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Contents

Chapter - 1
Emerging Multi Drug Resistant Bacteria in the Environment is Life-Threatening

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Chapter - 1

Emerging Multi Drug Resistant Bacteria in the Environment is Life-Threatening

Nagaraja Suryadevara, Yoke Ing Kwan, Ponmurugan P, Gnanendra Shanmugam and Balsubramani Gangathraprabhu

Abstract

Antibiotics have always been acknowledged as a wonder discovery of the 20th Century. Dating back to 1928, the introduction of first antibiotic-Penicillin by Alexander Fleming not only has changed the course of medicine but also well-improved the welfare of both human and animal husbandry. Unfortunately, the thought of any infectious disease is curable with antibiotics has encouraged people to abuse the use of antibiotics ever since the discovery of Penicillin. A selective pressure which act as driving force is asserted on the microorganisms, allowing them to evolve and alter their genetic materials to overcome the antimicrobial medicine administered. Hence, more microorganisms are becoming antimicrobial resistant especially towards two or more of current antibiotics-Multidrug Resistant (MDR). This global burden of latent MDR bacteria has caused disastrous impacts on the environmental bacteria particularly from river water and soil when the resistance genes are spread through discharges of hospital effluents, municipal wastes, sewage discharges, industrial, agricultural and farm wastes. The highly polluted ecosystem serves as reservoir for MDR bacteria transmission, facilitating intraspecies and interspecies horizontal gene transfer among the bacteria. From the studies, it is shown that the common MDR bacteria present in river water is mostly gram-positive *Staphylococcus spp*., wide range of MDR gram negative such as *Enterobacteriaceae*, *Aeromonas*, *Flavobacteriia*, *Acinetobacter*, *Pseudomonas* spp. and etc. where common MDR bacteria present in soil includes *Morganella morganii* and *Bacillus cereus*. These MDR bacteria commonly resistant to current antibiotics including Vancomycin, Tetracycline and Cephalosporins. As irresponsibility practices and lack of awareness from human being is the primary cause for the emergence of MDR bacteria in environment, education on the appropriate use antibiotics; proper management of treatment plant and disposal of wastes should be encouraged

in conjunction with enforcement of relative laws and policies to combat the MDR crisis at global level.

Keywords: multidrug resistant bacteria, antibiotics, *Enterobacteriaceae*, *Aeromonas*, *Flavobacteriia*, *Acinetobacter*, *Pseudomonas* spp.

Introduction

Antibiotics is one of the most successful breakthrough of therapy in medical history (Lin, *et al.*, 2015). Dating back to the discovery of penicillin in 1928 followed by production of many other antibiotics, antibiotics have been widely used to treat not only the deadly infections of human but also ensuring the welfare of animal husbandry and veterinary medicine. Unfortunately, people has been taking granted that any infectious disease is curable with treatment of antibiotics. There is an estimation of nearly 100,000 tons of antibiotics are produced annually worldwide (Economou and Gousia, 2015). The extensive and inappropriate use of antibiotics without restrictions has caused a profound impact on the life of bacteria on earth, resulting in the rise of antimicrobial resistance. Some are even resistant to more than one type of antibiotics and chemotherapeutic agents. This phenomenon has been known as multidrug resistance (Nikaido, 2009).

Bacteria is indicated as Multidrug Resistant (MDR) when a microorganism is insensitive or resistant to two or more of the given antimicrobial drugs (antibiotics) that are structurally unrelated and have different molecular targets despite earlier sensitivity to it, compromising the efficiency of the drugs to treatment of infections (Tanwar, *et al.*, 2014; Nikaido, 2009). The growing number of MDR bacteria is a wreaking havoc especially in people with common human infections leading to hospitalizations and even deaths. This has caused not only an elevation in the morbidity and mortality rates as well as the increase in treatment costs (Lin, *et al.*, 2015). Thus, MDR bacteria is well-recognized as "one of the primary human threats to public health worldwide" by both The Infectious Diseases Society of America (IDSA) and World Health Organization (WHO) who declared this critical situation as "combat drug resistance: no action today, no cure tomorrow" (Basak, *et al.*, 2015; Diun and Paterson, 2017). According to US Centers for Disease Control and Prevention (CDC), around two million of US people are infected by MDR bacteria, attributed to 23,000 deaths annually and an estimated of 700,000 deaths worldwide. In 2050, the number of deaths worldwide that are caused by MDR bacteria are expected to increase by another 10 million (Adrizain, *et al.*, 2018).

One of the most common MDR bacteria is methicillin-resistant *Staphylococcus aureus* (MRSA), which not only resistant to methicillin that was developed to fight against penicillinase-producing *S. aureus* but also aminoglycosides, macrolides, chloramphenicol, tetracycline, lincosamides and even disinfectants. Thus, MRSA has been recognized as a major source of hospital-acquired infections. Experts from worldwide are alarmed when a more serious threat has arisen, that is the emergence of gram-negative MDR bacteria especially the extended-spectrum beta-lactamase (ESBLs)-producing bacteria (Nikaido, 2009; Medina, 2016). According to the report from WHO, a critical rise in number of gram-negative MDR has been observed especially *Escherichia coli* (*E. coli*) which resistant to antibiotics such as cephalosporin and fluoroquinolones; *Klebsiella pneumoniae* (*K. pneumoniae*) resistant to cephalosporin and carbapenems; Nontyphoidal *Salmonella* and *Shigella* species which resistant to fluoroquinolones. Other MDR bacteria involved are *Streptococcus pneumoniae* (*S. pneumoniae*) which against penicillin, *Neisseria gonorrhoeae* (*N. gonorrhoeae*) against cephalosporin, and *Mycobacterium tuberculosis* (*M. tuberculosis*) that against isoniazid, rifampicin and fluoroquinolone. All of these MDR bacteria are responsible for common human infections such as urinary tract infections, bloodstream infections, pneumonia, and high percentage of hospital-acquired infections (Table 1) (Tanwar, *et al.*, 2014).

Table 1: MDR bacteria in common infections and the drugs resistant to (Nikaido, 2009; Tanwar, *et al*., 2014)

Name of Bacterium	Drug(s) resistant to	Infections Caused
Staphylococcus aureus	Methicillin, aminoglycosides, macrolides, chloramphenicol, tetracycline, lincosamides	Wound and blood stream infections
Escherichia coli	Cephalosporins and fluoroquinolones	Urinary tract infections and blood stream infections
Klebsiella pneumoniae	Cephalosporins and carbapenems	Pneumonia, blood stream, and urinary tract infections
Nontyphoidal Salmonella	Fluoroquinolones	Foodborne diarrhea, blood stream infections
Shigella species	Fluoroquinolones	Diarrhea (bacillary dysentery)
Streptococcus pneumoniae	Penicillin	Pneumonia, otitis and meningitis
Neisseria gonorrhoeae	Cephalosporins	Gonorrhoea
Mycobacterium tuberculosis	Isoniazid, rifampicin and fluoroquinolone	Tuberculosis

Mechanism of MDR

Bacteria responsible for the common infections can become multidrug resistant when the patients have repeated drug exposure, resulting in the evolution of bacteria to overcome the effectiveness of drugs. The survived bacteria with mutated genes can transmit the resistant genes to other bacteria, increasing number of drug resistance (Nikaido, 2009; Tanwar, *et al.*, 2014).

There are generally two mechanism for the generation of MDR bacteria. First, MDR bacteria accumulate multiple genes coding for resistance to an antibiotic by undergoing chromosomal mutations or exchange of extrachromosomal DNA elements such as conjugation or transformation (horizontal gene transfer). This accumulation generally occurs in resistance (R) plasmids. Exchange of genetic elements or mutations alters the cell membrane composition which in turn decrease the permeability and uptake of drugs into the cell (Nikaido, 2009; Tanwar, *et al*., 2014).

Another mechanism for the generation of MDR bacteria is by increasing the expression of genes that code for multidrug efflux pumps which can actively extrude a wide range of drugs administered (Nikaido, 2009). This mechanism is considered as the predominant mechanism of MDR. The overexpression of genes encoding for multidrug efflux pumps such as ATP-binding cassette (ABC) transporter membrane proteins which responsible for the export or expulsion of drugs out of the cell, affects the fluidity and permeability, leading to an ATP-dependent efflux of the antimicrobials and decreasing their intracellular concentration. Hence, making the antibiotics ineffective (Tanwar, *et al.*, 2014). Another common example seen is the coding of Resistance-Nodulation Division (RND) superfamily pumps by chromosomal genes which is overly expressed in gram-negative bacteria and caused a high resistance in most of the current use antibiotics (Nikaido, 2009).

Emergence of MDR bacteria in environment

According to studies, the emergence of wild-type resistance in bacteria are no longer restricted in antibiotic abuse for livestock breeding and medicine when the resistance genes are spread into environmental bacteria. There has been frequent reports indicating occurrence of many MDR bacteria and their resistant genes in environment such as river water, sewage, treated drinking water, soil and even air. The main source for the dissemination of resistant genes in environment is pollution caused by discharges of wastes including hospital effluents, municipal or residential wastes, sewage discharges, agricultural, industrial and farm wastes. These discharge of wastes will disrupt the environment quality and favoring the proliferation of MDR bacteria (Founou, *et al.*, 2018; Shi, *et al.*, 2013; Yuan, *et al.*, 2015).

Hospital effluents which contains 8 times more antibiotic resistant bacteria than domestic discharges and a particularly high antimicrobial selective pressure are the ultimate source of MDR bacteria especially ESBL-producing *Escherichia coli*, vancomycin-resistant Enterococci and *Pseudomonas aeruginosa* (Hocquet, *et al.*, 2016). Also, a study from Vaz-Moreira, *et al*. (2016) shown that an ubiquitous bacterial groups of MDR Aeromonas, Acinetobacter, Citrobacter, Enterobacter, Flavobacteriia, Klebsiella, and Pseudomonas have been detected in samples of hospital effluents. When disseminated to the environments, they become important vectors for multidrug resistance. For example, a study of wastewater soil of hospital in Aizawl, Mizoram, India has identified MDR bacteria including *Morganella morganii* and *Bacillus cereus* that are very resistant to wide range of antibiotics such as chloramphenicol and ciprofloxacin (Hauhnar, *et al.*, 2018). Also, according to Skariyachan, *et al.* (2013) and Berglund (2015), many studies have determined presence of high number of MDR bacteria in river water due to discharge of hospital effluents especially bacteria from group *Enterobacteriaceae* which strongly resistant to most of the Cephalosporins antibiotics while gram-positive *Staphylococcus* spp. showed resistance to vancomycin, methicillin, oxacillin and tetracycline. This highly polluted aquatic ecosystem serves as reservoir for MDR bacteria transmission where selective pressure will promote intraspecies and interspecies horizontal gene transfer (Hocquet, *et al.*, 2016).

Due to the variety of bacteria, nutrients and antibiotics mixtures, municipal or residential wastes water is also a favorable reservoir for both the survival and transfer of bacterial resistance. This is mostly due to urbanization and crowded population that results in high amount of municipal wastes discharged from residential areas. According to studies of river in Dhaka, one of the densely populated cities in the world, bacteria with very high antibiotic resistance have been determined including *Pseudomonas aeruginosa* which is found 100% resistant to Ampicillin (AMP) followed by 93.7% to both Tetracycline and Gentamycin and 71.8% to Co-trimoxazole (Nasreen, *et al.*, 2015). Similar studies performed by Haque, *et al.* (2014) in surface water of Dhaka river also high number of ESBL-producing *Enterobacteriaceae* such as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* with *E. coli* found to be the most prevalent among isolated from environmental water.

Urbanization also results in large amount of sewage discharges into the environment. According to study of Sidrach-Cardona, *et al.* (2014), abundance and spatial dynamics of MDR fecal bacteria such as *Escherichia coli*, *Enterococcus spp*. and *Total Coliforms* were determined in river samples

affected with antibiotic production plant and wastewater treatment plant discharges. 100% of bacterial isolates were resistant to penicillin and erythromycin and containing either blaTEM, blaCTX-M and blaSHV genes. Also, a study focuses on microbial pollution and antibiotic sensitivity profiling from River Cauvery, a major drinking water source in Karnataka, India has identified large number of fecal coliforms, 93.51% (n = 793) that are multidrug-resistant to most of the current use antibiotics predominantly *Escherichia coli* 96.46% (n = 273), followed by *Enterobacter cloacae*, 93.85% (n = 107), *Pseudomonas trivialis*, 94.49% (n = 103) and *Shigella sonnei*, 90.22% (n = 157) with high prevalence of blaTEM and dhfr genes. Other MDR gram-positive bacteria identified including methicillin resistant *Staphylococcus* spp. and vancomycin-resistant *Staphylococcus aureus* (Skariyachan, *et al.*, 2015).

Another source that promote the emergence of MDR bacteria in environment is discharge of farm wastes especially animal farms. The animals are usually fed with antibiotics and corresponding medicine to treat animal diseases and increase production rate. This results in a high number of MDR bacteria in animal. Also, to save cost most of the responsible parties will just discharge the wastes such as animal urine and feces that contains MDR bacteria into the environment particularly soil and river water due to convenience. This facilitates the dissemination of resistant genes and increase number MDR bacteria in environment predominantly ESBL-producing *Escherichia coli*, *Salmonella spp*. and *Shigella* spp. (Loh, *et al*., 2018). On the other hand, according to studies performed by Harnisz, *et al.* (2015) in water of Drwęca River, it is found that the discharges from a fish farm has also altered the water quality in the Drwęca River and caused a rise in diversity of tetracycline-resistance genes. Bacteria of genera *Aeromonas spp.* and *Acinetobacter spp.* shown to transfer 6 out of 13 tested tet genes into *Escherichia coli* in river water, promoting the spread of antibiotic resistance in the environment.

From the studies, it is well-recognized that the critical emerging of MDR bacteria and resistant genes are indisputable facts. From the increase of MDR gram-positive *Staphylococcus spp.* to wide range of MDR gram negative such as *Enterobacteriaceae*, *Aeromonas*, *Flavobacteriia*, *Acinetobacter*, *Pseudomonas spp*. and etc., the primary cause for this global health threat is none other than the irresponsibility practices and lack of awareness from human being. Not only are more bacteria getting multidrug resistant, the rise of many "Superbugs" nowadays has raise the urgency of need for new antibiotics. However, looking for cure is not the best way to solve the problem,

preventive measure must be taken. Other than organizing various awareness programs to educate the appropriate use antibiotics, there must be proper management of treatment plant and disposal of wastes especially from hospital parties. Appropriate laws and policies should be enforced upon the matter. With all the cooperative and effective actions taken at global level, there is hope to combat the MDR crisis in the future.

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Chapter - 2
Applications of RNA Interference (RNAi) Technology

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Chapter - 2

Applications of RNA Interference (RNAi) Technology

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Abstract

Ribonucleic acid interference (RNAi) is a naturally occurring phenomenon in living cells, whereby the introduction of double stranded RNA (dsRNA) into the cytoplasm of the cell results in the specific and efficient degradation of complementary mRNA and, therefore, reduced protein production. The discovery of RNAi has resulted in an explosion of research to understand and exploit it. The technique is now established via *in vitro* systems, and much work is focussed in adapting RNAi for *in vivo* application. The potential of the technology in understanding physiological and pathological processes is significant, while its development as a therapeutic agent holds much promise as targeted agents. RNAi is also being studied for applications regarding the management of pests and the control of plant diseases.

