TO STUDY AND EVALUATE THE ROLE OF GUT MODULATION ON THE TOXICITY AND EFFICACY OF DOXORUBICIN

A

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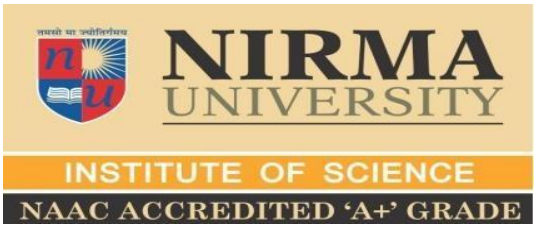

Under the Guidance of

Dr. Sriram Seshadri

DEDICATION SHEET

I Sincerely dedicate the Thesis to the Wistar rats who sacrificed their lives for our dissertation and Humankind.

NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
NAAC ACCREDITED 'A+' GRADE

CERTIFICATE

This is to certify that the thesis entitled **"To study and Evaluate the role of Gut modulation on the toxicity and efficacy of Doxorubicin"** submitted to the Institute of Science, Nirma university, in partial fulfillment of the requirement for the award of the degree of M.sc Biochemistry/Biotechnology/Microbiology, is record of Research work carried out by **Pariti Patel (20MBC008), Mohik Bhatt(20MBT042),Mausam Sankhala(20MBT042), Rashmi Solanki(20MMB027)** , Under the guidance of **Dr. Sriram Seshadri**. No part of the thesis has been submitted for any other Degree or Diploma work.

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“You must be prepared to work always without applause”

-Ernest Hemingway

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ABBREIVATIONS

WHO: World health Organization.

DXR/DOX: Doxorubicin

IBS: Irritable Bowel Syndrome

T2DM: Type 2 Diabetes Mellitus

PPI: Proton Pump Inhibition

IBD: Inflammatory bowel Syndrome

CRC: Colorectal Cancer

HSCT: Haematopoietic Stem cells

GI: Gastrointestinal

CD: Cardiovascular Diseases

BA: Bile Acids

TMAO: Trimethylamine -N- oxide

FMT: Faecal Microbiota Transplantation

NLR/NOD: Nod Like Receptors- Nucleotide- Binding Oligomerization Domain receptors

WTA: Wall- teichoic acids

LPA: Lipo- teichoic Acids

ANOVA: Analysis of Variance

GSH: Glutathione

CD: Crohn's diseases

UC: Ulcerative colitis

LDL: Low density Lipoprotein

LPS: Lipopolysaccharide

NF-kB: Nuclear Factor Kappa B

PBS: Phosphate Buffer Saline

SCFA: Short Chain Fatty Acid

AUC: Area under curve

HDL: High Density Lipoprotein

SGOT: Serum Glutamic Oxaloacetic Transaminase

SGPT: Serum Glutamic Pyruvic Transaminase

TG: Triglycerides

TLRs: Toll Like Receptors

ABSTRACT

ABSTRACT

Doxorubicin is one of the most effective chemotherapy drugs used against tumors in the treatment of several cancer types. Two different mechanisms (a) intercalation of doxorubicin into DNA and inhibition of topoisomerase II leading to changes in chromatin structure, (b) generation of free radicals and oxidative damage to biomolecules, have been proposed to explain the mode of action of this drug in cancer cells. Chemotherapeutic can further cause microbiome alterations that facilitate the emergence of antibiotic- resistant microorganisms and especially for pediatric patients, increase risk of dysbiosis- related health complication later in life (e.g., obesity, asthma, diabetes). Chemotherapeutic drugs are known to perturb the microbiome as well. Chitin is a polysaccharide obtained from crustaceans, fungus and insects that also is biocompatible and biodegradable. Chitosan is converted from chitin by deacetylation. Chitosan has been reported to have antioxidant, anticancer, anti-inflammatory, immunostimulant, wound healing cholesterol lowering, antibacterial and antifungal qualities, which can be used as an active component of the diet. Chitosan can be used as a prebiotic to boost the colonic mucosal populations of beneficial bacteria while suppressing pro-inflammatory bacteria, hence reducing gut microbiota imbalance and mucosal inflammation. Our hypothesis is to check that how Doxorubicin is involved in Gut micro floral alteration and does the co-administration if gut modulators influence the efficacy of doxorubicin. We have treated animals parallelly by giving chemotherapeutic Drug Doxorubicin and Chitosan microspheres. We observed that there are chances to restore Doxorubicin caused Micro floral dysbiosis by giving gut modulators such as chitosan microspheres. Futher this study can be useful to reduce the toxicity in the intestine by lowering the doses of chemotherapeutic drug and parallelly giving chitosan microsphere as gut modulator to restore gut biota.

INTRODUCTI0N AND REVIEW OF LITERATURE

INTRODUCTION AND REVIEW OF LITERATURE

Part 1: Doxorubicin

1.1 What is doxorubicin

Clinical professionals for curing cancer patients in the healthcare sector use this chemotherapy medicine. The generic name is doxorubicin; however, the medicine is sold under the name “Adriamycin” or “Rubex”. The other names that are also in the background of the medicine and are allocated in the chemistry modules are “HydroxydoxorubicinHydrochloride” and “Hydroxydaunomycin Hydrochloride” (Wang *et al.* 2019). The types of cancers that are cured by this chemotherapeutic drug are bladder cancer, “acute lymphocytic leukaemia”, breast cancer, Kaposi’s sarcoma and lymphoma. Other chemotherapeutic agents are given to the affected patients with the collaboration of doxorubicin. The medicine was originally derived from the bacterium named “*Streptomyces peucetius*”. The world health organisation (WHO) has suggested this medicine be on the essential list of the medicines that are needed in the healthcare domain.

1.2 Doxorubicin properties and chemical structure

Doxorubicin belongs to the family of anthracycline and antitumor antibiotics. Doxorubicin is an antineoplastic medicine in nature and this medicine directly interferes with the function of DNA in a different way. Doxorubicin should be dispensed only into the vein of the affected patient (Hujaya *et al.* 2018). However, it may trickle into encircling tissue driving severe itch or deterioration of tissue. This medicine is a 14-hydroxylated interpretation of “daunorubicin” and the nearest antecedent of DXR through the track of biosynthesis. This is a wild type strain of the bacteria streptomyces and is majorly used in the case of cancer curation.

Physical properties-

Table 1.1: physical properties of doxorubicin

Names	Property
Vapour pressure	2.5*10^-23 at 25ºC
Solubility	In water and dimethyl sulfoxide
Appearance	Appears as an orange-red thin needle
Solution property	Aqueous solution is yellow-orange at acidic pH value and purple-blue at pH ≥9

Chemical properties-

Table 1.2: Table of chemical properties of doxorubicin

Properties	Value
Molecular weight	543.5
Rotatable bond count	5
Hydrogen bond donor count	6
Exact Mass	543.17406074
Hydrogen Bond Acceptor Count	12
Colour and structure	Red, crystalline solid
Melting point	399-401ºF
Solubility	Soluble

Chemical structure-

The molecular formula of the medicine doxorubicin is C27H29NO11. Doxorubicin is a “deoxy hexoside”, an “anthracycline antibiotic”, and an “anthracycline” (Chhikara *et al.* 2019). This medicine can also be seen as an “aminoglycoside”, and it is a constituent of “tetracenequinones”, an associate of p-quinones, a “preparatory alpha-hydroxy ketone” and a “tertiary alpha-hydroxy ketone” (Pubchem.ncbi.nlm.nih.gov, 2020). It can metabolise the gut bacteria E. coli. It can be called a conjugate base of doxorubicin. It derives from a hydride of tetracene.

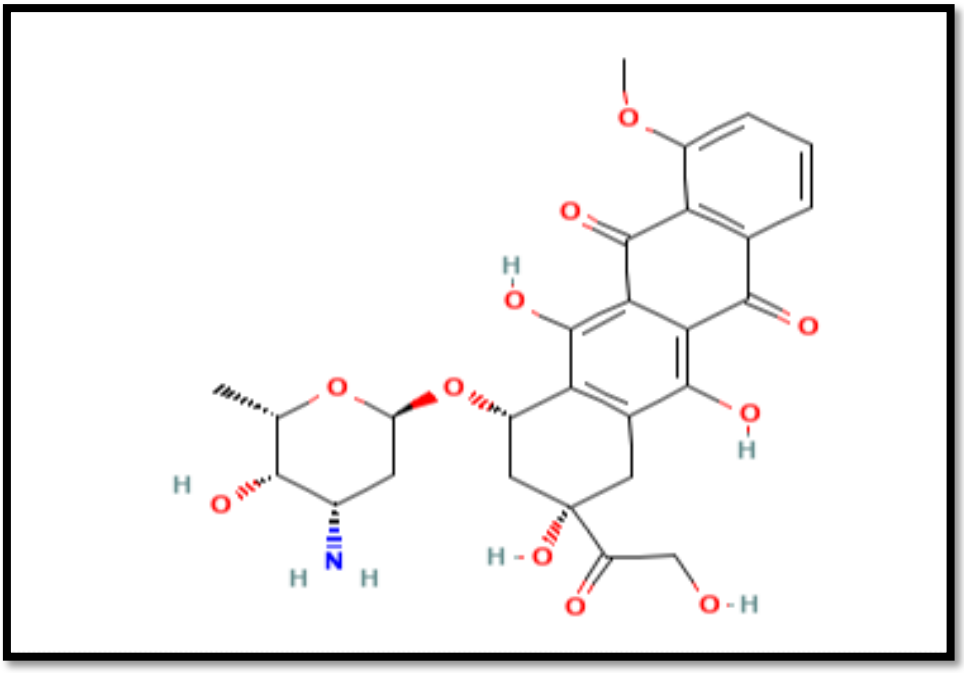


Figure 1.(A): Structure of doxorubicin

(Source: Pubchem.ncbi.nlm.nih.gov, 2020)

1.3 Mode of action of doxorubicin

The process can incorporate intercalation, that is squeezing between the base pairs of DNA, breaking the DNA strands and inhibiting the transcription transformation and replication of DNA with the help of the enzyme Topoisomerase II. Topoisomerase II helps in relaxing the supercoils of the DNA for transcription and DXR tries to stabilise the Topoisomerase II complex after the breaking scenario during the replication. This also prevents the release of DNA double helix and ultimately stops the replication process (Arruda *et al.* 2019). The production of free radicals such as quinone also is increased and for that, the cytotoxicity gets increased. Between two base pairs in the DNA aromatic chromophore part of the molecule tries to intercalate and, in that time, denopamine sugar with six-member sets into the minor groove and base pairs are flanked in that intercalation section. In that case, several crystalline structures are formed at that site of intercalation (Micallef and Baron, 2020). During the scenario of intercalation, the chemical component doxorubicin also induces histone eviction from chromatin, which is active in the transcription. In that case, the transcriptome and epigenome are deregulated in the cells that are doxorubicin-exposed. The action can be described in a systematic way and that is as follows-

- The quinone moiety foremost experiences a reduction process to develop free radicals such as hydrogen peroxide, superoxide, and hydroxyl in the existence of oxygen
- DOX-Top 2 beta interchange: As it represents high levels of “topoisomerase 2 beta enzyme”, it happens in “quiescent cells”, as “cardiomyocytes” (Arruda *et al.* 2019)
- DOX-Top 2 alpha dealings emerge in tumour cells, particularly in duplicating cells that represent a high level of “topoisomerase 2 alpha enzyme”.
- Probiotics and phytochemicals counteract free radicals and decrease harmful oxidation functions (Micallef and Baron, 2020)
- Polymers and nanoparticles undervalue DOX-induced toxicity from immediate drug delivery to tumour cells (Source: Arruda *et al.* 2019)

