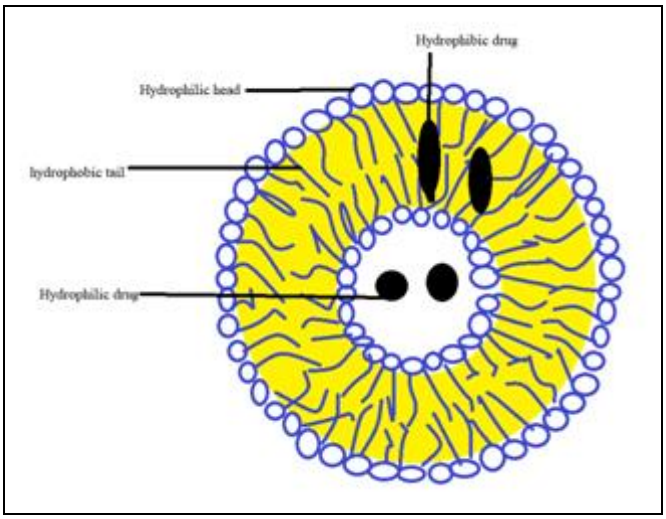

FIG. 5: STRUCTURE OF SPANLASTIC

Classification of Spanlastic: it is classified on the basic numbers of layers it is composed of[41, 42]

Multi-Lamellar Vesicles (MLV): MLVs structure consists of a number of the bilayer. The approx. size of MLVs is 0.5 to 1.0-micron diameter. It is commonly used, easy to make and remains stable upon storage for a long period.

Large Unilamellar Vesicles (LUV): The size range of LUVs is between 100 nm and 1 µm. LUVs have a high aqueous/ lipid component ratio and this ratio helps it to entrap a larger amount of drugs inside the core.

Small Unilamellar Vesicles (SUV): The size of SUVs is generally in the range of 20 nm to 50 µm. SUVs are prepared from multi-lamellar vesicles by the sonication method.

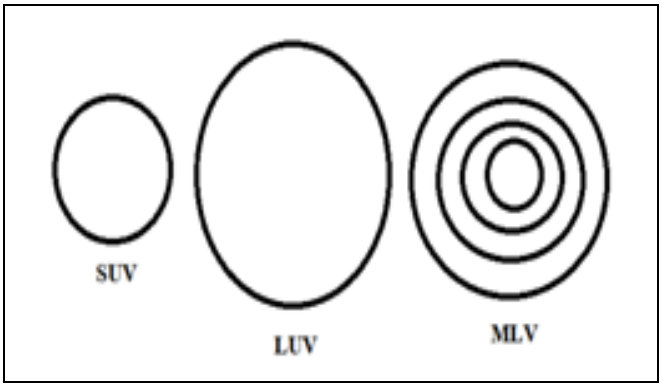

FIG. 6: DIFFERENT TYPES OF SPANLASTIC

The Spanlastic is composed of two parts, one is a non-ionic surfactant and another is an edge activator.

Non-ionic Surfactant: It is neutral on the head side. Surfactants play an important role in forming nano-emulsions by lowering the interfacial tension and Laplace pressure P (the difference in pressure between inside and outside the droplet), which reduces the stress needed to break the concentric structure of Spanlastic. Spans form concentric bilayers to form the vesicular structure of spanlastic. Different types of spans are used, such as Span 80 (mono-oleate), span 60 (mono-stearate), span 40 (mono-palmitate), and span 20 (mono-laurate), depending upon the fatty acid used with the association of poly-oxyethylene sorbitan [43].

Span 60 is often used because it is more stable than because Vesicles based on Span 80 and Span 40 are not often used because of their high degree of disruption, aggregation and instability. Uni-lamellar or multi-lamellar matrix vesicles are easily formed with the help of Span 60.