Keywords: RNA interference, RNAi, dsRNA, miRNA

Introduction

Ribonucleic acid interference (RNAi) is a naturally occurring intracellular mechanism, which causes sequence specific post-transcriptional gene silencing. The reaction is triggered by the introduction of double-stranded RNA (dsRNA) into the cytoplasm of the cell, and results in the specific targeted destruction of mRNA and a subsequent reduction in protein production [1]. When successfully manipulated, RNAi can result in the knockdown of single or multiple genes, so providing a quick and convenient method of analyzing gene function [2]. RNAi libraries have been developed as a useful screening method for assessing functional consequences of inhibiting multiple proteins within given pathways [3].

The natural role of RNA interference is thought to be the protection from invasion by viral pathogens[4]. On infection, RNA viruses generate double-stranded RNA (dsRNA) molecules either in activation or replication, and it is these molecules that are capable of activating the host RNAi

defence mechanism. This results in the specific degradation of the viral RNA, so preventing viral multiplication [5]. This phenomenon is highlighted by plants defective in RNAi demonstrating hypersensitivity to viral infection [6].

RNAi occurs in plants, animals, and humans. It is of great importance for the regulation of gene expression, participates in defence against viral infections, and keeps jumping genes under control. RNA interference is already being widely used in basic science as a method to study the function of genes and it may lead to novel therapies in the future [7].

Discovery of RNA interference

When researchers attempted to darken petunia flowers by over-expressing chalcone synthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS, they had some unexpected results. The introduced gene created a block in anthocyanin biosynthesis. Forty-two percent of plants with the introduced CHS gene produced totally white flowers and/or patterned flowers with white or pale non-clonal sectors on a wild-type pigmented background. None of the hundreds of transgenic control plants exhibited such phenotypes. The mechanism responsible for the reversible co-suppression of homologous genes in transgenic plants was unclear [8].

This mechanism was later uncovered when researchers, Andrew Fire and Craig Mellow published the results of their research work in 1998. They had been investigating the expression of genes in the nematode worm, *Caenorhabditis elegans*. Injecting mRNA molecules encoding a muscle protein led to no changes in the behaviour of the worms. The genetic code in mRNA is described as being the ‘sense’ sequence, and injecting ‘antisense’ RNA, which can pair with the mRNA, also had no effect. But when they injected sense and antisense RNA together, they observed that the worms displayed peculiar, twitching movements (Figure 1). Similar movements were seen in worms that completely lacked a functioning gene for the muscle protein. When sense and antisense RNA molecules meet, they bind to each other and form double-stranded RNA. It could be that such a double-stranded RNA molecule silences the gene carrying the same code as this particular RNA.

They tested this hypothesis by injecting double-stranded RNA molecules containing the genetic codes for several other worm proteins. In every experiment, injection of double-stranded RNA carrying a genetic code led to silencing of the gene containing that particular code. The protein

encoded by that gene was no longer formed. After a series of experiments, they deduced that double-stranded RNA can silence genes, that this RNA interference is specific for the gene whose code matches that of the injected RNA molecule, and that RNA interference can spread between cells and even be inherited [9]. It was enough to inject tiny amounts of double-stranded RNA to achieve an effect, and therefore, it was proposed that RNA interference (now commonly abbreviated to RNAi) is a catalytic process. Their discovery clarified many confusing and contradictory experimental observations and revealed a natural mechanism for controlling the flow of genetic information. This heralded the start of a new research field. The Nobel Prize in Physiology or Medicine for 2006 was jointly awarded to Fire and Mello for their discovery of RNA interference or gene silencing by double-stranded RNA, a fundamental mechanism for controlling the flow of genetic information [7].

RNAi machinery

The components of the RNAi machinery were identified following the discovery of RNAi. RNA interference takes place predominantly within the cytoplasm of the cell and is triggered by the introduction of a double-stranded oligonucleotide into the cell cytoplasm (Figure 2). The mechanism is mediated by the activation of 2 major molecules; the initial activity of the endonuclease Dicer (an RNase III family enzyme), followed by the activity of the RNA interfering silencing complex (RISC) [10]. An adenosine triphosphate (ATP) dependent reaction involving the endonuclease Dicer is responsible for cleaving the long ds nucleotide into short interfering (si) double-stranded RNAs, 21-23 nucleotides in length. RISC then unwinds the double-stranded siRNA, using a helicase, and subsequently binds to the free antisense strand. This complex is able to identify the specific complementary strand of mRNA and degrades it with the help of one of its major components, Argonaute 2 protein [11-12]. The result is destruction of the mRNA that is complementary to the antisense strand of the original dsRNA introduced into the cytoplasm, and prevention of translation and protein production [13].

As a result of numerous experiments, artificial manipulation of RNAi in cultured mammalian cells has been possible and its full potential for use in vivo hypothesized. It has prompted the need for mass production of synthetic siRNAs. The field of RNAi is still rapidly expanding and new discoveries are being made regularly. siRNAs are currently being used in gene function analysis, target identification and validation and as therapeutic agents. Their potential for use in evaluating target toxicity is significant. Although a viable

technique for in vitro experimentation, success can still be hampered by problems with intracellular siRNA delivery and effective gene silencing [13].

Regulation of gene expression by RNAi

Transcription and translation are the two major steps of gene expression. Any gene (DNA) that encodes a protein first synthesizes an mRNA. The code contained in this mRNA is then translated by a ribosome to synthesize the particular protein. RNA interference is used to regulate gene expression in eukaryotic cells. Hundreds of genes in the human genome encode small RNA molecules called microRNAs (miRNA). They contain pieces of the code of other genes. Such a miRNA molecule can form a double-stranded structure and activate the RNA interference machinery to block protein synthesis. The expression of that particular gene is silenced. It is now understood that genetic regulation by miRNAs plays an important role in the development of the organism and the control of cellular functions [7].

siRNA transfection methods

Whether *in vitro* or *in vivo*, the RNA interference mechanism can only be initiated once the siRNA has been transported into the cytoplasm of the cell. A successful transfection requires that the siRNA molecule, which carries a net negative charge under normal physiological conditions, must come into contact with and cross a cell membrane that also carries a net negative charge. Several methods of siRNA transfection have been developed for use *in vitro* and *in vivo* [13].

***In Vitro* Transfection**

Naked Delivery: The most basic approach involves the direct addition of naked siRNA to the cells without the use of a transfection reagent. High levels of cytotoxicity associated with methods using naked delivery means that they are generally reserved for the more difficult to transfect cell lines.

Chemical Transfection: Chemical transfection reagents are the most commonly used siRNA transfection vehicle, and of these cationic liposomes (CL) are the most popular.

Vector-Mediated Delivery: DNA-vector mediated, and virus-vector mediated mechanisms have been developed. The siRNA expressing vector works as a platform to produce a large amount of siRNA for a relatively long period. Retrovirus vectors have also been developed, and these use either oncoretrovirus or lentivirus vectors [13].

In vivo transfection

Hydrodynamic Delivery of siRNA: This introduces the nucleic acid via the intravascular route at high speed and volume, resulting in greater levels of transfection with a more diffuse distribution.

Chemical Modifications Enhancing Transfection *in vivo*: Transfection reagents are also a widely experimented technique for in vivo delivery of siRNA. Similar compounds developed for in vitro use have been trialled in vivo. Plasmid vectors have also been used for in vivo siRNA delivery. However, the relative ease of plasmid regeneration did not compensate for general poor transfection efficiency of the plasmid based vectors, and prompted the development of viral-/retroviral-based vectors for small hairpin RNA (shRNA) delivery [13].

Applications of RNAi technology

RNAi in pharmaceutical target validation and toxicology

Small interfering RNA (siRNA) has the potential to provide an early, specific, and relatively inexpensive method for studying a pharmacological mechanism in both preclinical models of efficacy and toxicity. This could result in reduction in mechanisms with efficacy and safety issues being progressed into late stages, diverting resource into more productive targets. In particular, the use of siRNA in toxicity screening has the potential to reduce the number of animals used in toxicity testing [13].

RNAi in mechanistic pathology

The understanding of pathogenesis of lesions frequently relies on the testing of generated hypotheses in vivo. This usually includes the exaggerated action or inhibition of cellular pathways via the over-expression or inhibition of cellular proteins. RNAi offers the potential for rapid development of the specific inhibition of protein expression in vivo to either mimic pathological findings or to investigate the toxicity of administered compounds in animals devoid of pathways suspected of initiating pathological findings [13].

RNAi in animal models of disease

Human diseases may be modelled in animals by the inhibition of key regulatory proteins. RNAi has the potential to produce rapid and cost effective models of human disease, especially for proteins whose inhibition in the embryo has significant effect on viability, precluding the use of gene knockout technology. This potential has been demonstrated by the recent use of adenovirus to introduce siRNA specifically targeting tyrosine hydroxylase

mRNA within neurones of the mid brain. This is a key enzyme in the production of dopamine, a molecule that is involved in regulating food intake, addiction, and movement control[14]. These animals are a model of Parkinson's disease [13].

RNAi as a therapeutic agent

There has been much research in animals into the potential of RNAi as a therapeutic. Initially, the prevention of liver disease was attempted by targeting genes linked to apoptosis control in the liver in two models of autoimmune hepatitis. siRNA was directed against caspase [15] and Fas [16] and was delivered hydrodynamically via the tail and portal vein respectively. In both cases this was successful in reducing hepatocyte necrosis and inflammation, and protected the mice from future chronic fibrosis.

In another experiment siRNA targeting Fas in the kidney was used to reduce ischaemia-reperfusion injury. Hydrodynamic and normal volume intrarenal delivery both successfully reduced Fas protein expression 4-fold. In addition, the pathology of the renal tissue following the treatment was reported to be much improved compared to the control [17]. Following this success one of the first examples of using RNAi to inhibit viruses in vivo was then attempted. Immunocompromised and immunocompetent mice were hydrodynamically injected with plasmids expressing hepatitis B virus (HBV) and shRNAs targeting HBV. This resulted in a 99% reduction in HBV detection using antibodies to detect HBV core antigen by Immunohistochemistry methods, and it was suggested that RNAi could be used to treat viral disease in the future18.

Since then, several experiments using RNA interference to target respiratory viruses have been attempted. Initially Influenza virus was chosen due to its significant public health issues and lack of a wholly effective vaccine[19]. Proteins were targeted that are highly conserved across several sub types of influenza and which are essential for viral replication. It was found that combined iv hydrodynamic and intranasal (in a lipid carrier) delivery was most effective at specifically inhibiting virus replication at the site of infection. It also reduced lung virus titres in infected animals and protected animals against lethal challenge. Another experiment using slow iv delivery of siRNA complexed with a polycation carrier, and its delivery using DNA vectors iv/intra nasally, also showed a dose dependant reduction in virus production [20].

Intranasal delivery was also found to be effective when targeting parainfluenza virus and Respiratory Syncytial virus. Administration of

siRNA with and without a vector (Transit TKO) was found to be both protective and therapeutic [21].

The ability to induce RNAi across mucosal surfaces is also being explored as a means of treating sexually transmitted disease. Intravaginal delivery of RNAi targeting two viral genes have been shown to protect the mice from the otherwise lethal Herpes simplex virus-2 [22].

RNAi has also been used to alleviate joint inflammation in experimental animals. siRNA targeting tumour necrosis factor alpha was injected into the knee joints of mice with collagen induced arthritis (CIA). This was followed by electroporation. The development of arthritis was scored by assessing the inflammation of joints in the mouse paw, and in mice with CIA, joint inflammation was successfully inhibited [23]. Finally, diminished pain responses have been observed in rats following intrathecal delivery of an siRNA directed against the pain related cation channel P2X3 [24]. Today, the use of RNAi as a therapeutic agent is of the greatest commercial interest [13].

RNAi technology in pest management

In RNAi-based crop protection, double-stranded RNA (dsRNA) is taken up by the target pest (e.g., through ingestion) and subsequently directs the degradation of specific, pest mRNA. Degradation of this mRNA prevents the synthesis of essential proteins in the pest, resulting in reduced pest growth or leading to pest mortality. RNAi biotechnology has been shown to be effective against an array of agricultural pests including insects and nematodes. The first commercially available RNAi-based product targeting an insect pest is genetically engineered maize that expresses a dsRNA pesticide within the plant's tissue (plant-incorporated protectant). However, RNAi based crop protection can also be achieved by applying exogenous dsRNA to crops [25].

Since the discovery of RNAi, research on the application of RNAi technology for insect pest management has led to great advancements. Two studies successfully demonstrated that insects feeding on transgenic plants engineered to produce specific dsRNA showed the suppression of the target gene expression, which led to increased mortality of the cotton bollworm, *Helicoverpa armigera* [26] and the western corn rootworm, *Diabrotica virgifera* [27]. RNAi technology provides a new avenue for insect pest management and has been applied to many insect groups including Diptera [28-29].

Aphids are one of the most economically important sap-sucking insect pests worldwide. There are over 4000 species that cause yield and financial

losses both from direct damage by feeding, thereby draining essential nutrients from the plant, and as major vectors for disease, transmitting over fifty percent of all plant viruses. Their fast reproduction rate involving parthenogenesis results in rapid development of resistance against different classes of insecticides. This makes it difficult to control them and also increases the cost of managing them. Bluegreen aphid, *Acyrthosiphon kondoj* is a major legume pest worldwide with particularly widespread distribution in North-America, Asia and Oceania. Researchers have described an artificial diet for rearing bluegreen aphid and also provide a proof of concept for the supplementation of the diet with RNAi molecules targeting the salivary gland transcript C002 and gap gene hunchback, resulting in bluegreen aphid mortality. Managing this pest via RNAi delivery through artificial feeding will be a major improvement to test bluegreen aphid candidate target genes for future pest control and gain significant insights into bluegreen aphid gene function [30].

Yellow stem borer (YSB), *Scirpophaga incertulas* is one the most destructive pests of rice and causes severe yield loss. Of the many approaches for managing insect pests, RNA interference (RNAi) is a viable option which can be employed by targeting the key insect genes that are involved in host-pest interactions. In a study, Acetylcholinesterase (AChE), a key gene of YSB was targeted for silencing through RNAi approach. The efficacy of dsRNA was evaluated through a systematic insect bioassay. Larval growth and mortality were observed for a period of fifteen days after the treatment through the cut stem assays. The reduced larval length and weight were observed in the dsRNA treated samples which were correlated with the low gene expression compared to the untreated controls. These results suggested that AChE is a potential target for RNAi approach in insects [31].

Phenacoccus solenopsis, commonly known as solenopsis mealybug, is a polyphagous pest, which infests major food and fibre crops in India. Twenty three genes involved in RNAi machinery identified through BLASTx search against NCBI nr database suggested the existence of robust RNAi in mealybug. RNAi in *P. solenopsis* was demonstrated through knockdown of IAP (Inhibitor of Apoptosis), AQP (Aquaporin), CAL (Calcitonin), V-ATPase (V-type proton ATPase subunit F 1), bursicon, chitin synthase, SNF7 and α-amylase by injecting sequence specific dsRNA of respective genes in adult female. Additionally, feeding RNAi was demonstrated in 2nd instar nymph through dsRNA uptake in plant. The knockdown of core RNAi machinery genes such as *Dicer*, *Argonaute* and *Staufen* significantly hampered RNAi efficiency in this insect [32].

Root-knot nematodes (RKNs) are devastating parasites that infect thousands of plants. RKN infection is facilitated by oesophageal gland effector genes. One of such effector genes is Mi-msp2. Based on domain analysis, the Mi-msp2 protein contains a ShKT domain, which is likely, involved in blocking potassium channels and may help in evading the plant defence response. Studies demonstrated that expression of the Mi-msp2 gene was higher in juveniles (parasitic stage of RKNs) than in eggs and adults. Stable homozygous transgenic *Arabidopsis* lines expressing Mi-msp2 dsRNA were generated, and the numbers of galls, females and egg masses were reduced by 52-54%, 60-66% and 84-95%, respectively, in two independent RNAi lines compared with control plants. Furthermore, expression analysis revealed a significant reduction in Mi-msp2 mRNA abundance (up to 88%) in female nematodes feeding on transgenic plants expressing dsRNA, and northern blot analysis confirmed expression of the Mi-msp2 siRNA in the transgenic plants. Interestingly, a significant reduction in the reproduction factor was observed (nearly forty fold). The data suggest that the Mi-msp2 gene can be used as a potential target for RKN management in crops of economic importance [33].