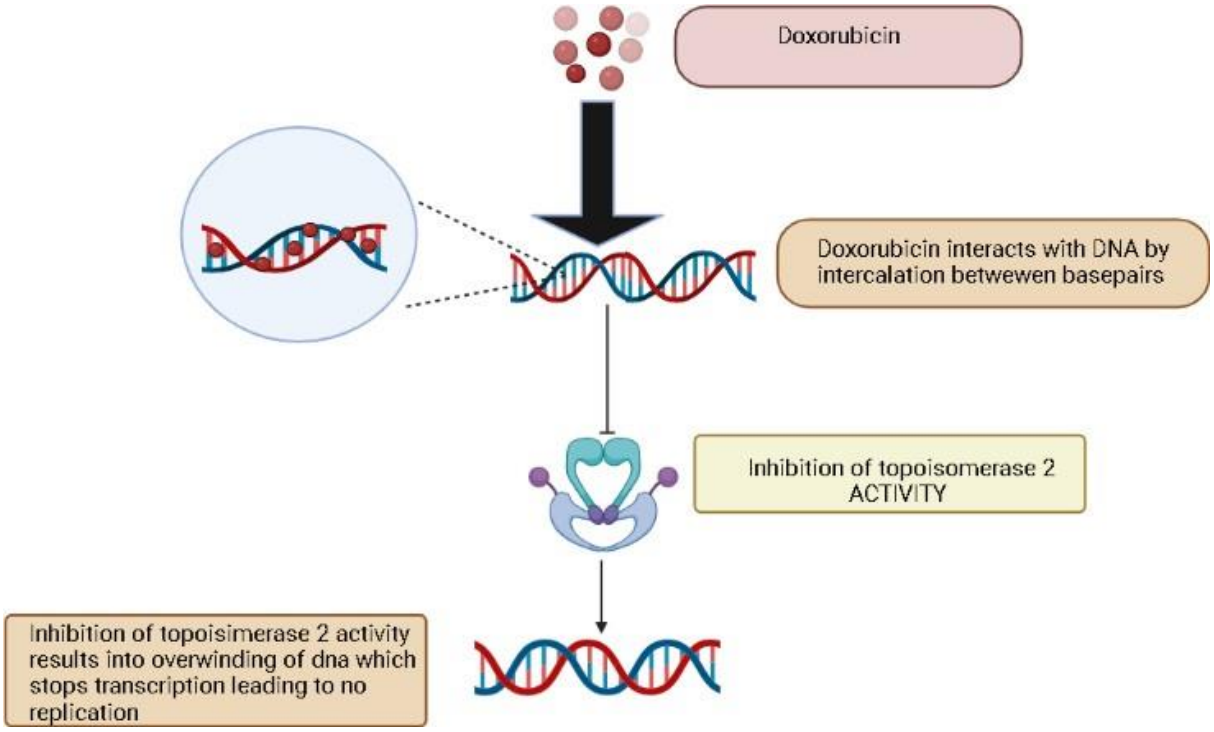


Figure 1(B): Mode of action of doxorubicin

1.4 Metabolic degeneration of doxorubicin

Doxorubicin is generally metabolised with a large no of metabolites in the in vivo process. These are interlinked with the scenario of cardiotoxicity. This chemotherapeutic drug needs to invade inside the tumour cells in order to produce cytotoxic effects (Bauckneht *et al*. 2019). In the case of humans, 50% of Doxorubicin is excreted from bodies in an unchanged version. The drug is processed through three major and important metabolic pathways-

- Two-electron reduction to the secondary alcohol metabolite
- Degycosylation to DOX aglycone
- One-electron reduction to DOX semiquinone radicals

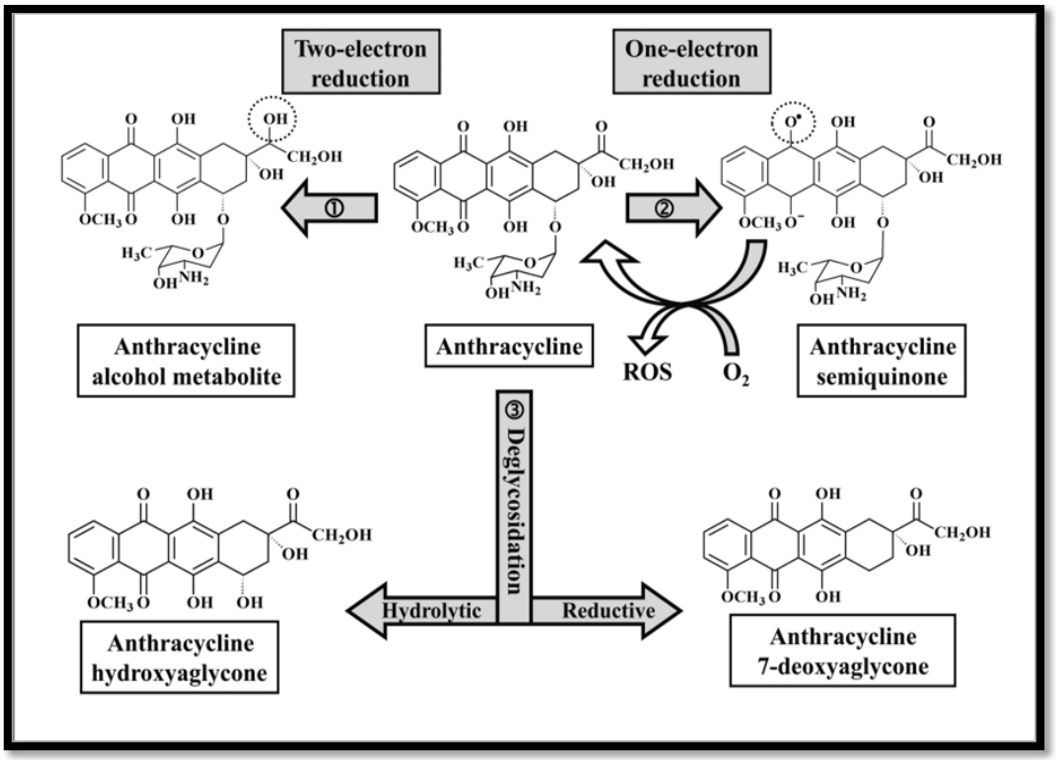


Figure 1(C): Metabolism of doxorubicin (Source: mordente *et al*. 2009)

1.5 Side effects of doxorubicin

Common side effects that are seen after applying cancer treatment are the drastic loss of hair, rash in the body, inflammation in the mouth and vomiting. On the other hand, the suspension of bone marrow can be a serious consequence of projecting doxorubicin to an affected person. Moreover, the critical side effects can include severe allergic reactions such as anaphylaxis, damage to tissue at the specific site of injecting, and treatment-related leukaemia with serious conditions of damage to the heart (Dostalova *et al.* 2018). Other than that, some of the patients can also experience discolouration of urine after injecting the medicine into the bloodstream of the patient. This can function by disintegrating the direct function of DNA. dilated cardiomyopathy can be a dangerous effect in case of injecting doxorubicin into the patients.

This can also cause oxidative stress and that can cause the downregulation of genes for the contractile proteins. When the overdose is given to the patients that are the dose exceeds 600 mg/m2 that situation can cause initiate cardiomyopathy. Typhlitis, which is known as acute bowel inflammation, can also happen to the patients (Gorini *et al.* 2018). Red colouration of the skin and skin eruption can also result in the person being injected with doxorubicin. The drug can also be leaked into the sole of the feet and palm of the hand and can cause tenderness

in the spots. This side effect can also cause the “Palmer Plantar Erythrodysesthesia” which is also known commonly as “hand-foot syndrome”.

1.6 Involvement of doxorubicin in dysbiosis

In the case of chemotherapy, intestinal mucositis can happen in case of people. In the case of the patients who are under chemotherapy often face the painful debilitating condition and from that, it can be seen that the chemotherapy or the chemotherapeutic medicines such as doxorubicin has a responsibility for creating inflammation in the intestine and shaping the intestinal microbiota (Zhao *et al.* 2018). The healthy cells are also affected by the chemotherapy and intestinal flora can be severely affected and cannot function normally in that condition (Wei *et al.* 2021). In this condition, intestinal mucositis and dysbiosis can happen and the affected patient can feel nauseous and also feel severe pain in the abdominal cavity with the facts of indigestion.

Part 2: Gut microbiota dysbiosis

2.1Basic idea of dysbiosis

Dysbiosis is the condition of the digestive system where the imbalance of several gut bacteria can cause digestive disturbances. Among the digestive complications the diarrhoea, constipation, stomach cramps bloating in the initial stage. In a more complex scenario, this issue can drag the problems such as irritable bowel syndrome (IBS), gastritis and inflammatory bowel disease. In this case, the microbial diversity gets reduced and the rate of beneficial bacteria, such as butyrate-producing bacteria or "Firmicutes 10" and bacteroid strains is lower (Betsaida, 2020). On the other hand, symbiotic bacteria such as "Pathobionts 12" can be increased that can become pathogenic under specific conditions.

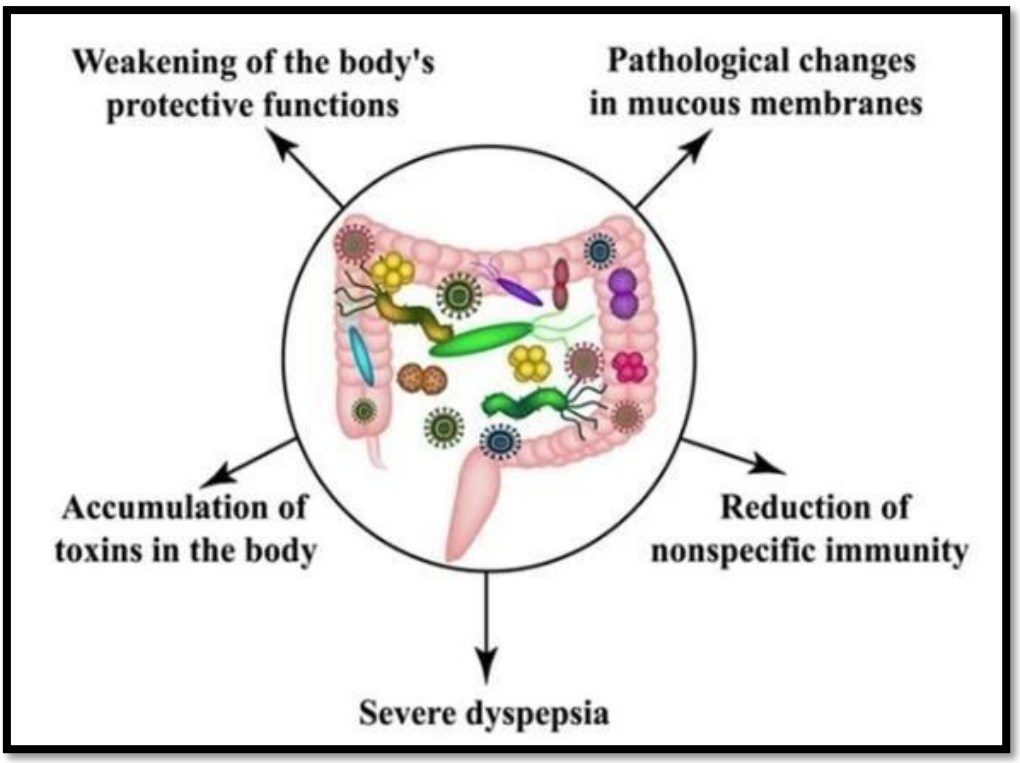


Figure 2(A): Consequences of dysbiosis (Source: Betsaida, 2021)

2.2 Cause of dysbiosis

The occurrence of Dysbiosis in the gastrointestinal tract can be happened due to a dietary imperfection or a sudden change of diet. The case of the change of diet includes the increment of sugar intake, protein or food additive intake. On the other hand, the accidental consumption of chemicals such as pesticides that remain in the unwashed fruits (Jewell, 2019). Per day intake of two or more alcoholic beverages can also increase the chance of dysbiosis in human beings or any kind of animal. Dysbiosis can also be caused by the declination of "mucin-degrading bacteria" like "*Lactobacillus*", "*Prevotella*" and "*Bifidobacteria*". The increase in the rate of Bacteroidetes and Clostridium can also initiate dysbiosis (Betsaida, 2021). The gut flora of an individual can also be affected by new antibiotics and for that, gut dysbiosis can happen.

2.3 What changes occurred in the body due to dysbiosis

The common changes that an individual can show for the occurrence of dysbiosis are upset stomach, diarrhoea, nausea, bloating, chest pain, and constipation. Other than that, there can be halitosis, difficulty in urinating, vaginal or rectal itching and indigestion that are common in this condition (Brüssow, 2020). Loss of intestinal permeability can also be seen along with the dysplasia of the mucosal place can also be visible as the change caused by dysbiosis. The congestion or over solicitation of the annexal organs such as the exocrine pancreas, liver and gallbladder can be seen.

2.4 Dysbiotic condition associated with disease.

Obesity: It has been seen that the dysbiotic condition can be derived from the obesity of the people. The increased rate of fast-food consumption can trigger oxidative stress and, in that case, the beneficial gut bacteria get disrupted in their duty and eventually result in the dysbiotic condition (Fukui, 2019). The modification or alteration of the gut flora can happen to the accidental weight gain and being obese. Obesity is related to the presence of a physiological tag such as interleukin-6 that emerges during inflammations, which directs to the premature growth of degenerative malfunctions (Vamanu and Rai, 2021).