Edge Activators: These are single-chain surfactants. Edge activators are bilayer softening components, such as biocompatible surfactant in which an amphiphilic drug is added to increase lipid bilayer flexibility and permeability. Edge activators generally have a high HLB (hydrophile-lipophile balance) value or hydrophilicity. It lowers the interfacial tension between the bilayer, and because of this, it increases the deformability. Tween 80 is a type of edge activator when incorporated; it increases the elastic nature of the vesicles.

The tween-80 temporarily increases the pore size of the biological membranes; due to this increase, any vesicle bigger than the pore size of the membrane can easily transfer from outside to inside. This also helps in the transfer of more amounts of drugs inside the vesicle as well as better drug penetration. Due to this property of edge activators, the Spanlastic loaded with EGCG can easily penetrate different kinds of tumor [44, 45].

Ethanol is also used because of its membrane condensing ability; it decreases the thickness of the vesicular membrane [46] and also it improves the drug entrapping efficiency of the spanlastic system. It also stabilizes the steric effect by modifying the net negative charge on the surface.

Cholesterol is also induced in spanlastic formulations, which improves the efficacy of the spanlastic particle due to the enhanced membrane fusion property of cholesterol.

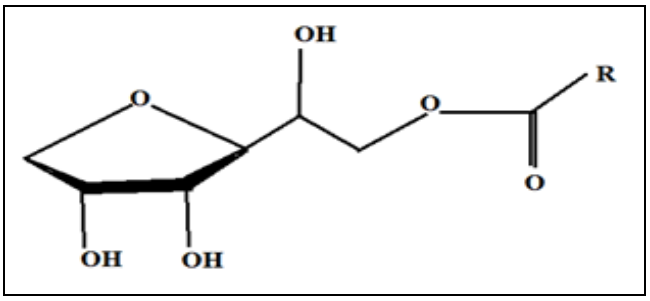

FIG. 7: CHEMICAL STRUCTURE OF SPAN-60 (R= SATURATED ALKYL CHAIN)

Mechanism of Penetration of Spanlastics Inside the Cell Membrane: The deformability of the vesicles is increased because the edge activators destabilize the lipid bilayers and the surfactant from which the vesicle is made up induces the pores in lipid structures so that the elastic vesicles under the influence of water gradient can pass themselves through intercellular regions. After entering the cell, the elastic cell releases the drug inside of the cell. There are two mechanisms for the penetration of drugs inside cells [47].

The epithelial cell membrane interacts with the elastic vesicle, and then the elastic vesicle act as penetration enhancers and afterward modifies the intercellular lipid lamellae.

The elastic vesicles can act as drug-carrier systems, whereby an intact vesicle that carries the drug passes through the intercellular spaces and reach across the biological membrane.

The nano-vesicular system was found safe from genotoxicity by ames test and cytotoxicity by MIT assay and acute dermal/eye irritation/corrosion and chronic eye irritation/corrosion tests (OECD guidelines) [48].

Spanlastic is also used in the treatment of Ocular mycosis (fungal infection of the eye) because of its better corneal permeability. It can reach both the anterior segment and posterior segment of an eye to target the choroid, retinal pigment epithelium and

vitreous cavity [49]. Also, the non-ionic surfactant has the lowest irritation power as compared to ionic or ampholytic. Hence, they are also not irritant to the eyes. The research also showed that the permeability of spanlastic is better than the niosomes [50, 51].