The RNAi mechanism works at the mRNA level by exploiting a sequence-dependent mode of action with high target specificity due to the design of complementary dsRNA molecules, allowing growers to target pests more precisely compared to conventional agrochemicals. The delivery of RNAi through transgenic plants is now a reality with some products currently in the market. Conversely, it is also expected that more RNA-based products reach the market as non-transformative alternatives. For instance, topically applied dsRNA/siRNA (SIGS-Spray Induced Gene Silencing) has attracted attention due to its feasibility and low cost compared to transgenic plants. Once on the leaf surface, dsRNAs can move directly to target pest cells (e.g., insects or pathogens) or can be taken up indirectly by plant cells to then be transferred into the pest cells. Water-soluble formulations containing pesticidal dsRNA provide alternatives, especially in some cases where plant transformation is not possible or takes years and cost millions to be developed (e.g., perennial crops). The ever-growing understanding of the RNAi mechanism and its limitations has allowed scientists to develop non-transgenic approaches such as trunk injection, soaking, and irrigation. While the technology has been considered promising for pest management, some issues such as RNAi efficiency, dsRNA degradation, environmental risk assessments, and resistance evolution still need to be addressed [34].

RNAi technology for *Aedes* mosquito control

Zika virus infection and dengue and chikungunya fevers are emerging viral diseases that have become public health threats. Their causative agents are transmitted by the bite of genus *Aedes* mosquitoes. Without effective therapies or vaccines, vector control is the main strategy for preventing the spread of these diseases. Increased insecticide resistance calls for novel strategies focused on control of the target vector population. The chitin required for larval survival structures is a good target for RNAi control. Chitin synthases A and B (CHS) are enzymes in the chitin synthesis pathway. Double-stranded RNA (dsRNA)-mediated gene silencing (RNAi) achieves specific knockdown of target proteins. A new RNAi-based bioinsecticide was developed as a potential strategy for mosquito population control. dsRNA molecules that target five different regions in the CHSA and B transcript sequences were produced *in vitro* and *in vivo* through expression in *E. coli* HT115 and tested by direct addition to larval breeding water. Mature and immature larvae treated with dsRNA targeting CHS catalytic sites showed significantly decreased viability associated with a reduction in CHS transcript levels. The few larval and adult survivors displayed an altered morphology and chitin content. In association with diflubenzuron, this bioinsecticide exhibited insecticidal adjuvant properties [35].

RNAi technology for managing plant diseases

A strategy to control fungal diseases is the host-induced gene silencing (HIGS) that has been used in transgenic plants to knockdown genes of several plant fungal pathogens such as *Blumeria graminis*, *Fusarium* spp., *Sclerotinia sclerotiorum*, *Puccinia striiformis*, *Aspergillus* spp. and *Magnaporthe oryzae*. The RNAi gene silencing technology has been successfully used to target several economically important plant viruses, such as barley yellow dwarf virus (BYDV), banana bract mosaic virus (BBrMV), bean golden mosaic virus (BGMV), potato virus Y (PVY), sugarcane mosaic virus (SCMV), cucumber green mottle mosaic virus (CGMMV), tomato yellow leaf curl virus (TYLCV), and rice tungro bacilliform virus (RTBV). Although most of the previous works have reported RNAi silencing-mediated resistance only against plant RNA viruses, it is worth to mention here that previous experiments have shown that this strategy is also effective in engineering resistance to DNA viruses [36].

Crown gall is a common plant disease caused by the soil-borne bacterium *Agrobacterium tumefaciens*. Crown gall disease management

strategy has been developed that targets the process of tumourogenesis (gall formation) by initiating RNAi of the iaaM and ipt oncogenes. Expression of these genes is a prerequisite for wild type tumour formation Transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* transformed with RNAi constructs, targeting iaaM and ipt genes showed resistance to crown gall disease. Transgenic plants generated through this technology contained a modified version of these two bacterial genes required to cause the disease and this was the first report about the management of a major bacterial disease through RNAi. The extra genes recognize and effectively shut down the expression of the corresponding bacterial gene during infection, thus preventing the spread of infection. The incoming bacteria could not make the hormones needed to cause tumours and plants deficient in silencing were hyper-susceptible to *A. tumefaciens* [37].

Agricultural applications of dsRNAs involve intrinsic problems in mass production (cost), delivery to the action site (survival), cell uptake, capture of target RNA and off-target side effects. Exogenous dsRNAs seem to be an excellent option against viral diseases. However, before they can be considered on a commercial basis, it is necessary to remove the intrinsic problems involved in their application. As per as cost is concerned, it is now possible to produce dsRNAs for about US $2 per gram, and spraying is predicted to require a minimum of 2gm per hectare, indicating that the goal for commercial use of dsRNAs is approaching. Considering such technological advancements for dsRNAs, it is safe to believe that they will become an efficient antiviral agent for the next generation [38].

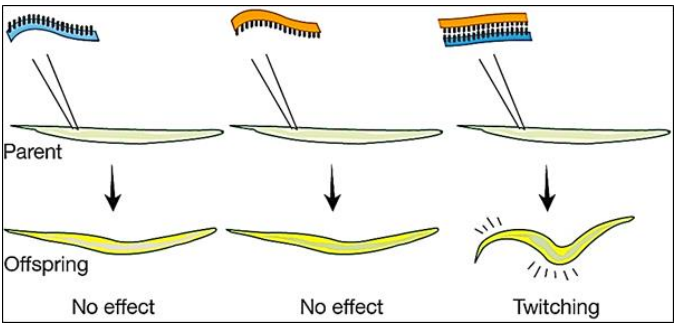

Fig 1: The experiment by Fire and Mello. RNA carrying the code for a muscle protein is injected into the worm C. elegans. Single stranded RNA has no effect, but when double stranded RNA is injected, the worm starts twitching in a similar way to worms carrying a defective gene for the muscle protein. From https://wwn .nobelprize.org/prizes/meclicine/2006/press-release/

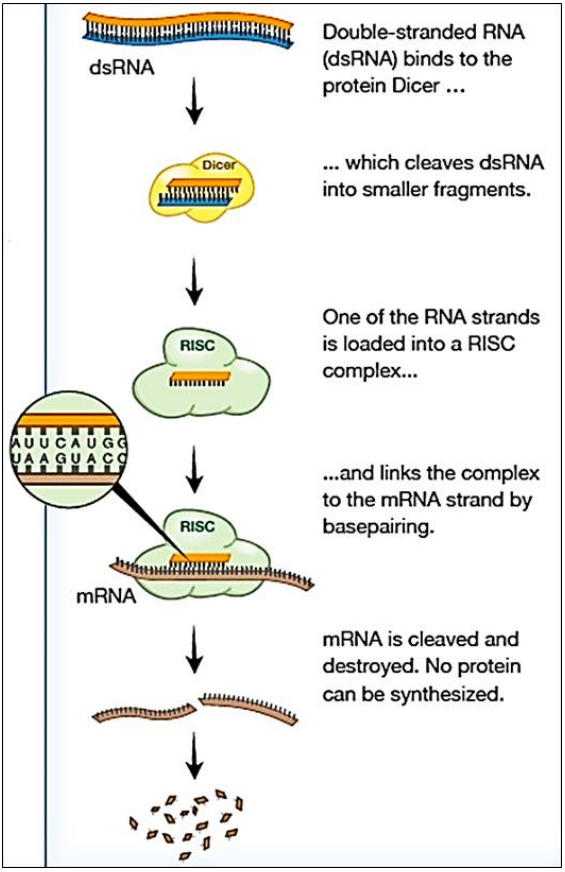

Fig 2: The RNAi Mechanism From https://www.nobelprize.org/prizes/medicine/2006/press-release/

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Chapter - 3
Antidiabetic and Biochemical Properties of Ethanolic Leaf Extract of Aegle Marmelos and Seeds Extract of Fenugreek in Alloxan-Induced Diabetic Mice

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Chapter - 3

Antidiabetic and Biochemical Properties of Ethanolic Leaf Extract of Aegle Marmelos and Seeds Extract of Fenugreek in Alloxan-Induced Diabetic Mice

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Abstract

Diabetes is a major metabolic disease of global concern. Ethanolic extract of A. Marmelos leaf and Seeds of Fenugreek were investigated for antidiabetic as well as other biochemical parameters activity in alloxan-induced diabetic mice. Mice (Swiss Albino) with body weight 25±35g were randomly selected into thirteen groups. Except Group I all other groups were made diabetic by administration of alloxan monohydrate (150 mg/kg, body weight i.p). Group II was diabetic untreated, group XIII was diabetic and treated with glibenclamide, while groups II to VII received the ethanolic extract of A. marmelos leaf at a dose of100, 200 mg/kg, 300 mg/kg 400 mg/Kg and 500 mg/kg body weight respectively and group VIII-XII received the ethanolic extract of Seeds of T. Foenum Graecum at a dose of 100 mg/kg, 200 mg/kg, 300 mg/kg 400 mg/Kg and 500 mg/kg body weight respectively. Phytochemical screening showed the presence of flavonoids, tannins, saponins, phlobatannins, anthraquinones, phenol and cardiac glycoside and saponin. The blood glucose of the alloxanized rats after 72 hours which ranged above 250 mg/dL were significantly (p<0.05) and progressively reduced in treated groups which compared favorably with the standard drug group. The significantly (p<0.05) elevated levels of serum blood glucose, reduced levels of body weight, (SGOT and SGPT), urea, creatinine, total cholesterol, VLDL-C, LDL-C, as well as reduced levels of total protein, albumin and HDL-C in the diabetic untreated mice were normalized upon treatment with ethanolic extract of both Plants. These results suggest that the ethanolic extract of A. Marmelos leaf and Seeds of Fenugreek possesses anti hyperglycemic property with no major side effect hence it could be considered safe for the management of diabetes.

Keywords: antioxidants, *Aegle marmelos, Alloxan T. fenugreek,* blood glucose, diabetes

1. Introduction

Diabetes mellitus has been recognized as one of the emerging health problems worldwide because of its high prevalence, adverse clinical outcomes, marked reduction in the quality of life of patients and high healthcare costs [1-3]. It is characterized by abnormalities in carbohydrate, lipid and lipoprotein metabolism. The disease not only leads to hyperglycemia but also causes many complications such as hyperlipidemia, hyperinsulinemia, hypertension and atherosclerosis [4, 5]. Global projections suggest that more than 350 million people will have diabetes by 2030 and the cost of treating diabetes and its complications could reach more than trillion dollars annually. Accordingly, it has become an adverse public health crisis. The percentage of deaths attributable to high blood glucose or diabetes that occurs prior to age 70 is higher in low and middle-income countries than in high-income countries. The disease is characterized by high blood glucose levels and abnormal metabolism of carbohydrates, proteins, and fat associated with a relative or absolute insufficiency of insulin secretion and with various degrees of insulin resistance. Such alterations result in increased blood glucose causing a chronic state of high blood glucose level (hyperglycemia) that results from an absolute or relative insulin deficiency and is associated with long-term complications affecting the eyes, kidneys, heart and nerves [5].

The pathogenesis of diabetes mellitus is managed by insulin and oral administration of hypoglycemic drugs such as sulfonylureas and biguanides which are not without a number of side effects. Moreover, none of the oral synthetic hypoglycemic agents has been successful in diabetes management and controlling long-term microvascular and macrovascular complications [10]. The toxicity of oral antidiabetic agents differs widely in clinical manifestations, severity, and treatment [11]. Optional therapies such as herbal preparations have been used for the management of diabetes. The benefits of these herbal medications are their efficacy, endogenous relativity, cost effectiveness and tolerability [12]. Various parts of medicinal trees have been employed in the third world traditional medicinal system and most have demonstrated pre-clinical or clinical normoglycemic activity [13]. Furthermore, World Health Organization has also recommended the evaluation of traditional plant treatments for diabetes [14]. A. Marmelos (Beal) and T. Fenugreek has been reported to be effective against a variety of disease. Secondary metabolites of A. Marmelos (Beal) and T. Fenugreek had laid foundation for many medicines or food supplements currently used to treat or prevent pathologic conditions. These suggestion from literature proved that A. Marmelos (Beal) and T. Fenugreek extract are good natural sources of

bioactive compounds further they had beneficial health effects for consumption.

2. Materials and methods

Alloxan monohydrate obtained is a product of Sigma Chemical Company, St. Louis, Mo, USA. Kit for the estimation of Total Glyceride, Total protein SGOT, SGPT, urea, creatinine and albumin, were produced by Randox Laboratories Ltd., Antrim, UK. All other chemicals were of analytical grades and prepared in all-glass apparatus using distilled water (BDH, UK).

2.1 Plant extract preparation

Raw and crisp leaves of Aegle marmelos were collected from Department of Food & Biotechnology and allied science, Faculty of Engineering and Technology, J.V.W. women's university, Jaipur (Rajasthan). The seeds of fenugreek (*Trigonella foenum-graecum* L.) were procured from Jobner Research Centre, Rajasthan. On the Basis of Taxonomical features the specimen samples were identified and registered at Herbarium in Botany Department, Rajasthan University, Jaipur. Aegle Marmelos was registered as RUBL-211737 and for Trigonella Foenum-graecum it was RUBL-211738.

The collected material of medicinal plant i.e. Leaves of Aegle Marmelos and Seeds of fenugreek was treated and prepared as shown below. Leaves and seeds were thoroughly cleaned in water to remove the unwanted particles. They were shaded dried in the laboratory for 20 days then they are grinded to fine powder using grinder, the powdered leaves and seeds separately were sieved into fine powered by using fine mesh sieve using the kitchen strainer and they were used for extract preparation. The leaves about 500g were mixed carefully in a solvent by continuous stirring which is formed of 700 ml ethanol (70%) and 300 ml distilled water at room temperature. The solution obtained was filtered Whitman filter paper No.41. The ethanol extract of the seeds was prepared by soaking 10 g of the powdered seed material in 70% alcohol for overnight and then filtered through Whitman filter paper No.41. Using a Soxhlet apparatus the powder was extracted in ethanol.

2.2 Experimental animals

Veterinary Research Institute, Bareilly, UP. They were housed under standard laboratory condition in our animal house at J.V.P.W. University, Jaipur and were used for the experiment. They were kept in polypropylene cages measuring 12”x10”x8” under controlled temperature (25±2 °C) with optimal light and dark cycle. Animals were fed on balanced diet and water ad libitum.

Animals were regularly checked throughout the investigation for any infection and if found infected, the animals were isolated and treated. A total check of cleanliness of the cages and general environmental of animal house was kept. Animals were treated intermittently with antibiotic and anthelminthic suspensions as a prophylactic measure.

2.3 Induction of diabetes

Weighed the selected mice, marked for individual identification and for overnight speed. Intraperitoneal (I.P) was given the alloxan monohydrate at a rate of 150mg/kg body weight to make the alloxan-induced diabetic mice model. For 24 hours after alloxan injection, 5% glucose solution was given to the alloxan treated animals to prevent the motility of hypoglycemia caused by the initial drug (Andallu and Varadachayulu, 2002). Diabetes was confirmed by blood samples obtained from the tip of the tail using a blood glucometer (Accusure, Taiwan). Blood glucose levels of these mice were measured at 72h after alloxan administration. Animals with blood glucose levels equal to or greater than 250mg/dl were considered diabetic and used throughout the experimental group.