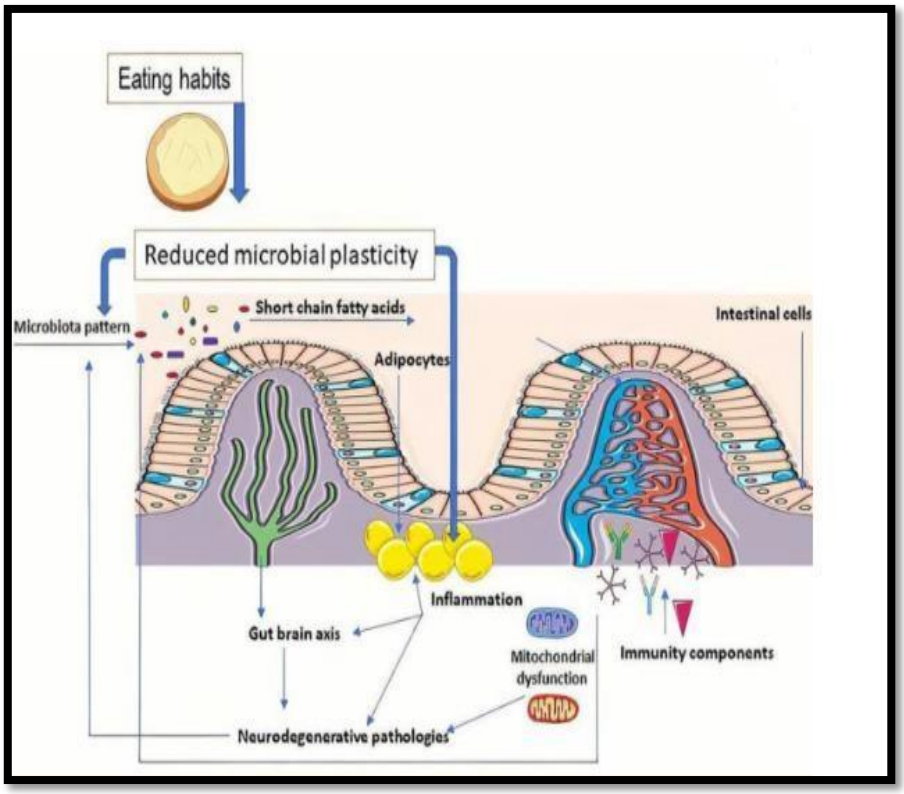


Figure 2.(B): Dysbiotic condition for obesity (Source: Vamanu and Rai, 2021)

Diabetes: Type 2 diabetes or Diabetes Mellitus (T2DM) is a multi-organ metabolic disorder that can cause an imbalance of the gut microbiome and also direct to dysbiosis. On the other hand, the sudden spiking up of glucose levels can alter the function of good bacteria in the gut and that can foster the increasing rate of retinopathy and nephropathy (Jazani *et al.* 2019). In this case, the gut bacteria integrity lowers with the higher rate of epithelial permeability that can trigger the hyperglycaemic situation that onsets diabetic retinopathy (Fernandes *et al.* 2019).

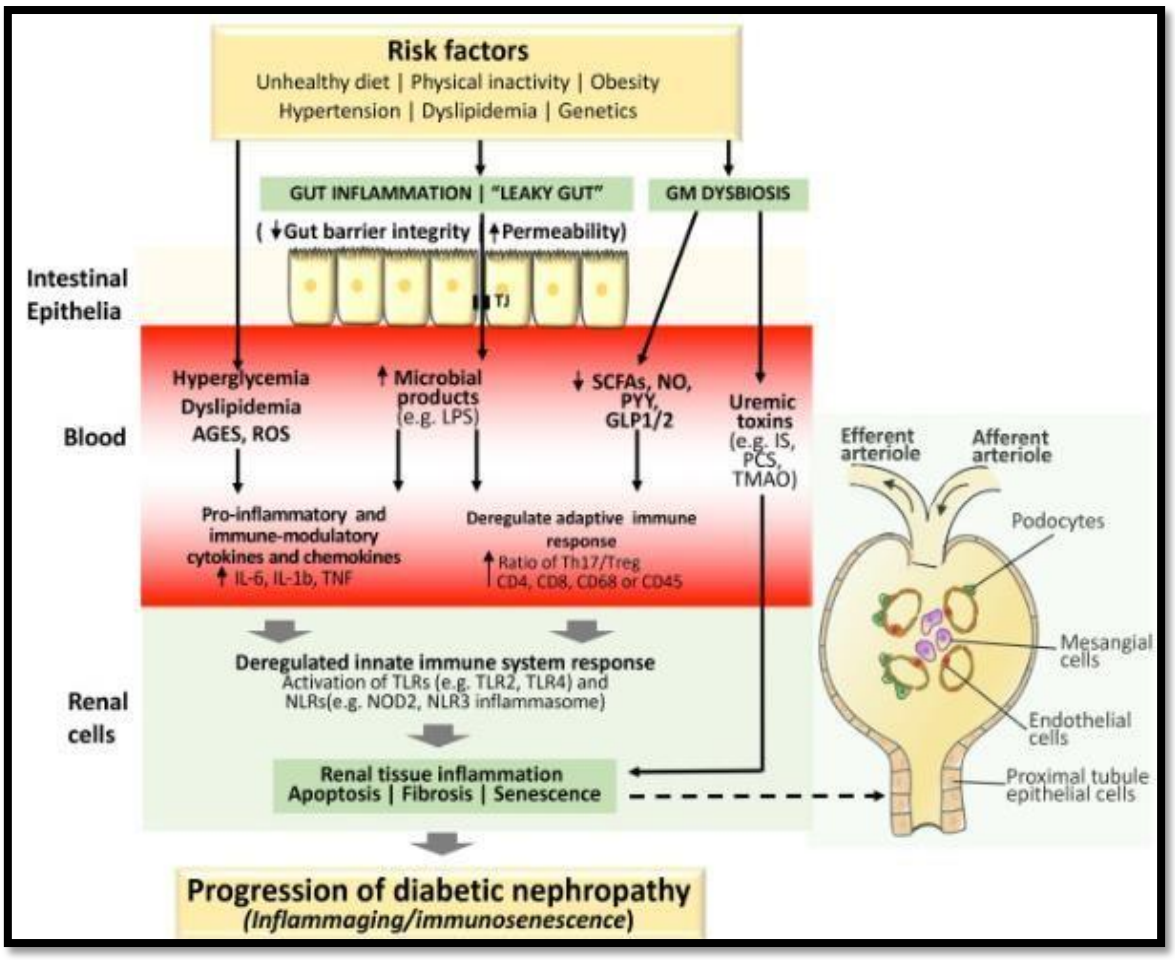


Figure 2(C): dysbiosis for diabetes (Source: Fernandes *et al.* 2019)

Medications: Sometimes the medications that are prescribed by the doctors for treating other complications of the body can trigger the initiation of dysbiosis. Some antibiotics are the main cause of processing of the drastic reduction of the beneficial gut flora and in that case, the digestive imperfection and gastrointestinal issues with mild to severe diarrhoea are also common. On the other hand, the medications such as metformin, antipsychotics, opioids, NSAIDs, and PPIs can disintegrate the condition of the gut bacteria (Sencio *et al.* 2021).

“Proton pump inhibitors” and “antipsychotic drugs” are associated with a reduction in “α diversity” in the stomach microbiome, while opioids can grow “α diversity”. NSAIDs and Metformin are not responsible for important modifications in “α diversity” (DeJong *et al.* 2020). “β diversity” was discovered to be extremely altered with all medications, excluding NSAIDs. PPI use was connected to a lowering in “Clotridiales” and an expansion in “Micrococcaceae”, “Actinomycetales” and “Streptococcaceae”, which are formerly incriminated in dysbiosis and augmented vulnerability to “Clostridium Difficile” disease (Ballway and Song, 2021).

IBD: Inflammatory bowel disease or IBD can also increase the condition regarding dysbiosis for the decolonising of the decent bacteria that help in digestion and food breaking (Rosa *et al.* 2018). In the case of pouchitis and ulcerative colitis, good bacteria such as E. coli degenerate in the intestinal tract therefore that condition can trigger the reduction of the decent effects of good bacteria. Thus, dysbiosis can happen to that individual.

Cancer: In the case of colorectal cancer the gut microbiota is disintegrated and, in that case, the affected person might die in a critical condition. On the other hand, CRC comprises a genetic backdrop and environmental risk elements, such as cholecystectomy, diabetes mellitus, high-fat diet, obesity, red meat and processed food. These reasons can cause the mismanagement of gut microbes (Da Silva *et al*. 2018). Bacteriassuch as “*Escherichia Coli*”, “*Fusobacterium nucleatum*” and “*Enterotoxigenic Bacteroides fragilis*” can be dead due to the occurrence of colorectal cancer.

Chemotherapeutic agents: Chemotherapy is typically utilised as “myeloablative conditioning treatment” to condition patients for transplantation of “haematopoietic stem cells” (HSCT). Chemotherapy directs to some side effects, with “gastrointestinal (GI) mucositis” that is the most frequent cause in the case of the human body (Brüssow, 2020). Existing models regarding “GI mucositis pathophysiology” are normally tight-lipped on the function of the “intestinal microbiome”. From the faecal samples collected from cancer-affected individuals, it has been seen that the bacteria such as Actinobacteria, Firmicutes and proteobacteria have significantly decreased.

Cardiac disease: Faecal microbial community transformations are related to multiple illness states, including cardiovascular disease (CVD). The richness of gut microbes is reduced with cardiovascular diseases (Brüssow, 2020). In this case, the patients with heart complications can

also face issues such as indigestion, nausea, bloating and abdominal cramping. The complicated host-micro biome relations control the synthesis and liberation of several metabolites, including" Bile Acids (BA)" "Trimethylamine-*N*-oxide (TMAO)", and "short-chain fatty acids (SCFA)", which influence the homeostasis of the host.

2.5 Which microbiota altered and change the normal function of the body due to these diseases mentioned above

In the case of obesity, the bacteria such as Firmicutes, Bacteroides and E. coli are affected and they change their normal function, which can cause the occurrence of dysbiosis in the body of human beings (Sencio *et al.* 2021). In the case of diabetes mellitus or type two diabetes, there is a significant increase of the bacteria such as Verucomicrobia, Proteobacteria and Actinobacteria and there is a significant decrease in Bacteroides. Moreover, the sudden decline of the Prevotella while "LachnospiraceaeIncertaeSedis", "Escherichia Shigella", Enterococcus, Subdoligranulum, and Klebsiella had distinct extents of multiplication in the Diabetes Mellitus group (Ballway and Song, 2021). In the case of cancer, an alteration of the bacterial function can be seen in the case of the Lactococcus, and Fusobacterium.

The restoration of the dysbiotic gut microbiome has emerged as a viable help and more effective treatment strategy. Prebiotics and probiotics, faecal microbiota transplantation (FMT), extracellular vesicles, immune modulation, microbial metabolites, dietary interventions and Phage's (Dixit et al., 2021).

- Chitosan has diverse biological and pharmacological effects and has been used as a new source of prebiotic. Chitosan regulates the gut microbiota. Chitosan prevented dysbiosis and inhibited activation of Toll-like and Nod-like receptor signaling in rats fed a high - fat diet (satitsri et al., 2020). Chitosan is significantly the growth of lactobacillus and Bifido bacteria, resulting in lower metabolic endotoxemia and inflammation. Chitosan treatment reduced the expression of innate immune receptors, such as TLRs and NLRs, which in turn reduced inflammation. The data show that chitosan may be able to slow the progression of T2D via altering gut microbe-mediated inflammation and insulin resistance (Prajapati et al., 2016).

3.1-Chitosan

Chitin is a polysaccharide obtained from crustaceans, fungus, and insects that also is biocompatible and biodegradable (Yan et al, 2020). Chitosan is converted from chitin by deacetylation (Saberpour et al, 2020). Antiviral, anticancer, and antifungal characteristics, as well as antibacterial qualities and a bacteriostatic effect on Gram-negative bacteria *Escherichia coli*, *Vibrio cholerae*, and *Shigella dysenteriae*, appear to be present in chitin or its derivatives (Piccolo et al., 2017).

Chemical deacetylation is the process of treating chitin with hydroxides at high temperatures, often above 80 °C [Lizardi-Mendoza et al., 2016]. Deacetylation occurs relatively quickly, within 2 hours, for treatments that use high NaOH concentrations (50-60 percent) and high temperatures (130-150 °C). However, under extreme conditions, the molecular weight of CS drops; thus, a balance between the deacetylation process and the final properties of CS must be discovered. CS is a random copolymer made by D-glucosamine and N-acetyl-D-glucosamine units joined by -1,4 glycosidic links created by alkaline deacetylation of chitin. The degree of deacetylation is measured as the ratio of the two units. (Bedian., et al., 2017) (A. Verlee, S. Mincke, C.V. Stevens, 2017)

Chitosan, a potential natural polymer exhibiting antimicrobial characteristics. Chitosan has been reported to have antioxidant, anticancer, anti-inflammatory, immunostimulant, wound healing, cholesterol-lowering, antibacterial, and antifungal qualities, which can be used as an active component of the diet to assist people to lose weight. Chitosan has also been related to lower blood pressure, arthritis control, diabetes mellitus treatment, and immunostimulant (Geerlings., et al., 2018) (Lee., et al., 2002) Furthermore, due to its antimicrobial characteristics, biocompatibility, and nontoxicity, chitosan has proven to be an appropriate component in medical science. (Matica., et al 2019) (Saberpour., et al 2020) the effects of chitin on the intestinal microflora, where it's been reported to benefit Gastro-Intestinal health due to its prebiotic function (Selenius., et al., 2018). Deacetylation of chitosan produces free amino groups, the Positive charges from chitosan amino groups interact electrostatically with negatively charged components on the microbial membrane, resulting in antimicrobial effects. The antimicrobial properties are mostly restricted to pH levels below 6 (Sahariah and Masson 2017). Chitosan can be used as a prebiotic to boost the colonic mucosal populations of beneficial bacteria while suppressing proinflammatory bacteria, hence reducing gut microbiota imbalance and mucosal inflammation (Gao., et al 2020). Chitosan and chitosan derivatives can be fermented by the gut microbiota, and the metabolites, such as short-chain fatty acids

(SCFAs), can promote probiotic growth (e.g., Bifidobacterium spp. and Lactobacillus spp.) and pathogen exclusion (Wang., et al 2019) (Prajapati., et al 2015).