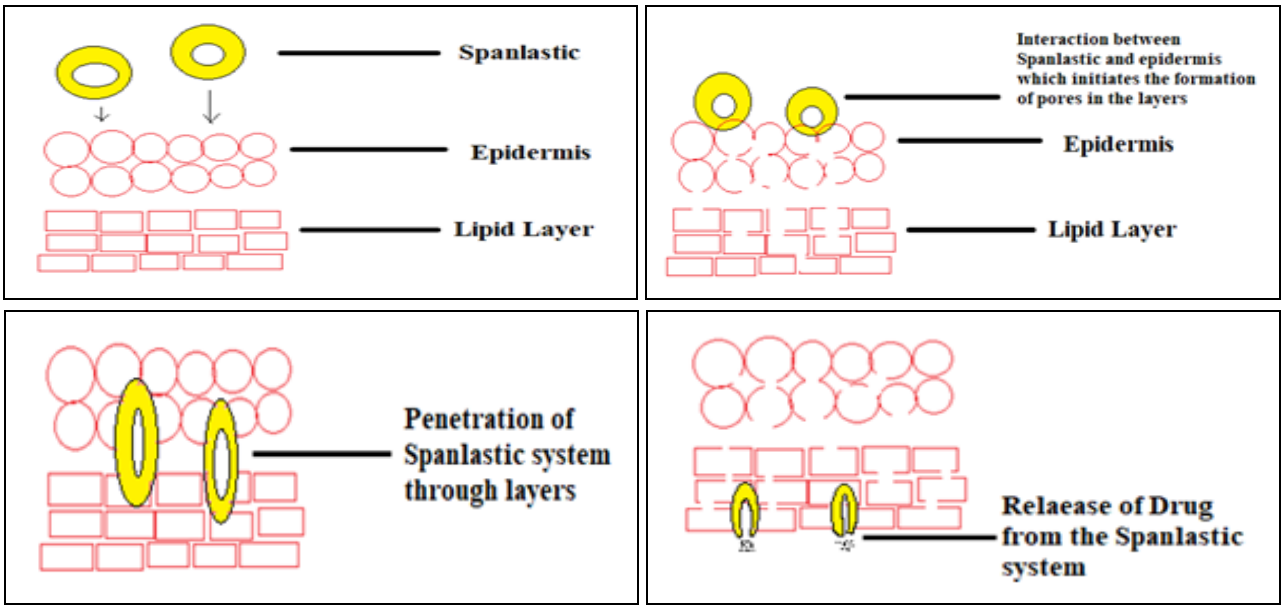

FIG. 8: MECHANISM OF PENETRATION OF SPANLASTIC SYSTEM INSIDE THE EPIDERMIS AND LIPID LAYER. (A) THE FIGURE REPRESENTS THE SPANLASTIC AND THE LAYERS OF THE ORGAN. (B) IN THIS FIGURE, WE CAN SEE THE INTERACTION BETWEEN THE SPANLASTIC SYSTEM AND THE EPIDERMIS AND THE FORMATION OF PORES INSIDE THE LAYERS OF THE ORGAN. (C) THE PENETRATION OF THE SPANLASTIC SYSTEM THROUGH THE LAYERS. (D) THE RELEASE OF DRUGS FROM THE SPANLASTIC SYSTEM INSIDE THE ORGAN. DUE TO THIS, THE DRUG ENTRAPPED INSIDE THE SYSTEM COMES DIRECTLY IN CONTACT WITH THE INFECTED PART.

Advantages of Spanlastic over Other Nanoparticles: [52]

- Spanlastics are biodegradable (eco-friendly).
- It shields the medication very efficiently.
- They are target-specific molecules.
- They are osmotically active and stable.
- Storage of surfactants is easy.
- Due to the presence of non-ionic surfactants on Spanlastic, they are highly compatible with biological systems.
- They provide better biological permeability as compared to the niosomal formulations as they are highly elastic and deformable.
- More efficient in entrapping of drugs (can entrap more amount of drugs).
- Zero or few side effects on the body.
- They are designed to achieve site-specific action.
- They are chemically stable as compared to liposomes.
- Economical as compared to others.
- Access to raw materials is convenient.
- The non-ionic surfactant is also the reason for the low toxicity character of Spanlastic.
- They can be easily transported to the site of action by topical, oral, and parenteral routes.
- Spanlastics are non-immunogenic in nature.

The best suggestion for the EGCG loaded in Spanlastic is the non-ionic surfactant made up of Span-60 (mono-stearate), and the edge activator made up of Tween-80.

The Technique to Prepare EGCG Loaded in Spanlastic System: Different steps involved in the preparation

1. Materials required
2. The preparation of the system
3. The preparation of cancer colony
4. The test of the system against cancer colonies and different pH for the detection of biostability.