2.4 Animal grouping and extract administration

Whole experiment was divided into several treatment groups. The experimental models were then administered the various plant extract and medicine treatment for 28 days. The control and experimental groups consisted of 6 animals each.

Group I: Control or Intact: The group consisted of non-diabetic mice without any alloxan induced diabetes induction. They received drug vehicle only i.e. normal saline water (2 ml/kg body wt./day) for 28 days orally.

Group II: Diabetic Control: The group contained alloxan induced diabetic mice. They received drug vehicle for 28 days according to their respective treatments without any plant extract administration.

Group III: Diabetic + Aegel marmelos treatment 70% Ethanol Solution 100mg/kg dose: The group consisted of alloxan induced diabetic mice which were given Aegel marmelos extract (100mg/kg body weight) treatment for 28 days.

Group IV: Diabetic + Aegel marmelos treatment 70% Ethanol Solution 200mg/kg dose: The group consisted of alloxan induced diabetic mice which were given Aegel marmelos extract (200mg/kg body weight) treatment for 28 days.

Group V: Diabetic + Aegel marmelos treatment 70% Ethanol Solution 300mg/kg dose: The group consist of alloxan induced diabetes mice which were given Aegel marmelos extract (300mg/kg body weight) treatment for 28 days.

Group VI: Diabetic + Aegel marmelos treatment 70% Ethanol Solution 400mg/kg dose: The group consist of alloxan induced diabetes mice which were given Aegel Marmelos extract (400mg/kg body weight) treatment for 28 days.

Group VII: Diabetic + Aegel marmelos treatment 70% Ethanol Solution 500mg/kg dose: The group consist of alloxan induced diabetes mice which were given Aegel Marmelos extract (500mg/kg body weight) treatment for 28 days.

Group VIII: Diabetic T. fenugreek treatment 70% Ethanol Solution 100mg/kg dose: The group consisted of alloxan induced diabetic mice, which were given T. fenugreek extract (100mg/kg body weight) treatment for 28 days.

Group IX: Diabetic T. fenugreek treatment 70% Ethanol Solution 200mg/kg dose: The group consisted of alloxan induced diabetic mice, which were given T. fenugreek extract (200mg/kg body weight) treatment for 28 days.

Group X: Diabetic T. fenugreek treatment 70% Ethanol Solution 300mg/kg dose: The group consisted of alloxan induced diabetic mice, which were given T. fenugreek extract (300mg/kg body weight) treatment for 28 days.

Group XI: Diabetic T. fenugreek treatment 70% Ethanol Solution 400mg/kg dose: The group consisted of alloxan induced diabetic mice, which were given T. fenugreek extract (400mg/kg body weight) treatment for 28 days.

Group XII: Diabetic + T. fenugreek treatment 70% Ethanol Solution 500mg/kg dose: The group consisted of alloxan induced diabetic mice which were given T. fenugreek extract (500mg/kg body weight) treatment for 28 days.

Group XIII: Diabetic + Glibenclamide treatment: Diabetes mice were given with Glibenclamide (5 mg/Kg body weight) in aqueous solution daily for 28 days. Food and water were provided the duration of treatment was 28 days.

2.5 Statistical analysis

The statistical significance was assessed using T Test using SPSS 12.0 version (SPSS, Cary, NC, USA). The values are expressed as mean ± SD and $p<0.05$ was considered significant.

3. Results

3.1 Phytochemical screening

Qualitative analysis of plant crude extract of Aegle marmelos, indicated the presence of alkaloids, carbohydrates, glycosides, flavonoids, phenols and saponins present in the Leaves. These results are similar to Chetna *et al*., (2014) [9] were it was reported that the phytochemical screening of water extract from the dried ripe fruits and Farina Mujeeb *et al*., (2014) [10] from leaves of Aegle marmelos revealed the presence of major bioactive compounds including phyto-sterols, carbohydrate, protein, alkaloids, glycosides, polyphenols, flavonoids and saponins, which may retain a wide range of pharmacological actions. Table 1 shows the results of the preliminary phytochemical analysis of the leaf extract. Analysis revealed the presence of alkaloids, flavonoids, tannins, saponins, carbohydrates, glycosides, polyphenol and saponin while Terpenoids, Steroids were not detected. The preliminary phytochemical analysis of alcoholic and aqueous extract of Fenugreek Seeds was performed and shown in table no 2. Below. It indicates the presence of alkaloids, carbohydrates, saponins glycosides, flavonoids, tannins steroids, triterpenoids. The presence of all above constituents were also similar as per following studies.

Table 1: Qualitative analysis of phytochemicals in aqueous and methanol extract of Bael (Aegle marmelos) leaves

Test	Ethanol extract	Aqueous extract
Alkaloids	+	-
Carbohydrates	+	-
Glycosides	+	+
Flavonoid	+	+
Polyphenols	+	+
Saponins	+	+
Steroids	-	-
Tannins	+	+
Terpenoids	-	-
+ Presence of Constituents	- Absence of Constituents	

Table 2: Qualitative analysis of phytochemicals in aqueous and methanol extract of Fenugreek Seeds

Test	Aqueous extract	Ethanol extract
Alkaloids	+	+
Carbohydrates	+	+
Glycosides	+	+
Flavonoids	+	+
Poly Phenols	+	+
Saponins	+	+
Steroids	+	+
Terpenoids	+	+
+ Presence of Constituents	- Absence of Constituents	

3.2 Glycemic effect of ethanolic extract of A. Marmelos leaf and Seeds of fenugreek of alloxan-induced diabetic mice

Table 3, 4 below presents the glycemic effects of ethanolic extract of A. marmelos leaf and seeds extract of Fenugreek in alloxan induced diabetic mice. Single dose of alloxan monohydrate (150 mg/kg) continuously increased the blood glucose from the first day of treatment until the third, while it get reduced to normalcy upon I.P. administration of ethanolic extract of both Plants and standard drug (Glibenclamide) for 28 days. A significant decrease ($P<0.05$) in blood glucose was observed particularly at the highest dose of 200, mg/kg of A. marmelos leaves extract and 300 mg/kg of Fenugreek seed extract.

Table 3: Changes in serum glucose level in 7, 14, 21 and 28 days treatment of various plant extracts in mice (mean of 6 value)

Sample	0 th Day	7 th Day	14 th Day	21 th Day	28 th Day
Group I (Untreated normal control mice)	111.68±1.6	113.56±0.99	114.19±1.5	115.51±0.77	116.49±1.25
Group II. (Untreated Diabetic control mice)	268.67±6.07	270.05±4.31	272.72±3.11	275.09±2.47	276.56±2.74
Group III. 100 mg/kg AME treated)	254.64±1.6	239.84±3.11	189.52±1.67	183.5±3.07	165.29±2.91
Group IV. 200 mg/kg AME treated)	273.54±0.99	241.28±2.47	210.98±2.18	175.61±2.71	148.01±3.03
Group V. 300 mg/kg AME treated)	255.76±1.5	215.84±2.74	181.38±3.99	161.37±2.36	144.47±1.74
Group VI. 400 mg/kg AME treated)	281.65±0.77	243.29±1.44	215.86±3.11	179.88±3.2	149.36±2.15
Group VII. 500 mg/kg AME treated)	273.52±1.25	241.25±1.15	207.65±1.98	176.07±3.55	149.1±3.08

Group VIII. 100 mg/kg TFK treated	263.44±3.63	232.09±3.38	195.88±2.11	162±0.89	142.58±1.18
Group IX. 200 mg/kg TFK treated	267.66±3.56	225.59±3.27	183.13±4.35	142.15±2.15	120.53±1.25
Group X. 300 mg/kg TFK treated	260.02±2.32	219.55±1.68	185.45±3.28	133.62±1.54	111.7±0.51
Group XI. 400 mg/kg TFK treated	262.13±3.96	219.97±2.17	185.13±2.82	142.02±3.71	110.11±2.43
Group XII. 500 mg/kg TFK treated	265.22±4.93	223.05±4.84	190.82±4.29	140.04±1.66	109.89±1.01
Group XIII. (Glibenclamide treated Diabetic control mice)	256.83±1.44	226.47±1.15	201.14±2.55	151.32±0.97	125.99±1.67

3.3 Effect of ethanolic leaf extract of *A. marmelos and Seed Extract of Fenugreek* on body weight of alloxan-induced diabetic mice

In diabetic mice, continuous reduction in body weight was observed as shown in Table**.** Glibenclamide (5 mg/kg) as well as the extract treatment groups at the dose of 200 mg/kg B.W. of leaves extract of A. Marmelos and 300 mg/kg B.W in T. Fenugreek showed improvement ($P<0.05$) improvement in body weight of diabetic mice.

Table 4: Changes in body weight level in 7, 14, 21 and 28 days treatment of various plant extracts in mice (mean of 6 value)

Sample	0 th Day	7 th Day	14 th Day	21 st Day	28 th Day
Group I (Untreated normal control mice)	27.65±0.48	28.02±0.5	28.37±0.54	29.04±0.51	29.92±0.58
Group II. (Untreated Diabetic control mice)	29.02±0.41	27.27±0.32	24.4±0.34	21.55±0.59	18.24±0.68
Group III. 100 mg/kg AME treated)	28.99±0.35	26.56±0.26	28.69±0.31	30.18±0.24	31.29±0.31
Group IV. 200 mg/kg AME treated)	28.25±0.35	26.46±0.37	28.39±0.3	30.93±0.24	32.21±0.12
Group V. 300 mg/kg AME treated)	28.22±0.49	26.06±0.45	27.82±0.39	31.12±0.17	32.24±0.36
Group VI. 400 mg/kg AME treated)	28.58±0.42	26.45±0.31	28.39±0.17	31.46±0.27	32.44±0.4
Group VII. 500 mg/kg AME treated)	28.31±0.42	26.7±0.63	28.62±0.31	31.55±0.11	32.94±0.19
Group VIII. 100 mg/kg TFK treated	28.1±0.54	25.91±0.74	26.63±0.66	29.33±0.77	32.03±0.74
Group IX. 200 mg/kg TFK treated	27.94±0.65	26.39±0.56	26.68±0.28	30.12±0.4	32.28±0.61
Group X. 300 mg/kg TFK treated	27.87±0.52	26.74±0.4	27.89±0.39	31.77±0.63	33.65±0.6
Group XI. 400 mg/kg TFK treated	28.33±0.65	27.19±0.6	29.8±0.58	32.31±0.63	34.36±0.68

Group XII. 500 mg/kg TFK treated	27.75±0.36	27.24±0.34	30.7±0.42	32.25±0.25	34.78±0.52
Group XIII. (Glibenclamide treated Diabetic control mice)	28.81±0.25	27.74±0.29	27.15±0.34	28.16±0.38	28.98±0.32

3.4 Effect of ethanolic leaf extract of *A. Marmelos and T Fenugreek* on liver function enzymes of alloxan-induced diabetic mice

The effect of ethanolic leaf extract of *A. Marmelos and Seeds extract of Fenugreek* on liver function enzymes is represented in Figure. SGOT and SGPT levels were significantly elevated in alloxan induced diabetes. The mice treated with ethanolic leaf extract of *A. Marmelos and Seeds extract of Fenugreek* showed significant ($P<0.05$) reduction in the activity of liver and serum SGOT and SGPT in the groups and standard drug (Glibenclamide) when compared with the control while there was no significant difference ($P>0.05$) in other treatment groups.

	SGOT	**SGOT**	**SGPT**	**SGPT**
	Initial	**Final**	**Initial**	**Final**
Group I (Untreated normal control mice)	40.91±0.47	44.1±0.49	32.44±0.86	32.86±1
Group II. (Untreated Diabetic control mice)	41.26±0.47	44.42±0.41	63.72±1.56	68.65±0.63
Group III. 100 mg/kg Aegel Marmelos extract treated diabetic mice)	47.27±0.29	28.46±0.11	72.5±0.69	34.63±1.16
Group IV. 200 mg/kg Aegel Marmelos extract treated diabetic mice)	48.07±0.53	28.72±0.06	76.86±0.61	33.16±0.28
Group V. 300 mg/kg Aegel Marmelos extract treated diabetic mice)	46.52±0.27	22.1±0.02	76.78±0.16	32.99±0.28
Group VI. 400 mg/kg Aegel Marmelos extract treated diabetic mice)	48.08±0.66	22.12±0.02	77.43±0.93	32.43±0.18
Group VII. 500 mg/kg Aegel Marmelos extract treated diabetic mice)	44.44±0.17	22.64±0.43	76.24±0.36	31.99±0.02
Group VIII. 100 mg/kg T. Fenugreek extract treated diabetic mice)	51.43±1.09	38.01±0.72	65.48±0.72	47.86±0.7
Group IX. 200 mg/kg T. Fenugreek extract treated diabetic mice)	51.12±1.11	38.32±0.77	65.96±0.45	45.09±0.66
Group X. 300 mg/kg T. Fenugreek extract treated diabetic mice)	51.18±1.21	42.82±0.85	64.89±0.38	42.98±0.21
Group XI. 400 mg/kg T. Fenugreek extract treated diabetic mice)	55.11±0.43	38.32±0.77	64.52±0.35	43.25±0.47
Group XII. 500 mg/kg T. Fenugreek extract treated diabetic mice)	52.83±0.7	38.01±0.72	64.49±0.53	45.26±0.46
Group XIII. (Glibenclamide treated Diabetic control mice)	40.97±0.3	21.99±0.28	61.41±1.22	31.29±0.35

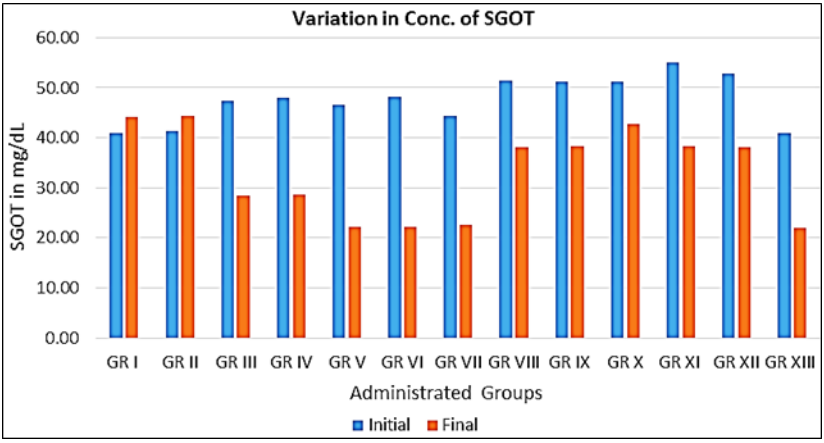

Fig 3.1: Variation of Concentration of SGOT in Mice for a duration of 28 days

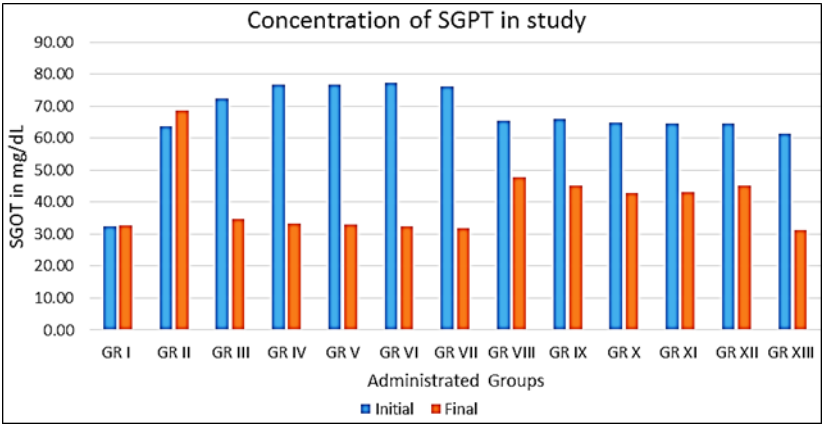

Fig 3.2: Variation of Concentration of SGPT in Mice for a duration of 28 days

3.5 Effect of ethanolic leaf extract of *A. marmelos and Seed extract of Fenugreek* on some kidney function indices and biochemical parameters of alloxan-induced diabetic mice

Figures show the effect of administration of ethanolic plant extract on, total protein, albumin and urea in alloxan induced diabetic mice. The concentration of both total Protein and albumin level in serum and liver was increased significantly ($P<0.05$) in diabetic untreated group compared to the control but was reduced upon administration of ethanolic leaf extract of A. Marmelos and Seed Extract of Fenugreek for 28 days.