Chitosan's antimicrobial property is determined by the presence of the amino groups of polymer chain These amino groups can be protonated, leading to a positive charge for chitosan (xing., et al 2015). As a result, chitosan becomes soluble in aqueous acidic solutions when the pH falls below 6.3, at which point the –NH2 groups are transformed to a soluble protonated form NH+3. (Wang., et al 2006) (Szymańska, Winnicka 2015). Gram-positive bacteria's cell wall is primarily comprised of a thick peptidoglycan layer containing teichoic acids, which give the bacterial surface a negative charge, whereas Gram-negative bacteria's cell wall is highly negatively charged due to lipopolysaccharides in the outer membrane layer. (Liu, et al. 2004)

Chitosan is also used to chelate cholesterol in meals, preventing from being absorbed by the human intestine (Tu., et al.,2015).

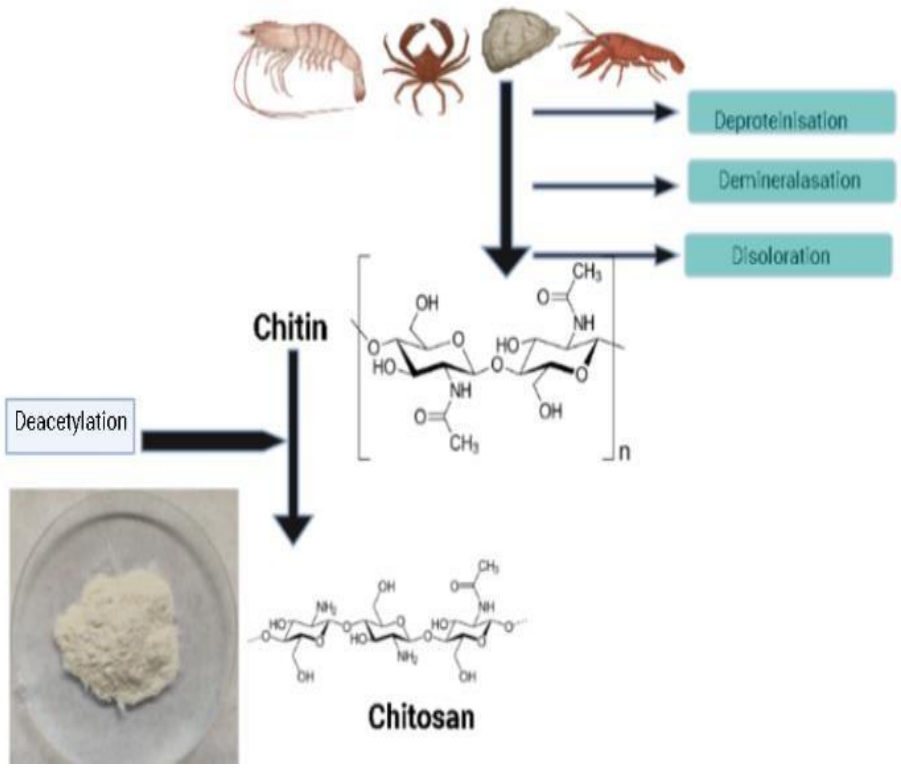


Figure 3. (A)Extraction of chitin and chitosan formation

3.2 Properties of Chitosan and its formulation from Chitin

Chitosan possesses a wide range of biological and chemical properties that are essential to its variety of applications. Chitosan's chemical characteristics include cationic nature, high charged density at pH 6.5, gel formation, adherence to negatively charged surfaces, high molecular weight, as well as the ability to chelate specific metals (Anon 2016) Chitosan's most useful property is chelation, which means that it can bind to beneficial metals such as chitosan, tumor cells, and fats. Chelation even has affinity for proteins such as maize germ agglutinin and trypsin. Chitosan also has some other properties, like the ability to inhibit tumor cells, possess antifungal properties, and assist in wound healing. Biological properties of chitosan like biocompatible, biologically degradable, non-toxic, adhesion to mucous i.e., Mucoadhesive, etc. Chitosan's features make it extremely versatile of applications, including pharmaceuticals, textiles, agriculture, and food. Chitosan's positive charge binds electrostatically with the negatively charged mucus layer. Chitosan's features make it extremely versatile of applications, including pharmaceuticals, textiles, agriculture, and food. Chitosan's positive charge binds electrostatic interactions with the negatively charged mucus layer. The properties of chitosan are as follows

1- Controlled drug delivery.

2- Wound healing.

3- Tissue regeneration.

4- Treatment of waste water.

5-Cosmetics.

6- As a Prebiotic.

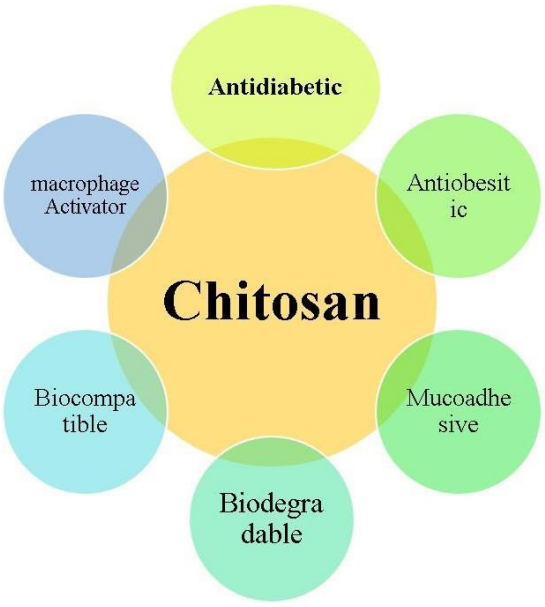


Figure 3. (B) Chitosan properties

3.3 Mode of action of chitosan microsphere on gut microbiota

Chitosan and its variants have different mechanisms and zone of action when it comes to bacteria (gram positive and gram negative).

The gram positive bacterial cell wall is made up of peptididoglycans, which are comprised of two types of teichoic acids.

1- Wall-teichoic acids (WTAs)

2. Lipo-teichoic acid (LTAs)

WTAs are covalently bonded to peptidoglycan, whereas LTAs are lipid-affixed to bacterial cell membranes (Brown et al .,2013). The presence of carboxyl and phosphate groups in teichoic acids recultivate in the negative charge of the cell wall of gram negative bacteria (Feng et al., 2021).

3.4 Interaction of chitosan with respect to Microbial DNA

Low molecular weight chitosan and chitosan hydrolysis product bind to microbial DNA and inhibit mRNA and protein translation in microorganisms (Yan et al ., 2021).

3.5 Chitosan:- A prebiotic

Chitosan in various forms has been tested as an antibacterial substance against a wide range of target species such as bacteria, yeast, and fungi. Chitosan is claimed to be bactericidal or bacteriostatic (obstructs bacterial growth). Chitosan when ingested as prebiotic can enhance the probiotics as prebiotics can only be digested by the intestinal microflora this results in the release of anti-inflamatory cytokines which reduces the pro-inflamatory cytokines which reduced the inflammation as well as restores the gut microbiota.

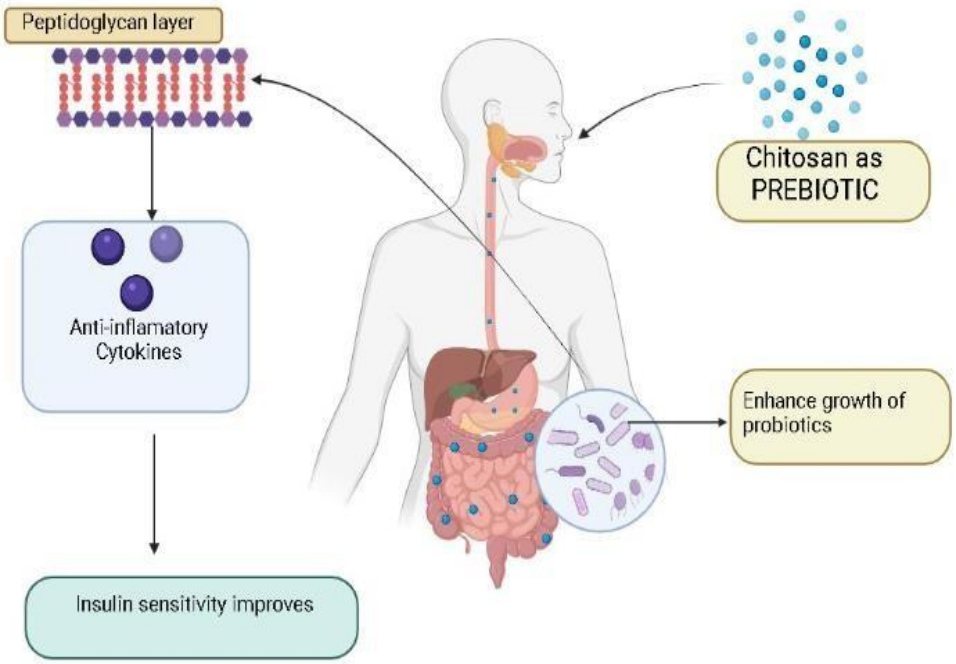


Figure 3(C)Chitosan's Antimicrobial Activity Against Bacteria

HYPOTHESIS AND OBJECTIVE

HYPOTHESIS

Hypothesis of our study was to determine whether the Doxorubicin was involved in the gut micro floral alteration. And to study that whether the co-administration of gut modulators influence the efficacy of Doxorubicin.

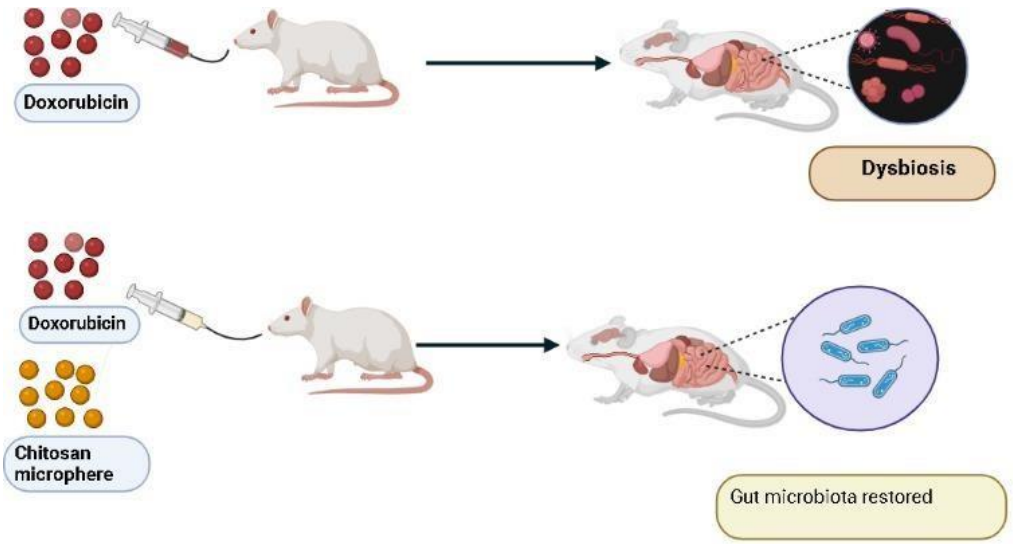


Figure: 4(A): Interdependence between Doxorubicin and chitosan

OBJECTIVES

A. To determine the interdependence between microflora and doxorubicin.

B. To evaluate the role of gut microflora modulators on the efficacy of Doxorubicin

MATERIALS AND METHODS

MATERIALS AND METHODS

ANIMAL EXPERIMENT

The animal's protocol number is (IS/PHD/27/2020/032). The animals were housed in a climate-controlled environment with optimal humidity and temperature. Based on their grouping, they were fed the appropriate meal and water, as shown in the table.

The animal studies were carried out in accordance with the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad's ethical guidelines for the care and use of laboratory animals, as well as the CPCSEA guidelines of the Ministry of Environment and Forest, New Delhi (Protocol no. IS/BT/FAC-13-1009). The animals were divided into the categories listed below.

ANIMAL GROUPING

Table 2.1 Animal Grouping

SR. NO.	GROUPS	NO. OF ANIMALS	DOSAGE REGIMEN
1)	CONTROL	04	-
2)	100% DOXORUBICIN	04	Orally single dose of Doxorubicin (1mg/kg) body weight and single dose of chitosan microspheres (40mg/kg) body weight
3)	75-25% DOXORUBICIN+MICROSPHERE	04	Orally single dose of Doxorubicin (1mg/kg) body weight and single dose of chitosan microspheres (40mg/kg) body weight
4)	50-50% DOXORUBICIN+MICROSPHERE	04	Orally single dose of Doxorubicin (1mg/kg) body weight and single dose of chitosan microspheres (40mg/kg) body weight

ANIMAL DIET

Animals of control group were fed with standard diet (Pellets) and animals of 100% Doxorubicin, 75-25% Doxorubicin-Chitosan microsphere, 50-50% Doxorubicin-Chitosan microsphere. Diet and water were continuously provided to rats.