1. Materials Required:

- EGCG (98%) pure

- Phosphoric acid
- Absolute ethyl alcohol
- $FeCl_3$
- Triton X-100 ($C_{14}H_{22}O$)
- DMSO
- Tween-80
- cholesterol
- Deionized water will be decarbonized by boiling and bubbling N_2
- Annexin-V-FLUOS Staining Kit
- Human Cancer Cell line (PC3)
- TPTZ (2,4,6-tri(2-pyridyl)-s-triazine)
- Methanol of HPLC grade
- 1% penicillin/streptomycin
- 10% FBS
- PBS
- 10% buffered formalin
- Stains used will be hematoxylin and eosin
- HCl
- NaOH
- NaCl
- Pepsin (Porcine)
- Bile extract (Porcine)
- Acetate buffer
- Propidium Iodide

2. Method of Preparation of EGCG Loaded Spanlastic:

- There are several ways to make the Spanlastic system; ethanol injection method will be used (ether injection method can also be used). The Ethanol injection method can fetch a great quality and quantity of Spanlastic.
- Span-60 (mono-stearate) and EGCG are dissolved in a solution and then sonicate the solution for a few minutes.
- The solution will then be shifted to preheated aqueous phase fixed ratio solution of edge activator (Tween-80) and cholesterol and then stirred on a magnetic stirrer at 1000-1400 rpm.
- The drawbacks to this method are: (1) Removal of residual ethanol is very difficult as it forms an azeotropic mixture with water. (2) Certain biological get inactivated in the presence of ethanol.
- The characterization is crucial in preparation; the Z-average diameter and polydispersity index (PDI) of the spanlastic will be determined by dynamic light scattering (DLS).
- This characterization will determine the shape and size of the spanlastic. The shape and size of the spanlastic may change during the digestive process.
- This change of shape and size can be seen under the TEM (transmission electron microscope) imaging.
- Encapsulation; one of the reasons for choosing Spanlastic over other different types of niosomal preparations is that Spanlastic can hold more amount of drugs inside its vesicular system.
- For the differentiation between the encapsulated EGCG and free EGCG Ultracentrifugation technique will be used and the dispersions will be released with Triton X-100.
- The amount of total encapsulated EGCG and free EGCG was quantified by reversed-phase high-performance liquid chromatography.
- The encapsulation will be determined by the formula of

$$EE\% = 1 - C\,(free) \times 100\% / C(total)$$

Where C (free) is the concentration of free EGCG and C(total) is total EGCG concentration prepared.

3. The Preparation of Cancer Colony and Testing it against the system:

- The cancer colony used will be PC3 cells. The formation of a cancer colony is necessary to analyze the effectiveness of the anti-cancer drugs against cancer cell proliferation.
- PC3 cells will be cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin.
- Then after 24 h the spanlastic loaded with EGCG will be added to the culture medium and incubated at 37°C for 14 days.

- The medium will be removed from the cells, followed by a wash with PBS.
- The fixing of colonies will be done with the help of 10% buffered formalin.
- The colonies will be stained with hematoxylin and eosin.
- A water bath will remove the excess strain.
- After all this, the cell culture will be observed under a light microscope.

4. Testing of the EGCG Loaded in Spanlastic on Different Criteria: Testing of the vesicular system against different pH. The EGCG is very unstable in the alkaline medium that's why it is loaded in Spanlastic. The vesicular system will be tested against pH 3.2 and 7.4; these 2 different pH will be chosen because 3.2 represents the pH of the stomach. Also, check the release of drugs from inside the vesicular system in acidic pH 7.4 represents the pH of the small intestine where the absorption of the vesicular system will occur. The vesicular system must have to be stable at that pH to deliver the drug to the target cells.

- **Testing of the Vesicular System against Different Temperatures:** The temperature of the body varies from 97°F (36.1°C) to 100.4°F (38°C) at normal. The vesicular system must be stable at this temperature.