The diabetic untreated mice group had decreased levels of serum and liver total protein, albumin and globulin when compared with normal control mice.

After treatment for 28 days, liver and serum total protein and albumin levels were restored to normalcy especially in the groups treated with 200 mg/kg body for Aegle Marmelos extract and 300 mg/kg for fenugreek the extract and reference drug (Glibenclamide).

Table 3.4: Conc. of biochemical parameters of mice under various experimental conditions of 28 days treatment

Sample No	T. Protein	T. Protein	Albumin	Albumin	Blood urea	Blood urea	S. creatinine	S. creatinine
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Group I: (Untreated normal control mice)	4.57±0.1	5.58±0.1	3.56±0.04	3.37±0.03	19.11	22.03	0.65	0.66
Group II: (Untreated Diabetic control mice)	4.54±0.08	3.86±0.06	3.53±0.04	3.3±0.02	30.10	41.83	1.50	1.60
Group III: 100 mg/kg AME treated)	4.59±0.04	5.46±0.01	3.64±0.03	3.94±0	23.67	22.72	1.44	0.84
Group IV: 200 mg/kg AME treated)	4.46±0.05	5.57±0.01	3.63±0.09	3.94±0	23.53	22.26	1.41	0.81
Group V: 300 mg/kg AME treated)	4.54±0.02	5.6±0.02	3.57±0.01	3.96±0.02	23.71	21.73	1.43	0.76
Group VI: 400 mg/kg AME treated)	4.63±0.09	5.72±0.01	3.61±0	3.99±0.01	23.66	21.04	1.52	0.72
Group VII: 500 mg/kg AME treated)	4.74±0.03	5.82±0.02	3.6±0.09	3.81±0.03	22.91	20.19	1.41	0.68
Group VIII: 100 mg/kg TFK treated	4.54±0.04	5.56±0.02	3.53±0.05	3.94±0.02	24.42	19.46	1.48	0.83
Group IX: 200 mg/kg TFK treated	4.61±0.05	5.67±0.1	3.54±0.02	3.97±0.02	24.04	20.50	1.46	0.78
Group X: 300 mg/kg TFK treated	4.69±0.02	5.89±0.07	3.58±0.04	3.97±0.04	23.78	18.49	1.41	0.77
Group XI: 400 mg/kg TFK treated	4.75±0.05	6.2±0.06	3.64±0.03	3.96±0.01	24.46	17.55	1.34	0.75
Group XII: 500 mg/kg TFK treated	4.83±0.02	6.37±0.07	3.64±0.03	4.05±0.01	24.13	17.61	1.29	0.71
Group XIII: (Glibenclamide)	4.66±0.03	6.06±0.13	3.55±0.02	3.97±0.01	28.64	21.36	1.32	0.80

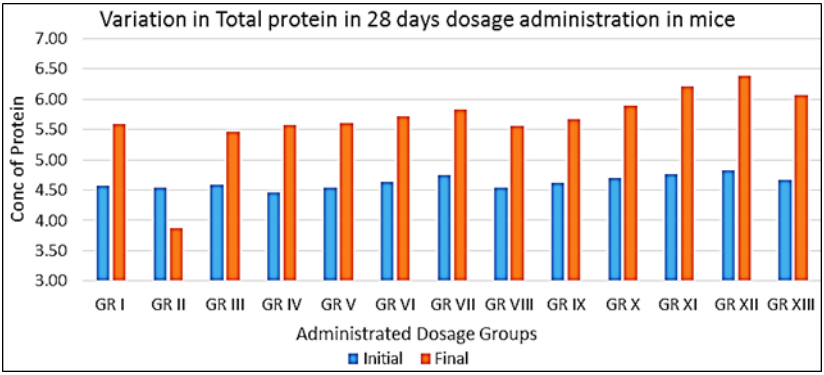

Fig 3.3: Variation of Concentration of Total Protein in Mice for a duration of 28 days

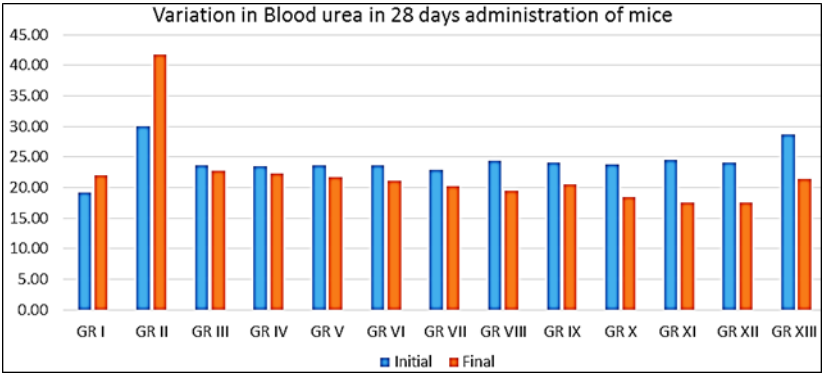

Fig 3.4: Variation of Concentration of Blood Urea in Mice for a duration of 28 days

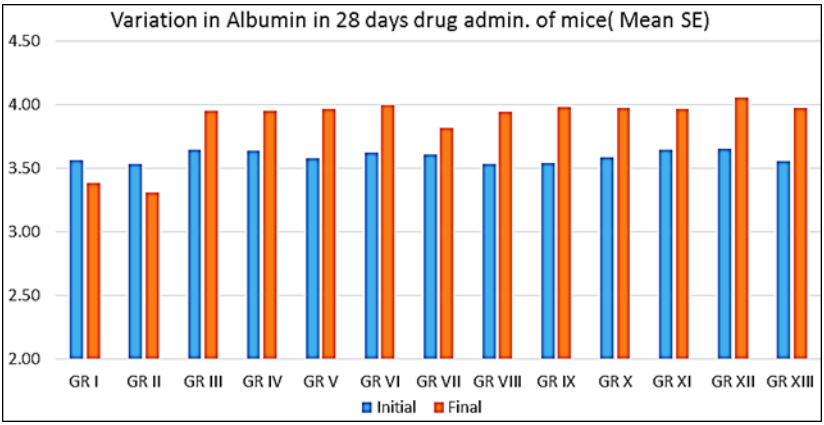

Fig 3.5: Variation of Concentration of Albumin in Mice for a duration of 28 days

The influence of administration of ethanolic leaf extract of *A. marmelos and Seeds Extract of Fenugreek* on kidney function indices is shown in Figure 5. In this study, urea and creatinine levels showed significant ($p<0.05$) increase in diabetic mice group when compared with the control but showed no significant ($p>0.05$) difference at all doses of treatment when compared with the control.

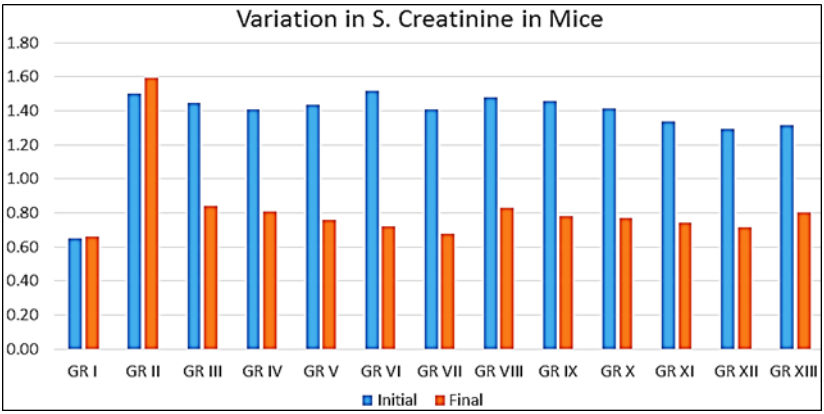

Fig 3.6: Variation of Concentration of S. creatinine in Mice for a duration of 28 days

3.6 Effect of administration of ethanolic leaf extract of *A. marmelos and Seeds Extract of Fenugreek* on liver lipid profile of alloxan-induced diabetic mice

The effect of I.P. administration of ethanolic leaf extract of *A. marmelos and Seeds Extract of Fenugreek* on the levels of total TC, HDL, LDL-C, and VLDL-C in the serum and liver of diabetic mice are shown in Figures. In alloxan-induced diabetic mice, TC, LDL, and VLDL levels were increased and HDL level was decreased significantly ($p<0.05$) when compared with normal control mice. In diabetic mice group, administration of ethanolic leaf extract of *A. marmelos and Seeds Extract of Fenugreek* at mg/kg body weight dose particularly, showed significant ($p<0.05$) reduction in elevated TC, LDL and VLDL levels while at doses 200 and 300 mg/kg body weight of the extract no significant ($p>0.05$) difference was observed when compared to diabetic mice group.

Table 3.6: Conc. of Lipid profile parameters of mice under various experimental conditions of 28 days treatment

Sample No	T. Cholestrol		HDL		LDL		VLDL	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Group I (Untreated normal control mice)	121.56 ±1.79	115.83 ±3.09	28.5± 0.25	30.05± 0.38	98.25± 1.03	98.95± 1.51	31.63± 0.23	27.44± 0.39
Group II. (Untreated Diabetic control mice)	150.92 ±2.67	184.47 ±2.48	28.15± 0.3	18.65± 0.77	120.01 ±1.01	145.28 ±1.72	31.59± 0.23	32.94± 0.09
Group III. 100 mg/kg AME treated)	177.83 ±1.8	143.78 ±1.06	27.04± 0.42	28.49± 0.4	116.52 ±0.17	106.97 ±0.69	30.91± 0.23	26.99± 0.12
Group IV. 200 mg/kg AME treated)	180.88 ±1.93	136.22 ±0.89	28.26± 0.16	29.06± 0.35	116.97 ±2.03	102.2± 1.21	36.31± 0.41	26.58± 0.14
Group V. 300 mg/kg AME treated)	174.77 ±1.65	127.05 ±0.63	27.41± 0.29	30.65± 0.57	112.78 ±1.39	96.99± 0.24	34.57± 0.2	26.64± 0.09
Group VI. 400 mg/kg AME treated)	180.25 ±2.77	125.45 ±0.46	27.7± 0.19	29.43± 0.43	116.45 ±0.2	96.79± 0.24	32.96± 0.66	26.69± 0.09
Group VII. 500 mg/kg AME treated)	180.27 ±0.95	124.71 ±0.3	28.21± 0.04	28.71± 0.21	105.81 ±0.72	97.3± 0.63	34.75± 0.26	26.67± 0.1
Group VIII. 100 mg/kg TFK treated	168.07 ±1.67	144.8 ±0.65	28.18± 0.37	30.48± 0.36	111.6± 1.61	103.43 ±2.46	29.41± 0.27	19.44± 0.18
Group IX. 200 mg/kg TFK treated	167.14 ±1.59	137.94 ±0.7	27.93± 0.46	30.73± 0.34	109.78 ±1.14	102.14 ±1.08	29.67± 0.28	19.22± 0.03
Group X. 300 mg/kg TFK treated	165.36 ±0.95	130.63 ±0.28	28.64± 0.34	32.78± 0.27	109.81 ±0.98	103.31 ±1.01	29.45± 0.41	17.99± 0.39
Group XI. 400 mg/kg TFK treated	167.23 ±1.71	128.31 ±0.51	29.07± 0.12	33.9± 0.44	111.26 ±2.34	102.3± 1.79	29.67± 0.28	17.92± 0.39
Group XII. 500 mg/kg TFK treated	169.94 ±0.89	126.17 ±0.91	28.89± 0.44	33.52± 0.27	113.64 ±1.87	100.17 ±1.27	29.41± 0.27	20.3± 0.8
Group XIII. (Glibenclamide)	159.25 ±0.88	126.75 ±0.85	26.21± 1.09	29.67± 0.84	104.83 ±2.2	89.89± 1.26	29.87± 0.42	14.94± 0.21

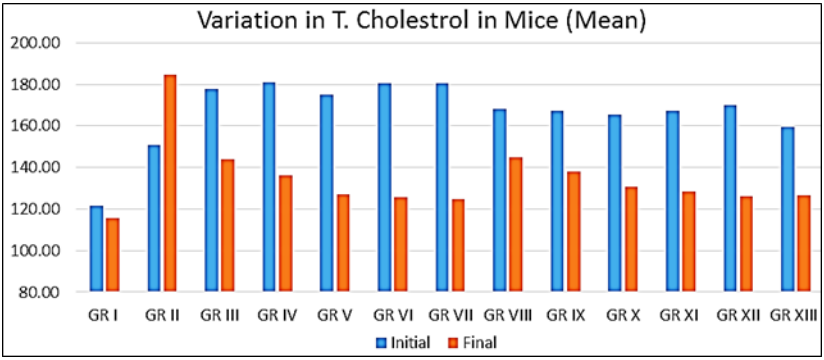

Fig 3.7: Variation of Concentration of total Cholesterol in Mice for a duration of 28 days

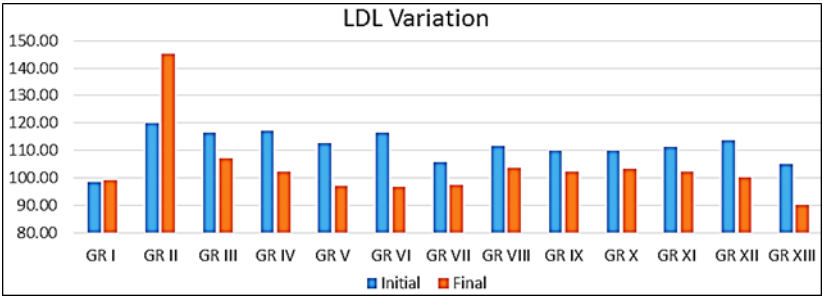

Fig 3.8: Variation of Concentration of LDL-C in Mice for a duration of 28 days

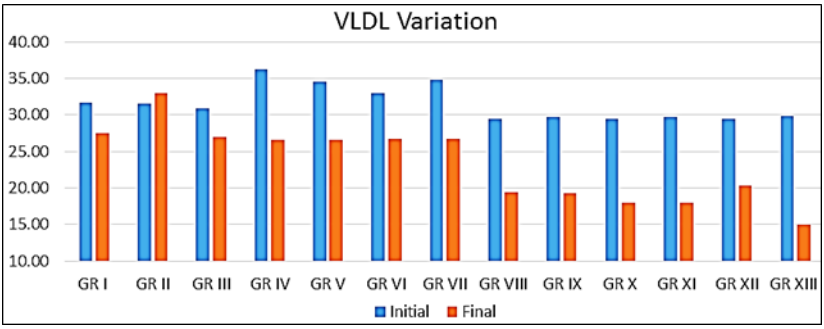

Fig 3.9: Variation of Concentration of VLDL-C in Mice for a duration of 28 days

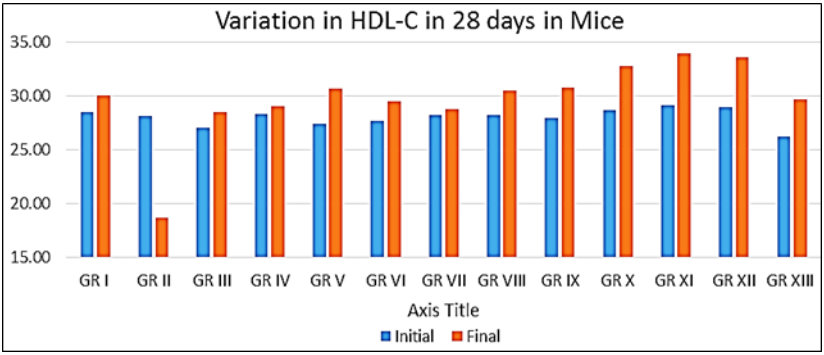

Fig 3.10: Variation of Concentration of HDL-C in Mice for a duration of 28 days

4. Discussion

The therapeutic cure for diabetes mellitus has remained elusive despite the discovery of an array of medications that can ameliorate the symptom's of the disease [18]. Phytotherapies have remained a veritable source for drug discovery the world over [19] and for some decades have played an important role in the management of diabetes especially in resource poor countries.