TREATMENT STRATERGY

Doxorubicin and chitosan microspheres were administered to rats. The doxorubicin dosage regimen was 1 mg/kg body weight, and the chitosan microsphere dosage regimen was 40 mg/kg body weight. The doxorubicin stock solution was prepared with distilled water and administered orally to the rats for 5 days. For 75 days, rats were given chitosan microspheres orally in PBS (Phosphate buffered saline).

CHITOSAN MICROSPHERE FORMULATION

Chitosan with appropriate amount was dissolved in 2% glacial acetic acid by putting on magnetic stirrer with continuous stirring. Chitosan solution along with the span 80 was then added dropwise to the low molecular weight paraffin oil and kept on the magnetic stirrer (1500 rpm). Glutaraldehyde was then added dropwise to the solution and stirring continued for 3-4 hours. The formulated microspheres were collected and filtered and dried in the hot air oven. The microspheres were crushed properly into minute pieces and stored in microfuge tube and at the time of dosing the microspheres were mixed with PBS (Phosphate buffer saline) and given. (Prajapati et.al, 2016).

ADMINISTRATION OF DOXORUBICIN AS STANDARD CHEMOTHERAPEUTIC DRUG

A group of Doxorubicin 100%, Doxorubicin 75%, Doxorubicin 50% was administered with Doxorubicin, a standard chemotherapeutic drug. Doxorubicin was administered for 5 consecutive days.

BODY WEIGHT AND ORGAN WEIGHT

Body weight was taken on alternative days throughout the treatment. Because of increase and decrease in body weight we were able to know the condition of our rats. During the time of

autopsy small intestine, Large intestine Liver and Adipose tissue of rats in each group were taken and weighed.

FECAL SAMPLE COLLECTION

Fresh fecal sample were collected based on 5 days of interval day from day of treatment to the end of the treatment period. Also, colonic fecal (cecal) sample were collected during autopsy and stored at -80ºC and were used further for microflora quantification.

AUTOPSY SCHEDULE

Autopsy for doxorubicin 100 % rats was performed on the 6th week after treatment for first 5 days and keeping it on observation then. Autopsy for 75-25% rats and 50-50 % rats were performed on the last day of treatment. After treatment organs (large intestine, small intestine, liver, adipose tissue) were collected and organ weight was measured.

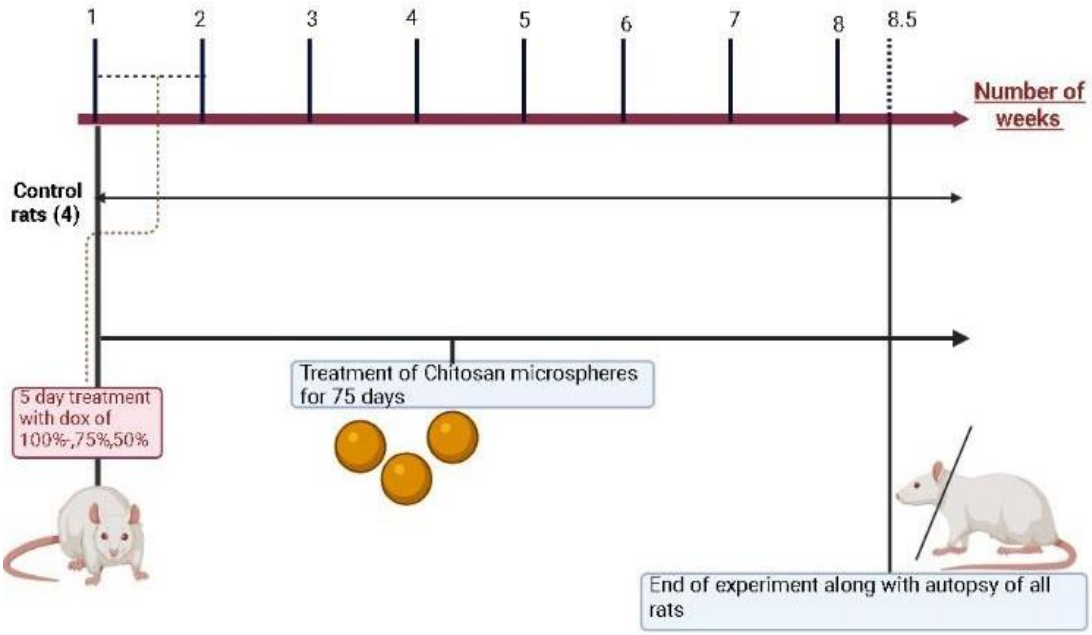


Figure.4 (B) Autopsy schedule

SERUM AND PLASMA COLLECTION

For the study and biochemical analysis, blood was collected by puncturing the hearts of animals. Two vials of blood were used to collect the blood (1.5 ml). After keeping the blood at room temperature for 4-5 hours, it was centrifuged, and the serum was collected. Accucare diagnostic kit was used to perform serum biochemical tests (SGPT, SGOT, TG, Glucose, HDL, LDL) and the procedure was followed according to the manufacturer's protocol.

HISTOPATHOLICAL ANALYSIS

After autopsy, a small section of small intestine, liver, and small intestine were removed from each group of rats and fixed in 10% formalin. A 5um section was cut with a microtome, and slides were prepared and stained with hematoxylin and eosin (H.E.) stain before being examined under a microscope and digital photographs were taken with a Cat-cam 3.0 MP.

ESTIMATION OF SHORT CHAIN FATTY ACIDS (SCFA)\

SCFAs (Acetate, Butyrate, and Propionate) were determined using HPLC from faecal samples of each group of animals. 1M phosphate buffer with pH 7.4 was used as the mobile phase, and 100 mg fresh pooled faecal samples from each experimental group were homogenised with mobile phase, centrifuged at 10000 rpm for 15 minutes at 4 °C, and then filtered using syringe filters in another Eppendorf which would then be analysed at a flow rate of 1mL at 254 °C. As well as the injection volume is 10uL. As standards, standard SCFAs (Acetate, Butyrate, Propionate, and a mix of all three) were used.

BIOCHEMICAL PARAMETERS ANALYSIS FROM SERUM

REAGNETS- Accucare serum glucose reagent set, Triglyceride's reagent set, HDL reagent set, LDL reagent set, SGOT reagent set, SGPT reagent set.

Serum biochemical parameters such as glucose, triglycerides, HDL (High density lipoprotein), LDL (Low density lipoprotein), SGPT (Serum Glutamic Pyruvic Transaminase), SGOT (Serum Glutamic-Oxaloacetic transaminase). Ultracentrifugation was used to separate serum from blood samples using Accucare reagents kits according to the manufacturer's protocol.

MICROBIOTA PROFILING BY PCR

PCR was used to characterize the fecal microbiota using specific primers targeting the 16 rRNA gene sequences of different bacterial Phylum and genera.

Table 2.2 - PCR primers for the 16S r RNA gene

Sr. No	Target Groups	Oligonucleotide sequence
A.	Bacteroides	F-CATGTGGTTTAATTCGATGAT R-AGCTGACGACAACCATGCAG
B.	Lactobacillus	F-TGAAACAGRTGCTAATACCG R-GTCCATTGTGGAAGATTCCC
C.	Firmicutes	F-ATGTGGTTTAATTCGAAGCA R-AGCTGACGACAACCATGCAC
D.	Bifidobacterium	F-GCGTGCTTAACACATGCAAGTC R-CACCCGTTTCCAGGAGATATT
E.	Escherichia Coli	F-CATGCCGCGTGTATGAA R-CGGGTAACGTCAATGAGC

TAKARA PCR kit was used to perform rum PCR according to the manufacturer's protocol.

GENE EXPRESSION ANALYSIS

TOTAL RNA ISOLATION

RNA isolation was performed from tissue samples obtained from animals. (Liver, Small intestine, large intestine).

100 mg of tissue was homogenized in a homogenizer tube with 1000ml of RNA Isoplus (Trizol Reagent). The homogenate was transferred to an Eppendorf tube and allowed to cool for 5 minutes before centrifugation at 12000rpm for 5 minutes at 4°C.Then these tubes were centrifuged. The supernatant was then transferred to a new Eppendorf tube (1.5 ml), and 200ml of chloroform was added and vigorously mixed to form a milky solution before being kept at room temperature for 5 minutes. Then these tubes were centrifuged for 15 minutes at 4°C at 12000 rpm. Place the upper layer (50-100µl) in a new centrifuge tube. The tube was then filled with chilled isopropanol (IPA), and 0.5-1.0x the volume of supernatant was added and gently stirred. The tubes were frozen after 10 minutes on ice and centrifuged at 12000 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet was washed with 100ul of ethanol. The sample was centrifuged at 8000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was air dried to let ethanol evaporate. The pellet was then dissolved in 50ul nuclear- free water. After, this pellet was loaded on gel and observed on UV spectrophotometer.

Nanodrop was used to check concentration and purity before using it for cDNA synthesis and gene expression studies.

PRIMER DESIGNINJG FOR GENE EXPRESSION STUDIES

Integrated DNA technologies were used to create primers for various genes. Primers for several genes, including TLR-2, TLR-4, and NF-kB, were created. The nucleotide sequence for each gene was blasted with the primer sequence to ensure that the primer sequence was completely aligned with the mRNA sequence of the respective genes.

PRIMERS FOR GENE EXPRESSION STUDIES

Table2.3 Primers for gene expression studies

Gene	FORWARD PRIMER	Tm °C	REVERSE PRIMER	Tm °C
TLR-2	TGCAGAGCAACGATGGAGAAA	68.1	ACAGGAGCGTCAGGGTGAAG	67.9
TLR-4	GGCTGTGGAGACAAAAATGACCTC	68.2	AGGCTTGGGCTTGAATGGAGTC	69.5
NF-kB	CCCCACGAGCTTGTAGGAAAG	66.8	CCAGGTTCTGGAAACTGTGGAT	66.2

The reverse transcriptase included in the cDNA synthesis kit was used to convert RNA to cDNA (Thermo specific, USA). NF-kB PCR was used to examine the transcriptional level expression of TLR-2 and TLR-4 (TAKARA).

DENSITOMETRIC ANALYSIS

Documentation for OmniDOC Gel The software version 1.3.3.4 was used to generate a graph of bands observed after PCR for both DNA and RNA, and the area under the curve was calculated and abundance was measured using statistical analysis.

STATISTICAL ANALYSIS

The data was provided as a mean ± SD. One way analysis of variance was used to investigate a statistical difference between the means of the various groups (ANOVA). The statistical significance was then assessed using GraphPad prism software's T-test (V 5.0).

RESULTS

RESULTS

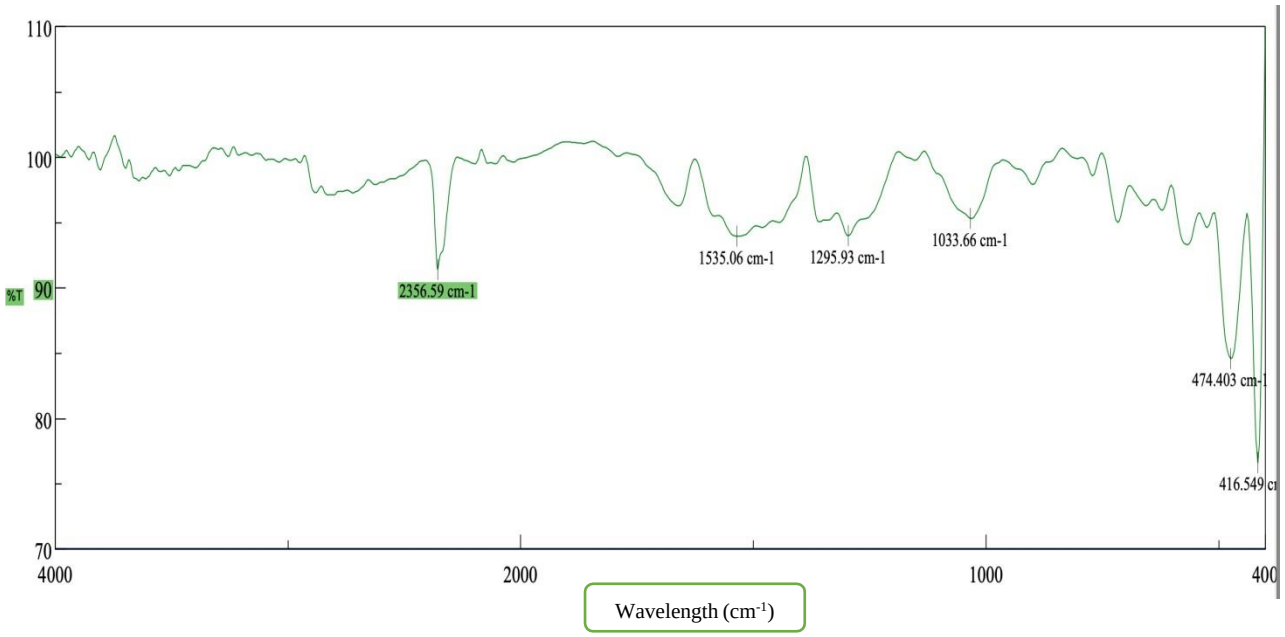
CRUDE CHITOSAN AND CHITOSAN MICROSPHERE CHARACTERIZATION.