- ***In-vitro* Digestion Stability:** The *in-vitro* digestion stability will be determined with the help of a simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The SGF will be prepared with NaCl, HCl, and pepsin in distilled water at a pH of 2.4. SIF will be prepared with bile salts and pancreatic juices in PBS at pH 7.4. The samples will be mixed with SGF and SIF in a fixed ratio. For calculation of Z-average diameter, samples will be taken out at different intervals. Also, HPLC will be performed. The remaining particles will be calculated by

$$\text{EGCG remaining\%} = C_{\text{interval}} \times 100\% \; C_{\text{initial}}$$

- **Antioxidant Activities during Digestion:** Ferric reducing antioxidant power (FRAP) will determine the antioxidant properties in SIF.

  To make FRAP reagent, acetate buffer will be mixed with TPTZ in HCl and $FeCl_3.6H_2O$. Then after the preparation of FRAP, the sample from SIF will be added to the FRAP, and the results will be observed under the UV−vis spectrophotometer.

- **Cellular Antioxidant Activity (CAA):** For the calculation of CAA, fluorescent readings will be observed. The formula for calculation of CAA is

$$\text{CAA unit} = 100 - (\int S - \int B) \times 100 / (\int C - \int B)$$

  Where ∫S is the integrated area under the sample fluorescence versus time curve, ∫B is the integrated area from the blank curve and ∫C is the integrated area under the control curve.

  MIT assay can also be used to detect the cytotoxic effects of the spanlastic system.

- **Detection of Apoptosis:** For the Detection of apoptosis, the Annexin V–FLUOS staining assay will be performed. In this, the cells are covered by Annexin-V-FLUOS labeling solution containing a diluted solution of Annexin V-FITC and Propidium Iodide. Then stored with Annexin V-FLUOS labeling reagent. In the dark, the differentiation will occur by using fluorescence microscopy. Propidium iodide will be taken up by Necrotic cells and stain red, while apoptotic cells stain green.

- **X-RAY Diffraction Method:** The X-ray diffraction method will determine the integrity of the vesicular system inside the gastric system. Also, the anions and cations stability will be observed at different bands.

- **Zeta-potential and Electrophoretic Mobility:** Both have to be calculated. If there is a relatively high surface charge of spanlastic then it will more likely interact with negatively charged cell membranes and be taken up by the cells easily.

RESULT: EGCG is prepared through ethanol injection with a non-ionic surfactant of Span 60 (mono-stearate) and edge activator of Tween-80

with additional cholesterol covering. The EGCG-spanlastic system then will be tested by TEM imaging for shape and size; its zeta potential and surface charge is also calculated PDI is calculated. Further, tested against SIF and SGF incubators for FRAP and CAA also tested at 37-40 ºC and pH of 7.4. MIT test is done for cytotoxic effects and Annexin V–FLUOS staining assay for detection of apoptosis and lastly X-ray diffraction test is done.

CONCLUSION: Spanlastic is quite better than any other niosomes in the category of stability and bio-permeability. If provided with a better edge activator, it may perform even better. The mechanism of entry of spanlastic loaded with the drug inside the cells is unique and it is because of its highly elastic and deformable nature, and also Spanlastic can entrap more amounts of drugs.

EGCG is quite good in the field of anti-proliferative and anti-inflammatory potential, but it is unstable at pH 7.4 and spanlastic is quite good in its field of the nanoparticle carrier. Hence, if EGCG is loaded inside the nano-vesicular system, it can easily deliver its anti-cancer potential more efficiently, with a few side effects. The fewer side effects and treatment will be of short duration and cheap as compared to traditional techniques. The technique is non-invasive, which means the patient acceptance will be high, and the post-operative problems will also decline. The half-life is also low and accumulation chances are very less hence the technique will change the way of treatment of cancer. Also, the Spanlastic-EGCG system has the potential to heal a lot of other fatal diseases due to high bio-stability and different properties of EGCG.

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