Alloxan acts as diabetogenic by the destruction of [β-cells of the islets of langerhans and causes massive reduction in insulin release, thereby inducing hyperglycemia [20]. Insulin deficiency leads to various metabolic alterations in the animals viz. increased blood glucosel, increased levels of alkaline phosphate and transaminases etc. [21].

Phytochemical investigation of ethanolic extract of *both plants* as shown in Table reveals the presences of alkaloids, flavonoids, tannins, saponins, phenol and glycoside and saponin. These secondary principles are known to be bioactive for the management of diabetes. It is well known that certain flavonoids exhibit hypoglycemic activity and pancreas beta cell regeneration ability. Thus, the significant antidiabetic effect of ethanolic plant extract may be due to the presence of more than one antihyperglycemic principle and their synergistic properties [22].

Single dose intra-peritoneal (I.P) treatment of mice with alloxan monohydrate (150 mg/kg) caused an increase in the blood glucose. Ethanolic plant extract and glibenclamide were found to reduce the elevated glucose level significantly in alloxan induced diabetes animals during the 28 days treatment. This suggests the hyperglycemic effect of the plant. As suggested by Ekpenyong *et al*., [23] that normal protein level reflects normal synthesis while high level is common in high protein diet.

The concentration of total protein and albumin may indicate the state of the liver and type of damage. Protein molecules that are regularly employed to assess the state of health of the liver are albumins and Total Proteins. The blood circulated albumin is the main carrier protein produced in the liver. The larger globulins are responsible for immunogenic activities [24]. Decreased serum albumin and globulin concentrations in the untreated diabetic mice suggests reduced synthetic function of the hepatic cells. Administration of ethanolic plant extract, however, normalized the serum albumin concentration. This is a further proof of the protective potential of ethanolic plant extract on the liver of diabetic mice.

Liver enzymes i.e. SGPT (ALT), and SGOT (ASAT) were increased in diabetic mice which is responsible for the liver damage. The elevated serum level of these enzymes was significantly reduced by plant extracts treatment suggesting the protective effect of the plant extract against diabetes-induced hepatocellular damage. The diabetic complications such as increased gluconeogenesis and ketogenesis may be due to elevated enzymes [26]. The restoration of transaminases to their normal levels also treatment also indicates revival of insulin secretion.

The kidney removes metabolic wastes such as urea and creatinine, the concentration of which are usually required to assess the normal functioning of different parts of the nephrons [27]. The serum creatinine and urea concentrations are widely interpreted as measures of the glomerular filtration rate (GFR) and are used as indices of renal function in clinical practice. The concentration of these metabolites increase in blood during renal damage associated with uncontrollable diabetes mellitus. On the contrary those treated with plant extract decrease in creatinine and urea levels, indicating ameliorative effect of the plant extract on kidney functions in diabetic mice. This may suggest that the damage caused on renal function indices by the disease had been restored by the plant extract, thus the proper function of the nephrons at the tubular and glomerular level.

Inbalances in serum lipid levels are usual occurrences in a diabetic state [28]. Since changes in lipoproteins concentrations is an inherent property of diabetes mellitus, such changes are usually triggered by diabetes induced obesity and renal complications [29]. As observed in this study administration of both plants extract led to a reduction in cholesterol, triglycerides and low density lipoprotein (LDL) concentrations while it led to the normalization of high density lipoprotein (HDL) concentration in diabetic mice when compared to the untreated diabetic group. The serum concentration of cholesterol is usually elevated in diabetes, and such an increase is a risk factor for cardiovascular diseases. The observed high concentration of serum cholesterol during diabetes is mainly attributable to pronounced mobilization of free fatty acids from the peripheral depots, because the hormone-sensitive lipase is usually inhibited by insulin [30]. Administration of plant extract to diabetic mice significantly decreased the plasma cholesterol level to near normalcy and therefore reduces the risk of cardiovascular disease [31]. An increase in the concentrations of LDL-cholesterol and reduced HDL-cholesterol as observed during diabetes are associated with raised risk of myocardial infarction [32]. Administration of plant extract led to an increased concentration of HDL-cholesterol and depleted VLD-cholesterol levels which are characteristic of reduced risk of myocardial infarction. Convincing evidence from laboratory, clinical and epidemiologic data have confirmed that increased serum concentration of triglyceride is a standalone risk factor for cardiovascular complications. Hyper triglyceridemia is a characteristic condition observed in diabetics, in this study, treatment with both plant extracts has prevented the elevation of triglycerides, signifying that myocardial membrane is intact and not damaged.

5. Conclusion

The present study showed that the ethanolic extract of *Aegle marmelos* leaves and Seed extract of Fenugreek exhibited antihyperglycemic and anti-lipidemic effects and there was no significant changes in the toxicological parameters evaluated hence it could be considered safe for use as an antidiabetic recipe.

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Chapter - 4
Banana Peel Mediated TiO2 Nanoparticles: A Promising Insecticidal and Antibacterial Agent

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Chapter - 4

Banana Peel Mediated TiO2 Nanoparticles: A Promising Insecticidal and Antibacterial Agent

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Abstract

Titanium nanoparticles have been synthesized with the aid of innovative, non-toxic, eco-friendly, biological material that is to say, banana peel extract (BPE). In this study, the researchers used banana peels extract as a new green synthesis method to synthesized titanium nanoparticles, this method has not been carried before. Titanium dioxide nanoparticles were biologically synthesized using an aqueous solution of banana peel extract as a bio reductant. This is interesting and novel route for synthesis of TiO2 nanoparticles. The synthesized titanium dioxide nanoparticles have characterized using ultraviolet-visible spectroscopy (UV-VIS), Atomic phase microscopy (AFM), X-Ray diffraction (X-R-D) and examined as antimicrobial agent against several pathogenic bacteria as well as insecticidal agent against *Musca domestica.* Titanium dioxide nanoparticles was successfully prepared with average diameter: 88.45 nm and volume 31.5nm. As resulted by AFM and XRD respectively. Moreover, gave inhibitory effect against several pathogenic bacteria, also prepared TiNPs caused high mortality percentages against three larval stages of house fly.

Keywords: banana peel extract (BPE), biomedical application, ecofriendly, *Musca domestica,* titanium nanoparticles

Introduction

The development of dependable investigational methods for the synthesis of nanoparticles over arrange of chemical conformation, high monodispersity and sizes is one of challenging issues in recent nanotechnology [1]. Nanotechnology is a fast emerging field with its application in technology and science. Nanoparticles are of great interest because of their optical, catalytic, and magnetic properties. Nanoparticles have unique properties thus applied in biosensing, catalysis, imaging, drug delivery, and in medicine [2, 3]. Nanoparticles Synthesis was broadly studied employing physical and

chemical methods, but the development of trustworthy natural technology for nanoparticles production is an significant aspect of nanotechnology [4]. Using enzymes And microorganisms, are suggested as probable natural alternatives [5]. These techniques employ lethal chemicals such as stabilizing agents, non-biodegradable reducing agents or organic solvents which are hazardous to the earth and organic systems, as well as time consuming and costly [6].

Biological control

Biological control consists of the introduction of beneficial predatory or parasitic species into cultivation systems where they were previously absent or present only at low population levels. This technique is designed to negatively affect specific target species that could otherwise become pests or infectious agents [7]. Susceptibility to pests is a general reflection of plant health, which can be negatively influenced by poor soil fertility management. One of the objectives of biological control is to assure that the beneficial organism to be introduced can complete its lifecycle at the site, and then reproduce with sufficient efficiency to become a permanent resident of the agrosystem. Frequently, however, the niche conditions available to the beneficial introduced organism do not fully satisfy its long-term needs, requiring its reintroduction [8]. Changes in production practices and the use of agricultural additives are often necessary for biological control to be successful. Integrated Pest Management (IPM) is an alternative to unilateral intervention strategies using agrochemicals, with a wider focus on the ecology of the insect pests as well as the crop plants, based on the use of complementary tactics and the adoption of cultivation techniques that favor plant diversity. Pest control in this type of approach is initially based on natural agents such as pathogens, parasites and predators, with the use of agro toxins being contemplated only as a last resort [9]. However, as biological pest control methods do not demonstrate immediate results in agro-industrial systems with large-scale production and commercialization goals (as agro toxins), commercial groups tend to avoid the costs and labor related to their development and perfection. Nonetheless, growing energy costs, environmental degradation, and inflation all reinforce the argument that immediate financial gains should not be the principal motivating force in agricultural production [8]. In spite of the strong economic pressure on agricultural production, many farmers are making the transition to practices that are more environmentally friendly and have the potential to contribute to long-term agricultural sustainability with biological control being one of the principal tools in this conversion process [7].

Microbial control is an aspect of biological insect control and consists of the rational use of pathogens to maintain pest balances in agricultural environments, with increases in the numbers of other natural enemies often being observed in fields where microbial control has been used [8].

The reduction of metal biologically can be useful for nontoxic application in green environment to the production of metals nanoparticles. Some microbes like yeast [10]. Fungi [11] and bacteria [12] were probably valuable in preparing metal nanoparticles using standard pressure and temperature. Lots of fungus were applied for nanoparticles fabrication, together with *Verticillium* spp. [13], *Aspergillus fumigates* [14] *Aspergillus niger* and *Fusarium oxysporum* [15]. Recently, mosquitoes defend the chemical pesticides; some bacterial toxins, *Bacillus thuringiensis* sub sp., (*Bacillus sphaericus* and *israelensis*) [16] are novel environmentally safer to have vectors at bottom level.

Plant (banana) extract

The extract of plants, which serve as capping and reducing agents for preparation of nanoparticles, are more beneficial over other biological procedures [17], because they avoid the elaborated techniques of culturing and continuing of the cell [18]. Moreover, nanoparticles from plant is preferred due to its properties as, ecofriendly, cost-effective, a single-step biosynthesis process and human friendly [19].

Classically, Different parts of plant materials such as extracts [20], fruit [21], fruit peels [22], bark [23], callus [24] and root [25] these parts have been investigated so far for the synthesis of gold, silver, titanium nanoparticles in various shapes and sizes [26] Banana (*Musa paradisiaca*), belongs to the family Musaceae and is a standout amongst the most vital tropical fruits in the world market [27].

Banana is used up all over the world, after pulp consumption, the peels are usually discarded [28]. Banana peels forms approximately 18-33% of the whole fruit and presently, they aren‘t being utilized for any other purposes if not dumped as solid waste to the environment or used as animals food to a limited extent. It is consequently critical and even vital to discover applications for these peels as they can add to genuine ecological topics [6]. Literature assessment has shown that naturally obtainable agricultural wastes have not been examined for nanoparticles synthesis. Banana peel is a classic illustration of such an abundantly offered natural material. We hypothesized that banana peels that are innately rich in pectin, lignin and hemicellulose [29]. Could be used in the green synthesis of nanoparticles, furthermore, Banana

peels contains large amounts of phenolic compounds which assists in creation of metallic nanoparticles [30].

Titanium dioxide nanoparticles

Titanium dioxide (TiO2) is an inert, nontoxic and low-cost metal, whose high absorbance ability to UV light and high refractive index make it an interesting eco-friendly catalyst [31]. The nano sized Titanium dioxide particles are broadly used to provide whiteness and opaqueness to products such as sunscreen lotions, paints, food colorants, papers, plastics, inks and toothpastes [32]. TiO2 nanoparticles have been created using many plants like *Nyctanthes arbor tristis* extract [33], *Eclipta prostrate* aqueous leaf extract. [34] and peel extract of *Annona squamosa* L. [35].

In the current paper, titanium dioxide nanoparticles have been created using a previously unreported biological method for the synthesis using an water extract obtained from banana peels as anon-toxic, inexpensive and eco-friendly protocol for the assembly and synthesis of nanoparticles.

The prepared nanoparticles have been characterized by several analysis (UV-visible spectroscopy, XRD and AFM). An application of these biologically created nanoparticles as antibacterial and insecticidal agents against several pathogenic bacteria and (*Muscaedomesticae*) respectively has also been examined and discussed.

TiO2 nanoparticles description

The formation of titanium nanoparticles confirmed through visual assessment. The reaction mixture turned to white greyish color from fine white color within 30min. indicated the synthesis of titanium nanoparticles figure (1).

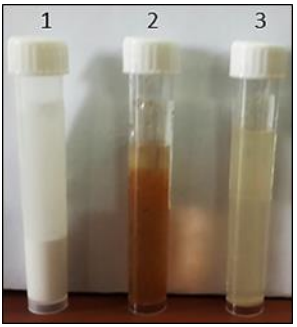

Fig 1: Comparison between three solutions, 1: Titanium Dioxide bulk solution, 2: water extract of banana peel, 3: synthesized Titanium Dioxide nanoparticles

UV-Visible spectral

Synthesized TiO2 nanoparticles sample was examined under UV-Visible Spectrophotometer within wave length range (200-800) nm. The results showed that TiO2 appears within the absorbance spectra (4.2) at wave length 208 nm, indicating the presence of titanium nanoparticles. Figure (2). In addition, the value of energy gap (E.g.) of prepared titanium nanoparticles was 4.7(ev). Figure (3). These results are agreed with [40].

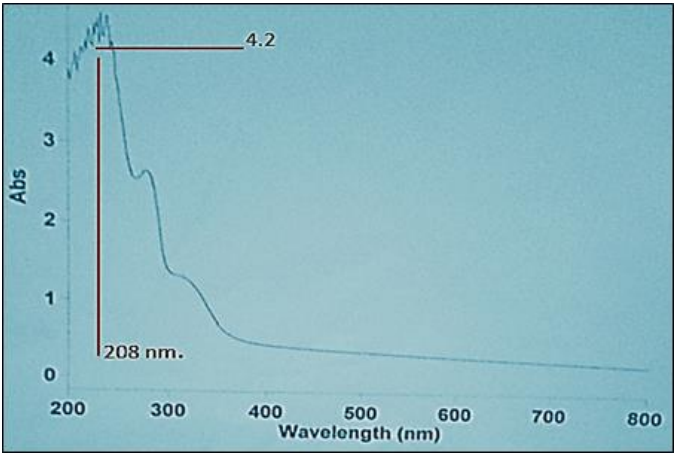

Fig 2: Absorbance spectrum of synthesized (Ti NPs)

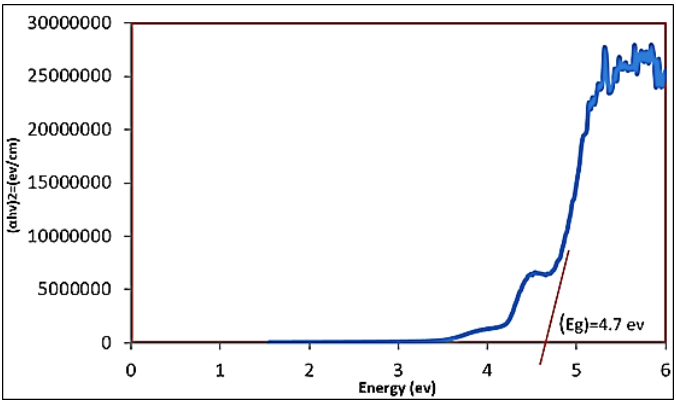

Fig 3: $(\alpha hv)^2$ versus photon energy plot of synthesized (TiNPs) thin film

Atomic force microscopy (AFM)

AFM results revealed that prepared titanium nanoparticles Size was ranged between (65-115) nm with average diameter: 88.45 nm, Roughness average (RA) and Root mean square (Sq.): 1.53 nm and 1.77 nm, respectively.