(i) ZETA potential and Particle size analysis-

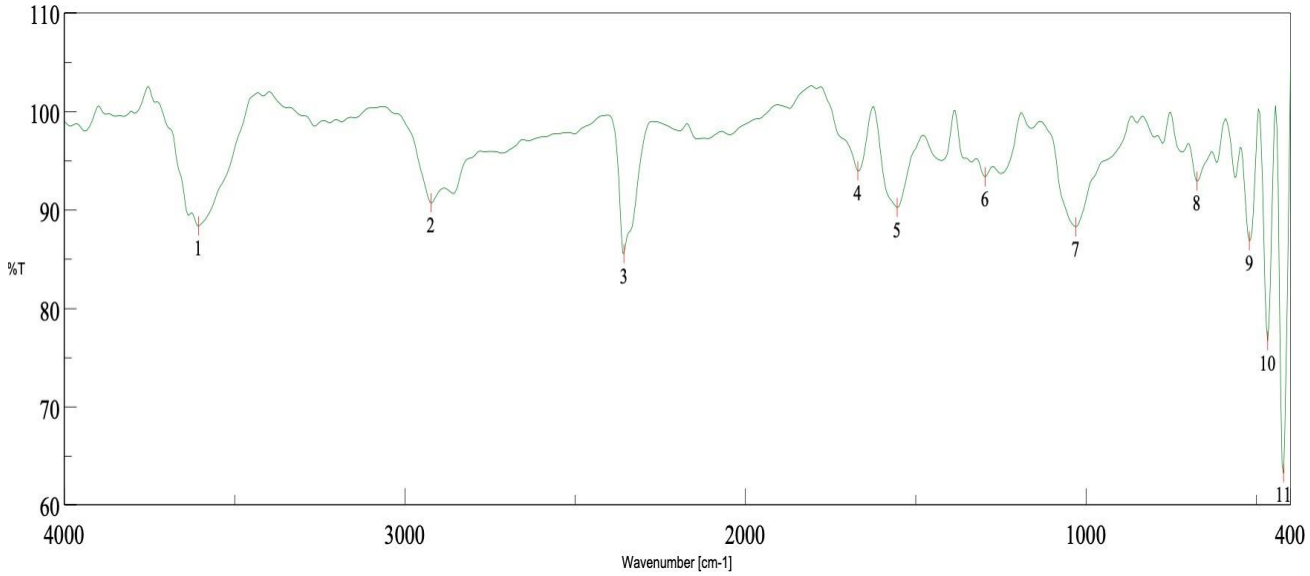
Data of ZETA potential and particle size analysis have shown that the chitosan microsphere is positively charged with size of molecule 24.5 nm.

(ii) Fourier- transform Infrared Spectroscopy (FTIR)

The FT-IR was carried out to check the possible bonds present between chitosan and crude microsphere. IR spectrum of crude chitosan was characterized by absorption peaks at 2000cm^{-1} (Alkyne), 1500 cm^{-1} (C=C), 2850 cm^{-1} (C-H), 1000 cm^{-1} – 1300^{-1} (C-O), 3230 cm^{-1} – 3550 cm^{-1} (Acids). IR spectrum of Chitosan microsphere was characterized by absorption peaks at 2356 cm^{-1} (NH component), 1535.06 cm^{-1} (Amide group), 1295.93 cm^{-1} (C=N, C=C).



Graph – 1 – FT-IR Of Crude Chitosan with Absorption peaks at different wavelength

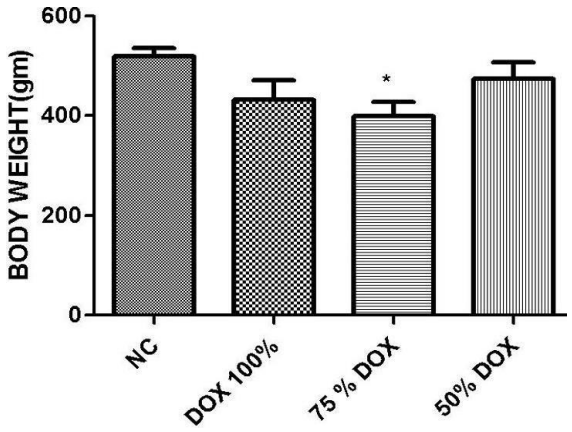


Graph-2 FT-IR Of Chitosan Microsphere with absorption peaks at different wavelengths.

1.3606.23cm^{-1}, 2. 2923.56cm$^{-1,}$ 3. 2356.59cm$^{-1,}$ 4. 1670.05 cm^{-1} , 5. 1554.34 cm^{-1} , 6. 1295.93cm^{-1} , 7. 1029.8cm^{-1} , 8. 674.963cm^{-1} , 9. 520.686cm^{-1} , 10 466.689cm^{-1}, 11. 420.406cm^{-1} .

CHANGE IN PERCENTAGE BODY WEIGHT

There was significant change in weight of the animals of Doxorubicin 100%, Doxorubicin 75% and Doxorubicin 50% because of the fluctuations seen during comparison of body weight in these animals. Weight loss was observed in these animals compared to normal.

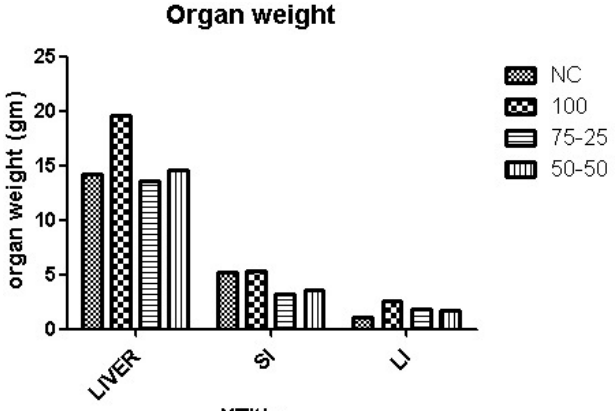


Graph-3 Body weight graph of NC, 100% Doxorubicin, 75-25 % Doxorubicin+MS, 50-50% Doxorubicin+MS (*- $p<0.05$).

Graph 1- % change in Body weight of animals of all groups, NC- Normal control, 100% Doxorubicin, 75-25% Doxorubicin + MS, 50-50% Doxorubicin+MS

AVERAGE ORGAN WEIGHT

During Autopsy small intestine, large intestine, liver of each rat was taken and weighed.

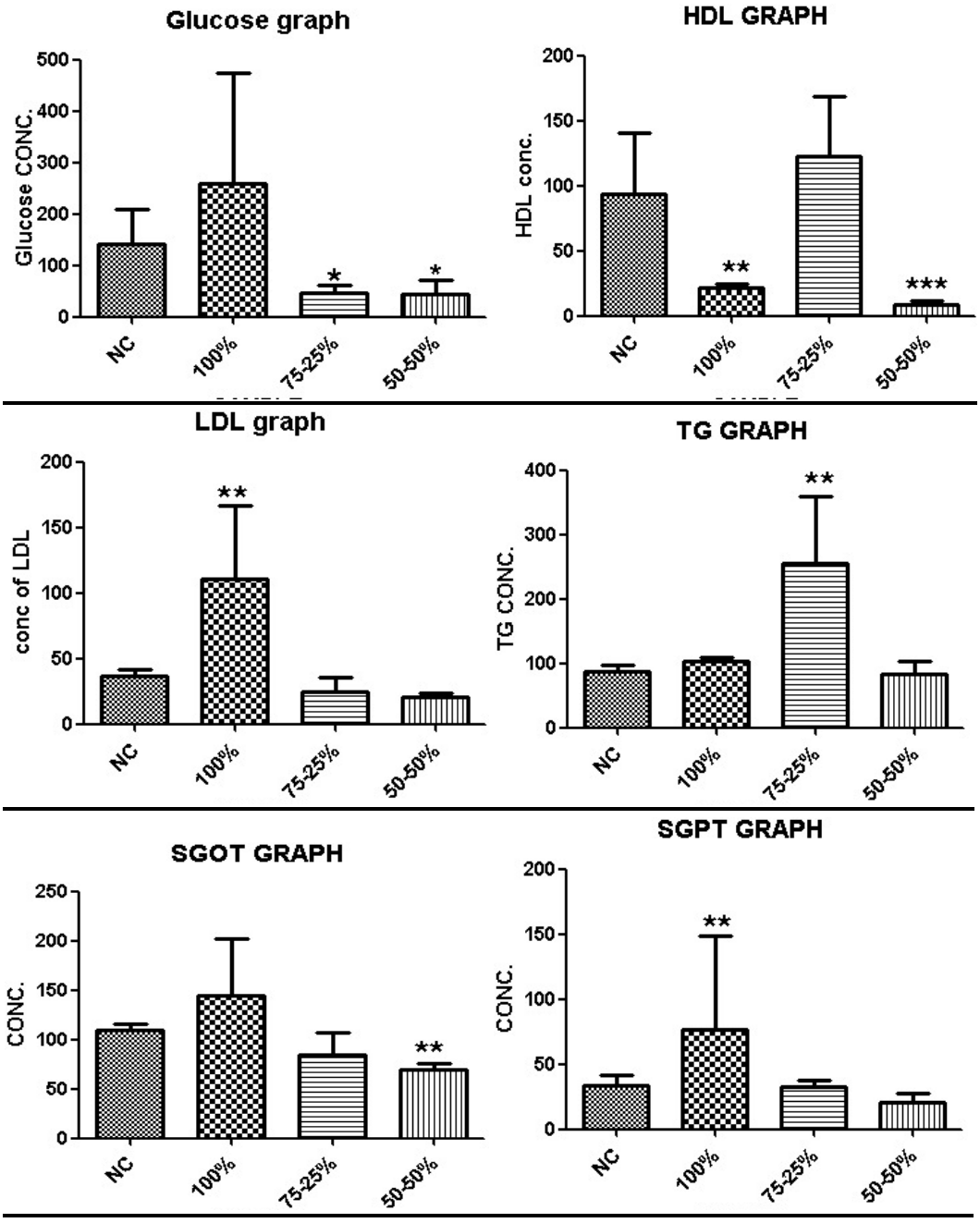


Graph -4 Organ weight was taken for NC (Normal control), 100% Doxorubicin, 75-25% Doxorubicin+MS, 50-50% Doxorubicin+MS for Liver, Small Intestine, Large Intestine

BIOCHEMICAL PARAMETERS

Glucose, Triglyceride, LDL, HDL, SGOT and SGPT

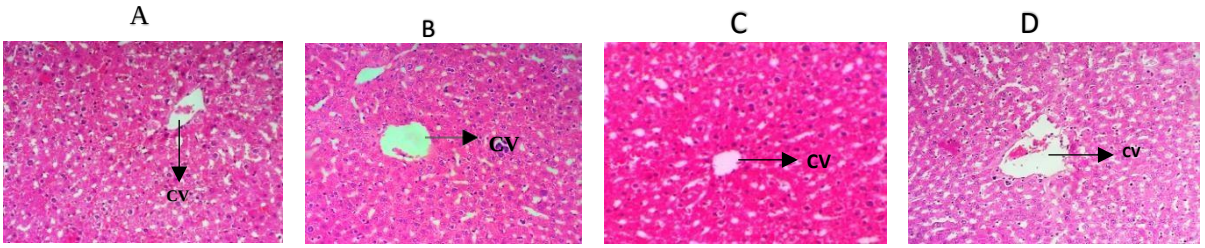
As shown in the Graph level of Glucose had decreased significantly in Doxorubicin+MS 75-25%, 50-50% compared to NC. Triglyceride (TG) are type of fat found in blood, we noticed increase in level of TG in Doxorubicin+MS 75-25 and 50-50% compared with NC. HDL (good cholesterol) absorbs cholesterol and carries it to liver, we noticed significant increase in Doxorubicin+MS 75-25% compared to NC and in 100% Doxorubicin and 50-50 % Doxorubicin+MS decreased level of HDL were observed. LDL (bad cholesterol) high LDL levels leads to build up of cholesterol in arteries which results in formation of plaque, we noticed level of LDL has increased in 100% Doxorubicin compared to NC, which indicates risk of cardiovascular diseases. SGOT (AST) measures the level of AST- AST is one of the enzymes that assist the liver to convert food into energy, the level of SGOT have increased in 100% Doxorubicin which indicates that liver is injured or irritated and there was significant decrease in 75-25 and 50-50% Doxorubicin+MS when compared with NC. SGPT(ALT) measures the amount of Glutamate pyruvate transaminase (GPT), as observed the level of SGPT in 100 % Doxorubicin has increased compared to NC which indicates that the liver is damaged.



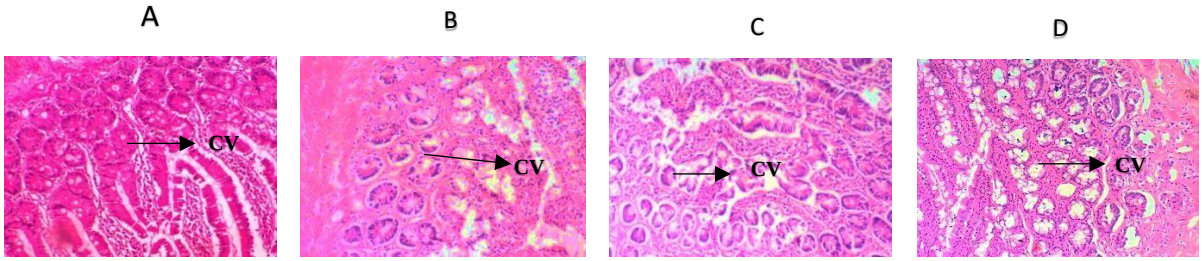
Graph 5- These are the graphs obtained using Graph pad prism (V 5.0) by significant values obtained from Biochemical analysis of Glucose, TG(Triglyceride), HDL, LDL, SGPT, SGOT. Values with different number of (*) differs significantly. p value (*)- $p<0.05$, (**)- $p<0.01$, (***)- $p<0.001$

HISTOPATHOLOGICAL ANALYSIS

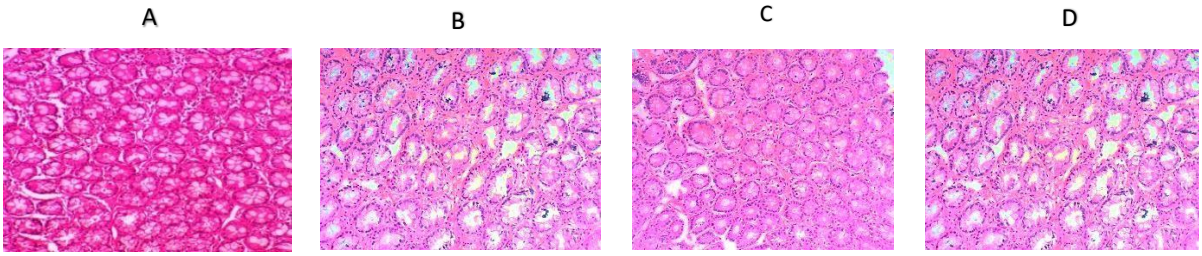
After autopsy, histopathological analysis of the liver, small intestine, and large intestine was performed to assess the effect of Doxorubicin and Chitosan microsphere. We observed from the slides that in Liver 100% Doxorubicin and 50-50% Doxorubicin, hepatic cells have bursted out in the central vein as compared with the fine structured cells of NC group. In Large intestine, Inflammation has been observed, the crypts and the goblet cells are distorted and bursted out in 100% doxorubicin, 75-25% Doxorubicin+MS, 50-50% Doxorubicin+MS as compared with the fine structured of NC cells. In Small intestine, the 100% Doxorubicin, 75-25% Doxorubicin+MS, 50-50% Doxorubicin+MS the crypts and villi are distorted as compared to NC.



LIVER: A. NC, B. 100% Doxorubicin, C. 75-25% Doxorubicin+MS, D.50-50% Doxorubicin+MS (CV-Central vein)



SMALL INTESTINE: A. NC, B. 100% Doxorubicin, B. 75-25% Doxorubicin+MS, D. 50-50% Doxorubicin+MS (CC-Crypt Cell)



LARGE INTESTINE : A. NC, B. 100% Doxorubicin, C. 75-25% Doxorubicin+MS, D. 50-50% Doxorubicin+MS

Figure: 4(C) Histopathological analysis of Liver, Small intestine, large intestine

GENE EXPRESSION STUDIES

Liver gene expression research was carried out. NF-kB, TLR-2, TLR-4 expression was checked using Densitometric analysis. NF-kB induces various pro-inflammatory responses but no results was obtained. TLR-2 is a receptor for peptidoglycan present in cell wall of Gram positive bacteria, but no results were obtained. TLR-4 is a receptor for lipopolysaccharide present in cell wall of Gram negative bacteria and no results were obtained for it.

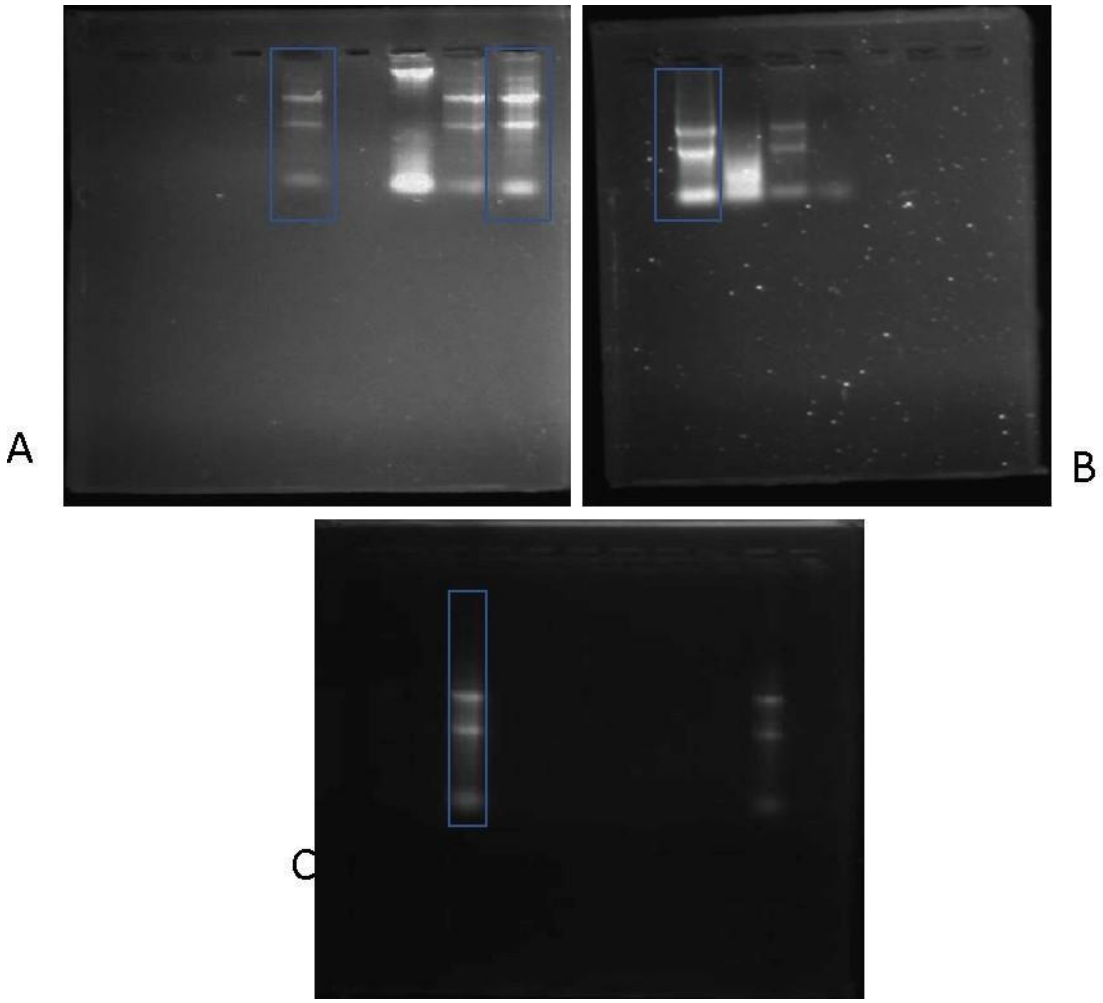


Figure: 5(A) RNA Isolation from NC, Doxorubicin 100%, Doxorubicin+MS 75-25%, Doxorubicin+MS 50-50%. A. LANE-1 : NC, LANE-4: Doxorubicin-100% B. LANE-1: 75-25% Doxorubicin C. LANE-1: 50-50% Doxorubicin+MS

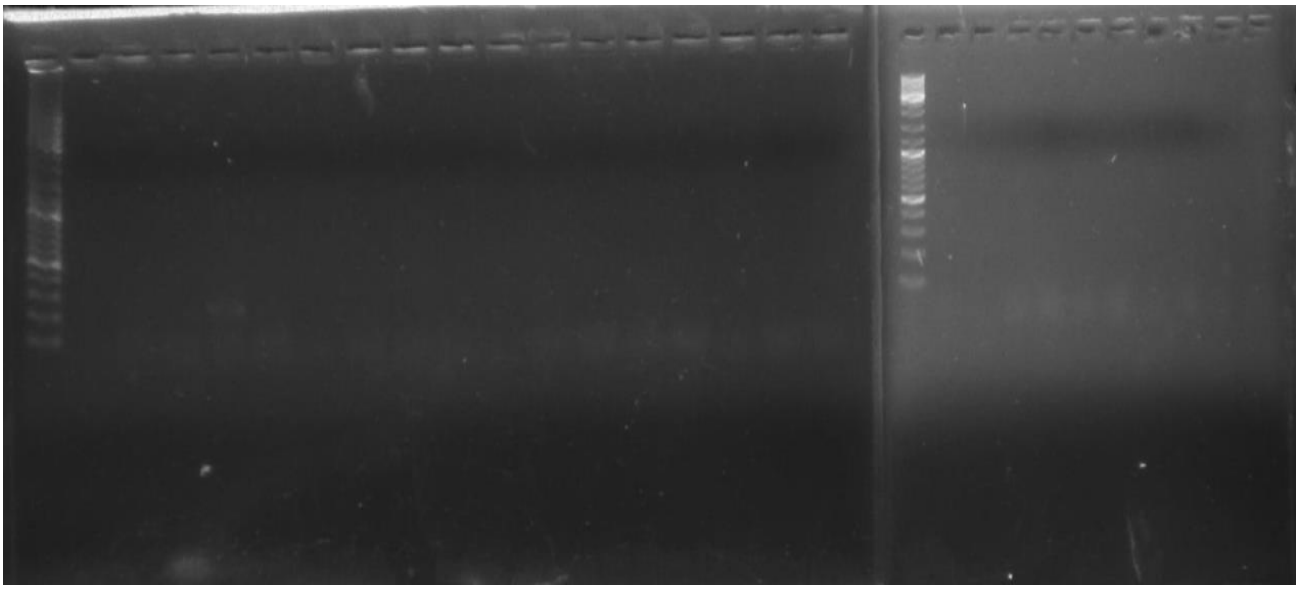


Figure:5(B) Gene expression for NF-kB, TLR-4, TLR-2 was carried out from cDNA extracted from liver. LANE-1: Ladder, LANE 2-7: NF-kB, Next Gel- TLR-4. No results were observed.

MICROBIOTA PROFILING

A faecal sample collected during the study was used to examine the common population of Gut microbiota. Data analysed showed increase in the number of E.coli(Gram-negative) and Bifidobacterium(Gram-postive) in 100% Doxorubicin group compared to NC. The results of 75-25% and 50-50% were not attained due to the time constraint.

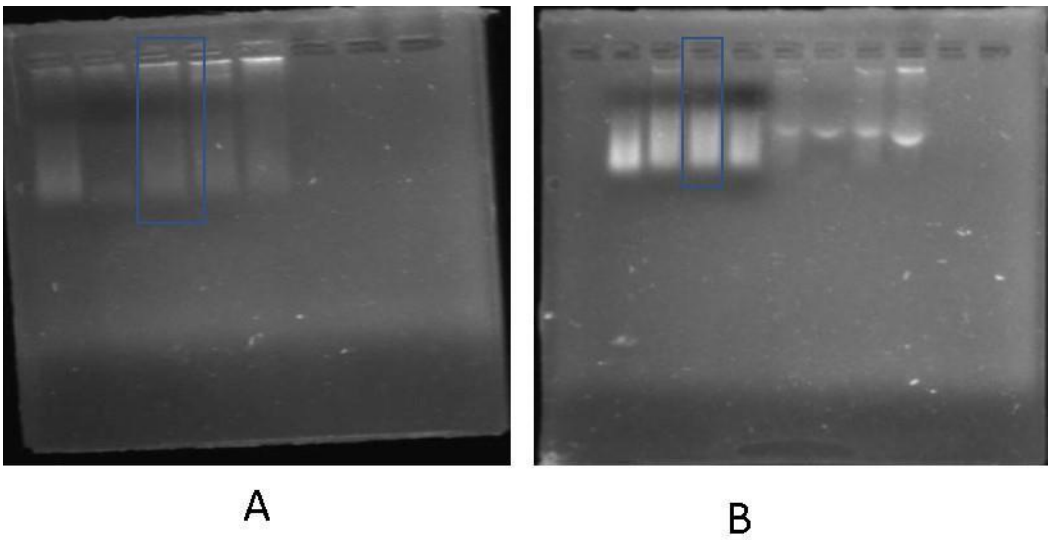
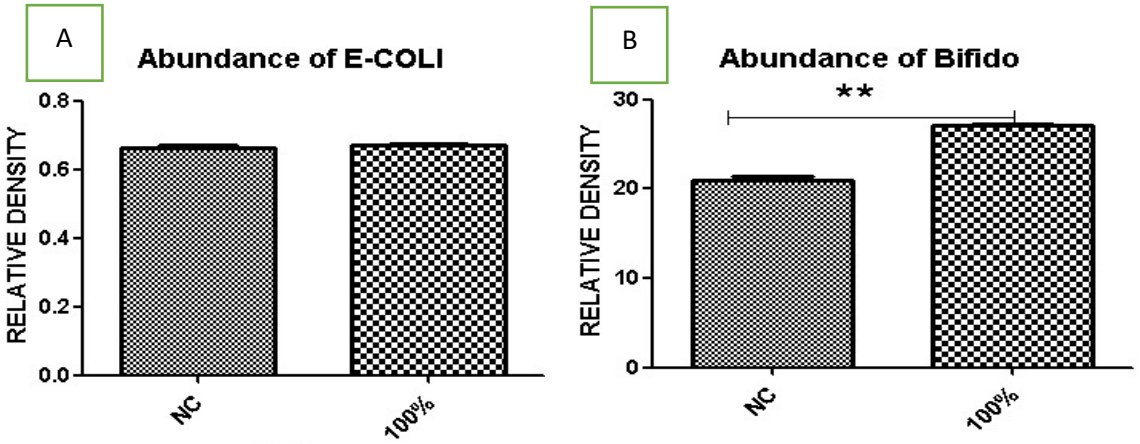