Figure (4). Shows AFM topographic images of biosynthesis TiO2 NPs. also figure (5) shows Granularity volume distribution of TiO2 NPs synthesized by banana peel extract.

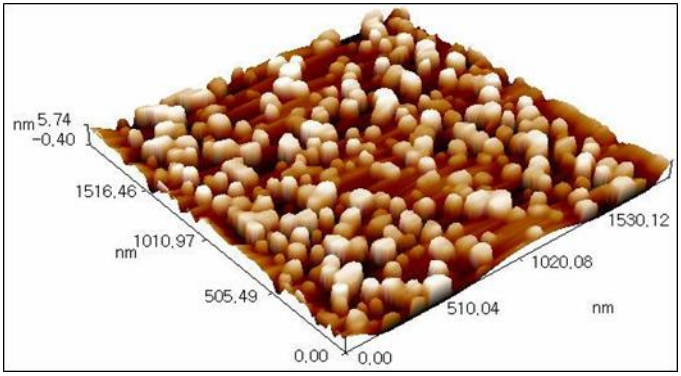

Fig 4: AFM topographic images

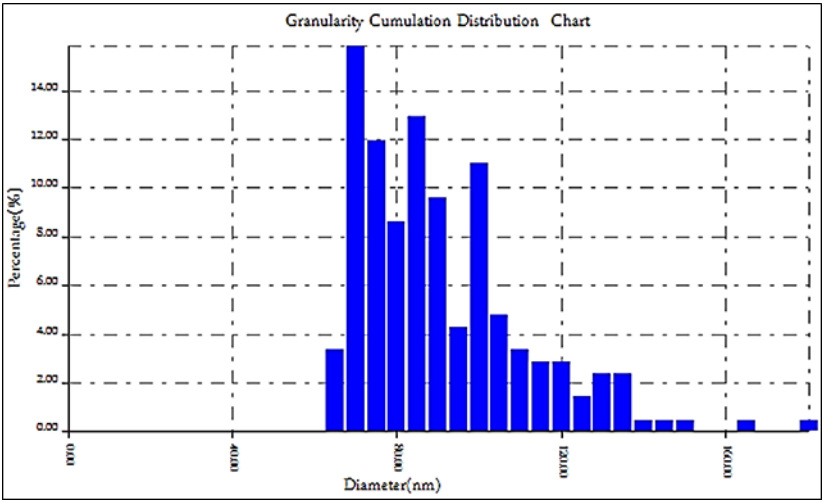

Fig 5: Granularity volume distribution of TiO2 NPs synthesized by banana peel extract

X-Ray Diffraction (XRD)

The results of XRD indicating the presence of three peaks. Strong diffraction peaks: 2-theta 28.2956°, 2-theta 40.4901° and 2-theta 50.1821°, the average crystallite size of NPs was calculated by Scherer's equation, they were 31.5nm. Figure (6).

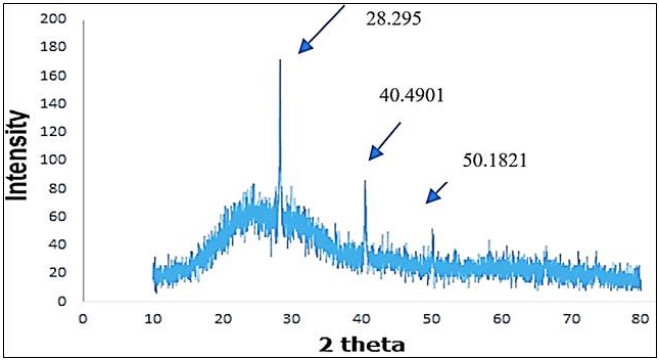

Fig 6: X-Ray pattern of synthesized TiO2 from BPE

Antimicrobial analysis of prepared titanium nanoparticles

The results of antimicrobial activity of prepared titanium nanoparticles showed that these nanoparticles exhibited antimicrobial impact against tested pathogenic microorganisms, with fluctuated degrees, suggested by inhibition zone diameter, while BPE did not show any activity against studied bacteria (Fig. 7 and 8). Titanium nanoparticles showed antibacterial effect for all tested concentrations against The Gram positive bacteria (*Staphylococcus aureus*) and (*Streptococcus* sp.). For other bacterial species (*Staphylococcus epidermidis, Escherichia coli, Klebsiella* sp., *Bacillus* sp.) only concentration (2) give an inhibition zone and there was no inhibition zones estimated for all examined synthesized titanium nanoparticles concentrations in *Pseudomonas aeruginosa*. Also the results showed that there was no antimicrobial activity for banana Peel (sample 4) extract against all tested pathogens.

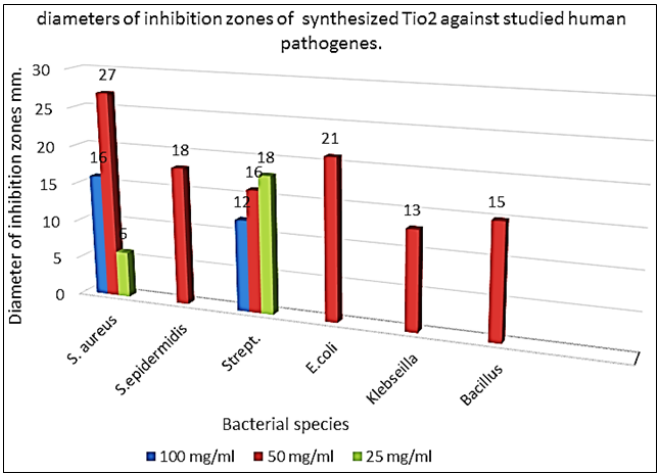

Fig 7: Diameters of inhibition zones of synthesized TiNPs against studied human pathogens

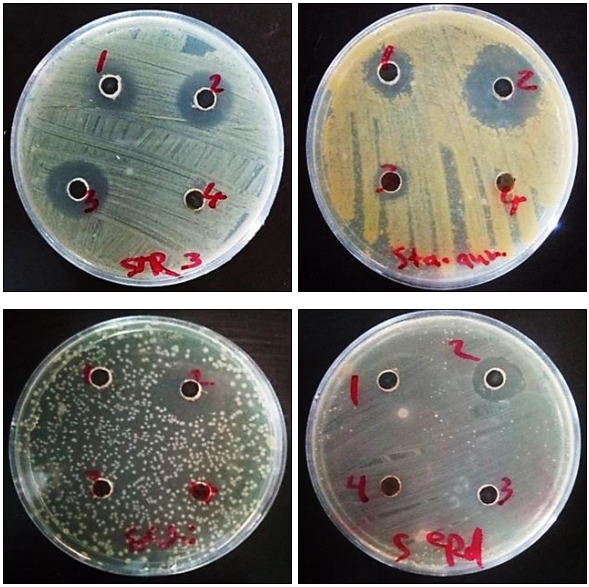

Fig 8: Zone of inhibition of prepared titanium nanoparticles against different bacteria (sample no. 1: 100 mg/ml, sample no. 2:50 mg/ml, sample no. 3: 25 mg/ml, sample no. 4: banana peel extract only)

Antibacterial effect of synthesized TiO2 nanoparticles: results revealed that BPE + TiO2 affect both experimented Gram positive and Gram Negative bacteria. The cellular wall of Gram positive bacteria characterized with possessing a thick peptidoglycan layer, consisting of linear chains of polysaccharide cross linked by short peptides, thus creating more firm structure causing difficult penetration of [36], in spite of, TiO2 that synthesized in current study resulted larger inhibition zone for *Staphylococcus aureus* (27mm.) then (18mm.) in *Staphylococcus epidermidis* and *Streptococcus* sp., and give less inhibition zone against *Klebsiella* and no inhibition zone against *Pseudomonas* sp. These results clearly agreed with [37], that NPs inhibited growth of the gram-positive by 90% but gram-negative was much more resistant.

A number of surveys have proposed the possible mechanisms involving the nanoparticles interaction with the biomolecules. It is established that microorganisms are negatively charge while metal oxides are positively charge. This generates an “electromagnetic” attraction between the treated surface and microbe. The contact between them leading to microbial oxidation and rapid death [38]. It is believed that nanoparticles release ions, which react with the thiol group (-SH) of the surface protein molecules of bacteria. Such

proteins extend beyond the cellular membrane of bacteria, allowing nutrients to transport over the cell wall. Nanomaterials deactivate the proteins, decreasing the permeability of membrane and finally leading to cellular death [39].

The mechanism of microbial toxicity of titanium nanoparticles is poorly known [40], reported that the main mechanism of antimicrobial effect of nano-TiO2 against *Salmonella typhimurium* and *E. coli* is rupture of bacterial inner wall, and releasing of cytoplasmic content after 5 min of treatment in a dose-dependent manner. Nanoparticles are attracting a great contract of attention because of their potential of accomplishing specific processes and selectivity, mainly in pharmaceutical and biological applications [41].

Larvicidal effect of titanium nanoparticles

During larvicidal analysis, larval development and was monitored and the larvae death was scored after 24 hr. then mortality rate measured. The results revealed that there was marked mortality recorded during the development of (*Musca domestica*) larvae in dose dependent manner in each cup treated with prepared titanium nanoparticles as showed in figure (6). Highest mortality was observed for 1stfollowed by 2nd then 3rd larval stage when treated with synthesized titanium nanoparticles with phenotypic variation observed in 2nd and 3rd larval stage treated with 100 mg/ml synthesized titanium nanoparticles as presented in figure (9). In addition, Normal development was observed in the control and in vials treated with banana peel extract (BPE) during development of second and third larval stage with no mortality observed. While negligible mortality observed in 1st larval stage vials treated with water and BPE (6.6%, 6.6%) as showed in figure (10).

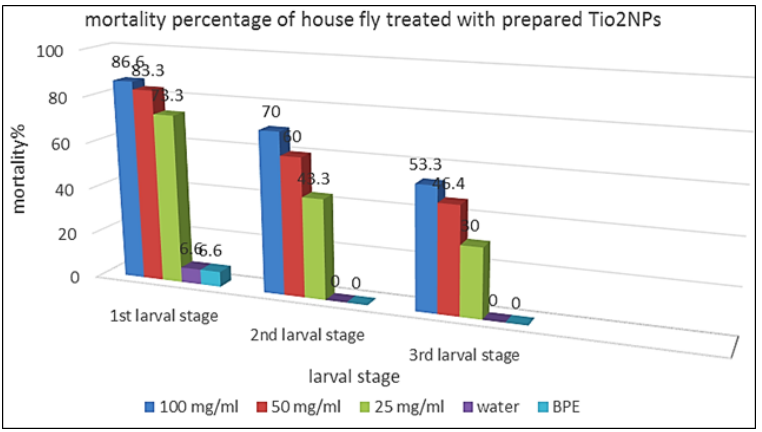

Fig 9: Mortality percentage of house fly larvae treated with synthesized TiO2 NPs

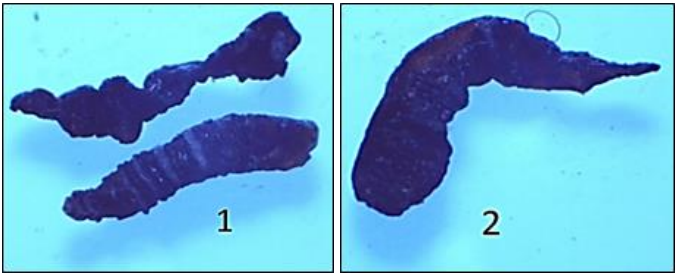

Fig 10: Phenotypic variation observed in (1): 2nd larval stage, (2): 3rd larval stage of *Musca domestica* treated with prepared titanium nanoparticles (100 mg/ml)

The findings of larvicidal activity noticeably indicate that the mortality % being proportional to the prepared nanoparticles concentration. This proves that concentration plays essential role in larvicidal activity. Each test included a control group with three replicates for each concentration. All the tested elements that showed lethal effect and mortality were progressively dose-dependent.

Larvicidal results in current study agreed with [42]. The TiO2 NPs are administered by feeding. After hatching from the egg, the larva begin feeding with treated food. Once these NPs pass in larval gut, it interferes with the developmental process creating atypical larval behavior and leading to unusual phenotypes. Larvae when fed with TiO2 NPs shows neuronal defect, then the larvae death is detected.

As reported by [43]. The possible mechanism behind the death of house fly is due to the dispersion of metal nanoparticles via oral route or through rupturing of the cuticle membrane, thereby entering into the cavity of insect body. TiO2 nanoparticles has earlier been informed to affect the surviving ability of the being by generating ROS (reactive oxygen species), these particles are causative agent for oxidative stress. A similar process is expected by [44]. Oxidative stress is reported to cause developmental putting back of numerous insects. One probable mechanism is interference of eggs with TiO2 found within the food material. Leadings to eggs damage due to its toxic tension, then the egg do not proceed on its next developmental stages. An analogous mechanism was recorded by magnetite NPs effect against *Drosophila melanogaster* [45] and silver NPs against *Culex pipiens* [46].

In producing nanoparticles using whole or part of plant, concentrates is integrated with metal salt solution at ambient conditions. Concentration of the plant extract, its Nature and concentration of the metal salt, temperature at which the reaction is carried out, reaction time and pH are recognized to effect

of nanoparticles production rate in terms of their quantity and characteristics [47, 48].

Banana peels are primarily consisting of cellulose, hemicelluloses and pectin and the functional groups linked with these polymers in addition to the proteinaceous material may thus be involved in reducing the titanium [49]. Biological components are known to cooperate with metal salts by these functional groups and mediate their reduction to nanoparticles [50, 51]. (23) Used Banana peel waste concentrate as an effective green material for quick production of gold and palladium nanoparticles. These nanomaterials displayed antifungal antibacterial activities towards pathogenic fungi and bacteria cultures [52].

A research by [53] have also used aqueous extract of banana peel for quick synthesis of different nanoparticles. They used different reaction conditions to produce silver nanoparticles that are used in designing of newer drugs. Thus, the use of banana peels extract has been shown to be simple, non-toxic, eco-friendly and low cost method of synthesizing nanoparticles and could replace the cost intensive conventional protocols of producing nanoparticles [54, 55]. In addition, the banana peels are abundantly available agricultural waste materials that are usually discarded [6, 56].

In this study, the researchers used banana peels extract as a new green synthesis method to synthesized titanium nanoparticles, this method has not been carried before. Prepared titanium nanoparticles displayed good antibacterial applications against many serious human pathogens. In addition, good larvicidal applications against *Musca domestica* resulted. Thus, these results give new insight for using this method to prepare titanium nanoparticles and use it in many other biomedical applications.