Figure: 5(B)DNA Isolation from pooled fecal sample of Doxorubicin 100% and Normal control. GEL-A LANE-3 NC, GEL-2 LANE-3 DOXORUBICIN 100%

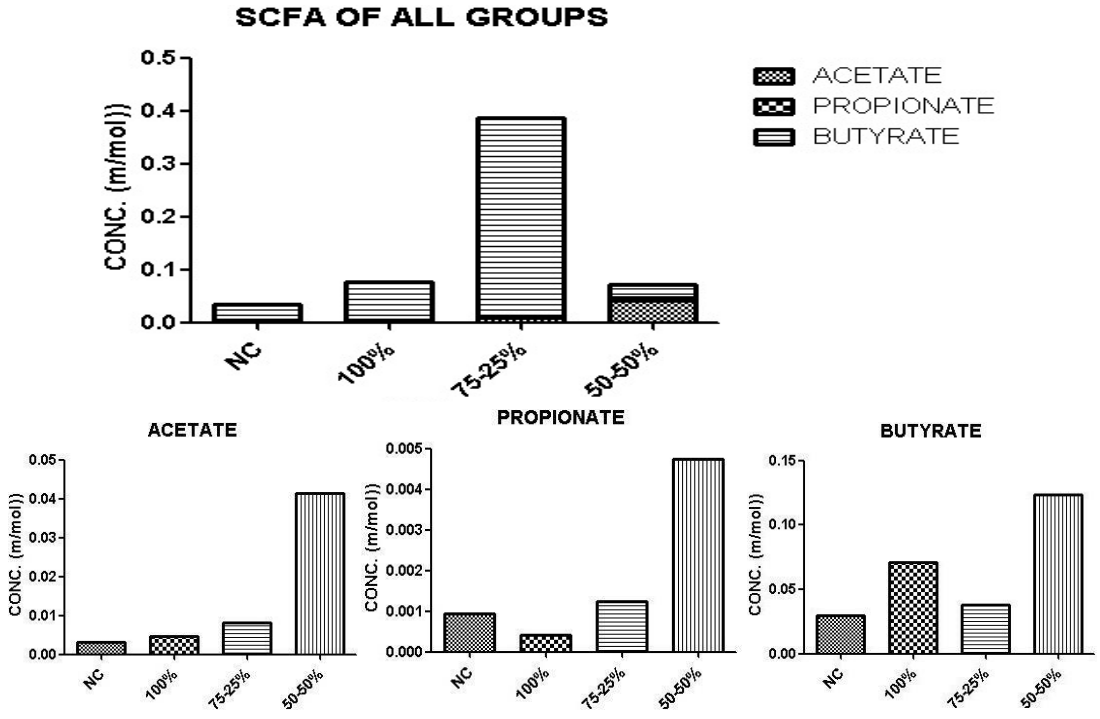


Graph-6: A.Abundance of E.Coli, B. Abundance of Bifidobacterium- The abundance of E.coli is same in 100% doxorubicin as that in NC. The abundance of bifidobacterium has significantly increased in 100% doxorubicin compared to NC. Values are represented as mean. (**) $p<0.01$.

SHORT CHAIN FATTY ACID (SCFAs)ANALYSIS

Prior to autopsy, faecal samples from each group were collected and used for SCFA estimation by HPLC. The concentration of Acetate, Propionate, and Butyrate in mmol/ml was calculated and plotted in a bar graph based on the standard sample peak, retention time, and peak area.

Butyrate, Propionate and Acetate are important SCFAs produced by the Gut microflora. The level of these SCFA shows the activity of microbial population. Butyrate is higher in the Doxorubicin+MS 75-25% , comapred to Doxorubicin 100% and Doxorubicin50-50%, Acetate and propionate concentration is low as compared to butyrate concentaration in Doxorubicin 75-25% and these shows the increase in number of gram positive bacteria producing butyrate.



Graph-7: HPLC of SCFA from fecal sample. Graph represents the concentration (mmol/ml) of Butyrate, Acetate, Propionate in NC, Doxorubicin 100%, Dororubicin+MS 75-25%, Doxorubicin+MS 50-50%

DISCUSSION

DISCUSSION

The intestinal microbial population that occupies the human gut has approximately 380 billion microorganisms with a biomass of nearly 1.8 kg, with Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia dominating. Many physiological and pathological processes in the human body, such as drug metabolism and vitamin production, are influenced by the gut microbiota. IMD (intestinal microbiota dysbiosis) is not only linked to the occurrence and progression of cancer, but it also influences treatment impact. Chemotherapeutic medicines can produce IMD, and the intestinal microbiota can alter chemotherapy's effectiveness and toxicity. The presence of specific bacteria in tumour tissues and their potential to regulate the response to chemotherapy drugs [Wei et al., 2021].

They also have an indirect and direct effects on the host's chemotherapeutic metabolism by altering the host's microenvironment. The intestinal flora develops in symbiosis with the host, so its composition and function are highly individualized. During the course of cancer treatment, the community structure of the intestinal flora is affected by several factors. Host environment and diet, surgery, use of antibiotics, and the effects of chemotherapy administered many of these factors cause dysbiosis, which is the destruction of microbial communities that disrupts symbiotic relationships with the host. Interaction with the immune system, heterogous metabolism, and changes in the community structure all cause unwanted side effects and can compromise the outcome of chemotherapy. Therefore, analysis and manipulation of the intestinal flora can be an important factor in the development of personalized and effective cancer treatments. [Pouncey et al., 2018].

The effects of chemotherapy treatment on gut microbiota in paediatric patients with acute myeloid leukaemia was using polymerase chain reaction/denaturing gradient gel electrophoresis and fluorescent in situ hybridization to analyse and quantify bacterial populations: during chemotherapy, the number of bacteria in fecal samples was 100-fold lower than in healthy control samples.

The gut microbiota profile to patients with non-lymphoma Hodgkin's was analysed before and after 5-day myeloablative chemotherapy regimen of high-dose carmustine, etoposide, aracytine

and melphalan: a significant decrease in Firmicutes and Actinobacteria abundance and a significant increase in proteobacteria abundance were documented when compared to samples collected before chemotherapy [Panebicano et al., 2018].

The effects of chemotherapeutic drugs on gut microflora shred some characteristics, most notably: (1) the overall quantity and diversity of microbiota in animal feces were frequently reduced, particularly in test with earlier sampling times. (2) increased abundance of firmicutes and decreased abundance of Bacteroidetes, despites conflicting results; (3) increased abundance of gram-negative bacteria, including potentially pathogenic microbes such as E.coli and Pseudomonas, and decreased abundance of gram - positive bacteria such as Bifidobacterium and lactobacillus. Furthermore, some intestinal bacteria were revealed to have been translocated to the mesenteric lymph nodes and spleen [Wei et al., 2021].

Doxorubicin is involved in weight loss of body. Doxorubicin caused damaged and reduces liver cells regeneration by increasing oxidative stress caused by radical production by oxidation in hepatocytes the produced radical reduces GSH levels, degrades DNA, and acts as a secondary metabolite in several metabolic pathways, including cell proliferation and cell death. Because of this effect, liver cells are occasionally unable to heal from DOX-induced damage, resulting in hepatotoxicity.

DOX-induced cardiotoxicity shows itself equally acute and chronically. The patient may experience arrhythmia, hypotension, and super ventricular tachycardia within one week of starting the medication. These abnormalities are usually reversible in short period of time. Only 1.7 percent of patients experience chronic symptoms, with a 50% mortality rate. A study found that DOX has a long-term effect, such as congestive heart failure [Ajaykumar and Chittipolu., 2020].

Doxorubicin causes considerable, but brief, increase in apoptosis in the jejunum’s stem cells zone, followed by mucosal damage characterised by a decrease in crypt proliferation, crypt number, and villus height [Rigby et al., 2016].

Dysbiosis is caused by oral mucositis, which reduced the numbers of Streptococcus, Actinomyces, Gemella, Granulicatella, and Vellionella bacteria while boosting the amounts of other gram-negative bacteria such as Fusobacterium nucleatum and Prevotella oris.

Fusobacterium nucleatum has pro-inflammatory and pro-apoptotic activities, which contributes to mucosal injury [Singh et al., 2015].

Dysbiosis and gastrointestinal disease related diseases: Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowels characterized by chronic recurrent inflammation of the intestinal lining, metabolic disorders, obesity, and type 2 diabetes. It is also associated with several other GI-related disease and irritable bowel syndrome (IBS), celiac diseases and colon cancer.

Many studies shows that dysbiosis can cause many disorders, so it is necessary to restore dysbiosis [Carding, et al., 2015].

The mucosal barrier on the epithelial surface of a intestine is composed up in part by colonial microbial flora, which may contribute to the defensive gut barrier and shield from additional pathogen invasion, decreasing the possibility of bacterial translocation (Gori et al., 2011).

To be classified as a prebiotic, a substance need not be hydrolysed or absorbed in the anterior gastrointestinal tract, must be preferentially fermented by one or a small number of beneficial bacteria in the intestine, and must be capable of changing the colonic microbiota toward a healthier composition (Gibson and Roberfroid, 1995; Looijer-van Langen and Dieleman, 2009;Roberfroid, 2007). Short-chain fatty acids (SCFA) are generated during the fermentation of prebiotics in the colon, which assist in the proliferation of the colonic microbiota (Yanahira et al., 1995). Oligosaccharides (OS) predominate as substances that fulfill the definition of a prebiotic (Roberfroid, 2007) Different prebiotics may have a wide range of biological implications, including alterations in gut morphology(Leforestier et al., 2009). Chemotherapy can influence microbial activity that may lead transition towards a more pathogenic makeup(Stringer et al., 2009) Several studies have already shown that prebiotics can change the makeup of commensal microbiota to be more beneficial. A prebiotic oligosaccharide mixture, for example, enhanced gut microbiota composition in people, as indicated by an increase in *Bifidobacteria* and a decrease in pathogenic Clostridia-related species (Gori et al.,2011).In our studies we used Chitosan a prebiotic oligosaccharide which significantly reduces the effects of microflora alteration caused by Chemotherapeutic drug Doxorubicin.

SGPT and SGOT tests were performed which indicate the affect of Doxorubicin on liver damage. When treated with chitosan the effects were significantly reduced in other two groups.

Body weight reduction is a visible sign that shows the effect of chemotherapy , thus by treating with chitosan bodyweight of groups treated with respective concentration showed a significant measurement when compared with the one treated with only chemotherapy drug.

SCFA analysis showed that the concentration of Butyrate has significantly increased in groups treated with chitosan microspheres which shows the concentration of Gram-positive bacteria has significantly increased thus by maintain the microflora.

CONCLUSION AND FUTURE PROSPECTS

CONCLUSION

The Doxorubicin, a modified chemotherapeutic drug, settles into the gut, creating dysbiosis, gut microbiota restoration is an option to minimize the effects of chemotherapy- mediated toxic effects. This dysbiosis can be restored in a variety way, including antibiotics, probiotics, dietary changes, and prebiotics. So, by using prebiotics and Doxorubicin at the same time, we saw significant changes, indicating that restoring gut microbiota may be a novel way to treat the side effects of Doxorubicin.

FUTURE PROSPECTS

A. We can reduce the toxicity in the intestine by lowering the doses of chemotherapy medicines and parallelly giving chitosan microsphere as a gut modulator to restore the Gut microbiome.
B. Specific drug combination will also be developed to support the formation of healthy flora in the gut and aid in removal of chemotherapeutic- mediated toxicity.
C. By developing new ways to effectively monitor chemotherapy levels, this research will lead to direct and significant benefits, including fewer side effects and restoration in gut.

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