Conclusion

Our study implied a green, eco-friendly and rapid synthetic method as a new approach to produce TiO2 NPs by using agricultural waste material (banana peel) at optimum parameters of reaction that can easily attained to obtain nanoparticles with unique characteristics. Moreover, various applications of green synthesized nanoparticles against infectious bacteria, and health threatening insect (house fly) were also examined that denote the promising uses of BPE to produce titanium nanoparticles to be applied as a new approach to control these heaths frightening problems.

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Chapter - 5
Aquatic Toxicology

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Chapter - 5

Aquatic Toxicology

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Abstract

Toxicology is a scientific discipline which includes biology, chemistry, pharmacology and medicines which studies the adverse effects of chemical substances on living organisms. It also includes the practice of diagnosing and treating exposures to toxins and toxicants. Therefore, *aquatic toxicology* is the study of the effects of harmful substances such as various chemicals and other materials and activities caused by humans and nature on various categories of aquatic organisms. This chapter concludes with an idea about aquatic toxicology, test involved in identifying the source and the importance of available resources. Aquatic toxicology is one of the key problems faced in our modern world. Though there have been massive technological advancement in these recent days, it has also invited negative effects to the environment. It is the duty of every individual to save our mother nature.

Keywords: Toxicology, aquatic toxicology, acute, chronic, marine

Introduction

Toxicology is a scientific discipline which includes biology, chemistry, pharmacology and medicines which studies the adverse effects of chemical substances on living organisms [1]. It also includes the practice of diagnosing and treating exposures to toxins and toxicants [2]. Therefore, *aquatic toxicology* is the study of the effects of harmful substances such as various chemicals and other materials and activities caused by humans and nature on various categories of aquatic organisms [3]. This field of study includes freshwater, marine water and sediment environments [4].

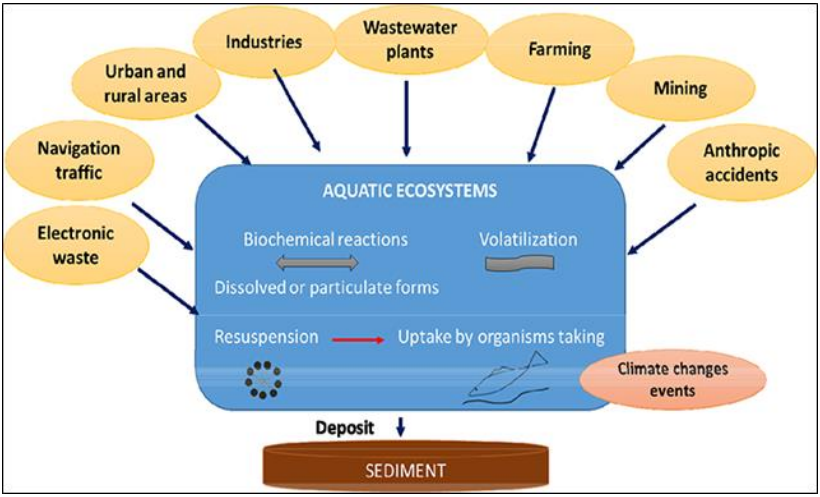

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Fig 1: Sources of various aquatic contaminants

Marine pollution occurs due to the incorporation or entry of various chemicals, particles, agricultural, residential and industrial wastes, or the invasive organisms into the marine ecosystem [5]. Statistic shows about 80 percent of the marine pollutants come from land [6]. Polluted air also contributes in increasing toxicity in the aquatic environment as it carries chemicals in the form of dirt and pesticides. Oil pollution toxicity to marine ecology is an outcome of oil spills that contain *polycyclic aromatic hydrocarbons*, a classic example is the oil spill from *Exxon Valdez* [7]. Another contributing factor is the water flowing as surface run off which carries lot of harmful chemicals with it [8]. Cyanotoxins are another very harmful pollutant found in lakes and oceans where there is high concentration of phosphorous [9]. They are produced by cyanobacteria (or blue-green algae) which multiply exponentially in the presence of phosphorous [10]. It is so harmful that it can kill the animals and humans [11].

Toxicological effects

The toxicity of a specific substance varies in different fish species and can change with water temperature, pH and ion concentration [12]. In re-circulation systems, waste materials and microbial degradation can reach toxic levels [11]. Chemical intoxication and other environmental stressors can lead to infectious disease in aquatic species [13]. There are a number of effects that occur when an organism is simultaneously exposed to two or more

toxicants [14]. These effects include *additive effects*, *Synergistic effects*, *Potentiation effects* and *antagonistic* effects.

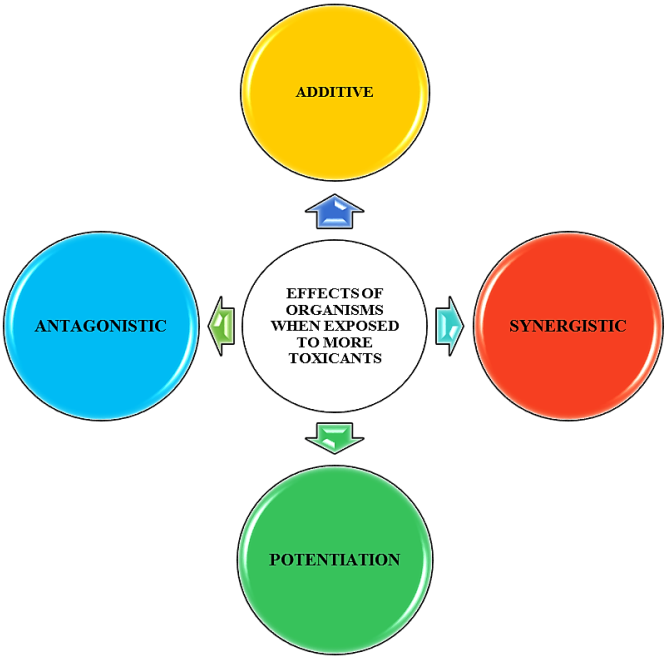


Fig 2: Sources of various aquatic contaminants

Potentiation is an effect that occurs when an individual chemical has no effect is added to a toxicant and the combination has a greater effect than just the toxicant alone [15]. Finally, an antagonistic effect occurs when a combination of chemicals has less of an effect than the sum of their individual effects [16]. The use of acute toxicity testing on fish was first established around 1930s and in a couple of decades, this became a public issue which eventually lead to increased efforts being taken to standardize toxicity testing techniques [17].

Aquatic toxicity tests

Aquatic toxicology tests provide us the qualitative and quantitative data on adverse effects on aquatic organisms from a toxicant [18]. These tests can be performed in the field or in the laboratory where field tests generally refer to multiple species exposures and lab tests generally refer to single species exposure [19]. Due to differences in metabolism, rate of excretion, age, sex, health, accessibility, genetic factors, dietary factors and stress level of the organisms, different species differ in their susceptibility to chemicals [20].

Common standard test species are the fathead minnow (*Pimephales promelas*), daphnids (*Daphnia magna, D. pulex, D. pulicaria, Ceriodaphnia dubia*), midge (*Chironomus tentans, C. riparius*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnow (*Cyprinodon variegatu*), zebra fish (*Danio rerio*), mysids (*Mysidopsis*), oyster (*Crassostrea*) and mussels (*Mytilus galloprovincialis*) [21]. As defined by ASTM, these species are routinely selected on the basis of availability, commercial, recreational, and ecological importance, past successful use, and regulatory use.

There are a variety of standardized test methods which are published by various agencies namely, the American Public Health Association, US Environmental Protection Agency (EPA), ASTM International, International Organization for Standardization, Environment and Climate Change Canada, and Organisation for Economic Co-operation and development [22, 23]. These standardized tests help to compare the results between laboratories. There are various toxicity tests that are widely accepted in scientific literature and regulatory agencies. The type of test used depends on many factors such as specific regulatory agency conducting the test, resources available, physical and chemical characteristics of the environment, type of toxicant, test species available, laboratory vs. field testing, end-point selection, and time and resources available to conduct the assays are some of the most common influencing factors on test design [24]. Before going into the tests, we should have a brief idea on what is exposure science, as the organisms, while being tested, has to be exposed to different environment [25].

Exposure system

Exposure science is the study of an organism's contact with chemical, physical, biological agents or other health risk (e.g. accidental) occurring in their environments, and advances knowledge of the mechanisms and dynamics of events either causing or preventing adverse health outcomes [26]. Exposure science plays a fundamental role in the development and application of epidemiology, toxicology, and risk assessment [27]. It provides critical information for protecting the ecosystem health [28]. There are different methods in which the control and test organisms are exposed to test solutions [29]. These are *static, recirculation, renewal* and *flow-through*.

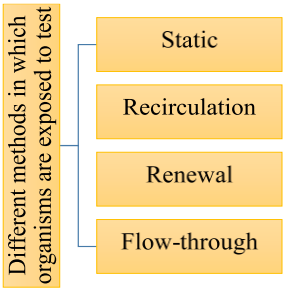


Fig 3: Methods exposed to test solutions

Types of tests

Static: In this method the organisms are exposed in still water where the toxicants are added to achieve the desired concentration. The control and test organisms are the placed in the test solutions and the water is not changed for the entire duration of the test [30].

Recirculation: A recirculation test exposes the organism to the toxicant in a similar manner as the static test, except that the test solutions are pumped through a filter to maintain water quality, but not reduce the concentration of the toxicant in the water. The water is circulated through the test chamber continuously with the help of aerator. There are high possibilities that the toxicant may be affected due to the usage of aerator or the filter [31].

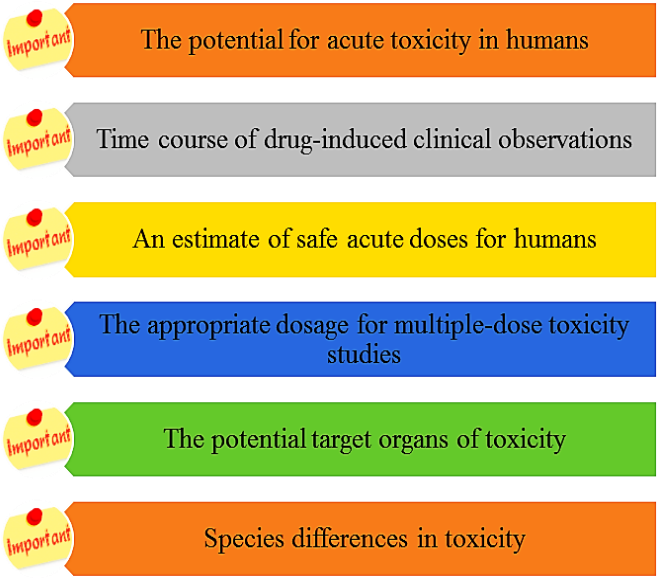
Renewal: This method also exposes the organism to the toxicant in a similar manner as the static test because it is in still water. However, in a renewal test the test solution is renewed after constant intervals by transferring the organism to a fresh test chamber keeping the concentration of the toxicant same [32].

Flow-through: In this method, as the name suggests, the organisms are exposed to the toxicant with a flow into the test chambers and then out of the test chambers. This can be either intermittent or continuous flow. A stock solution of the correct concentrations of contaminant must be prepared beforehand. Metering pumps or diluters will control the flow and the volume of the test solution, and the proper proportions of water and contaminant will be mixed [33]. There are various types of tests that can be performed on organisms which are discussed below as follows:

Acute toxicity tests: These tests are short-term exposure tests, usually for 24 hrs, and generally use lethality as an endpoint. In acute exposures, organisms come into contact with higher doses of the toxicant in a single

event or in multiple events over a short period of time and usually produce immediate effects, depending on absorption time of the toxicant [34]. These tests are generally conducted on organisms during a specific time period of the organism's life cycle, and are considered partial life cycle tests.

This test provides information on the following:



Please note that acute test is not valid if the mortality in the control sample is greater than 10%. The results are reported in EC50, or concentration that will affect fifty percent of the sample size [35]. The diagram below indicates the various aquatic toxicity test grouped into the following categories.

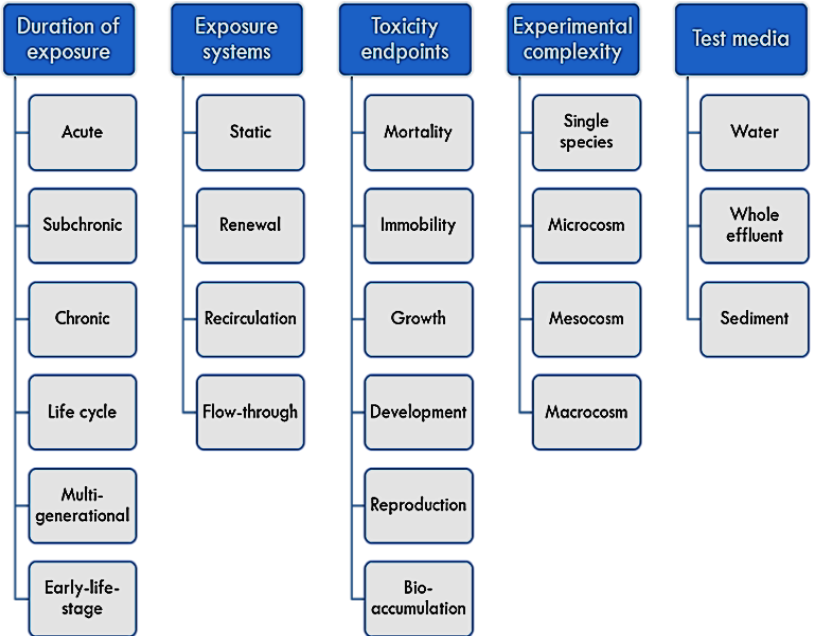


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Fig 4: Aquatic toxicity tests grouped into categories

Chronic tests: Chronic toxicity is in contrast to acute toxicity, which occurs over a shorter period of time to higher concentrations. These tests are carried out on the organisms in repeated manner or in continuous administration of the test sample for a major part of their life span [36]. Tests are long-term tests (weeks, months years), relative to the test organism's life span (>10% of life span), and generally use sub-lethal endpoints. The study design and endpoints evaluated are similar to the sub chronic toxicity [37, 38]. For this longer duration study, the numbers of animals per treatment group are larger to account for possible losses over the course of the study and to improve statistical power.

In chronic exposures, organisms come into contact with low, continuous doses of a toxicant. Results of aquatic chronic toxicity tests can be used to determine water quality guidelines and regulations for protection of aquatic organisms [39]. Short-term sub lethal tests are used to evaluate the toxicity of effluents to aquatic organisms. These methods are developed by the EPA, and only focus on the most sensitive life stages. Endpoints for these tests include changes in growth, reproduction and survival. NOECs, LOECs and EC50s are reported in these tests [40, 41]. Chronic tests are not considered

valid if mortality in the control sample is greater than 20%. These results are generally reported in NOECs (No observed effects level) and LOECs (Lowest observed effects level).

Early life stage tests are considered as sub chronic exposures that are less than a complete reproductive life cycle and include exposure during early, sensitive life stages of an organism. These exposures are also called critical life stage, embryo-larval, or egg-fry tests. Early life stage tests are not considered valid if mortality in the control sample is greater than 30%.

Bioaccumulation tests: Bioaccumulation is defined as the increase of contaminant concentrations in aquatic organisms following uptake from the ambient environmental medium. Different sources of exposure contribute to contaminant bioaccumulation. Bioaccumulation refers to how pollutants enter a food chain and relates to the accumulation of contaminants, in biological tissues by aquatic organisms, from sources such as water, food, and particles of suspended sediment [42, 43]. It is the net result of all uptake and loss processes, such as respiratory and dietary uptake, and loss by egestion, passive diffusion, metabolism, transfer to offspring and growth.

Bioaccumulation tests are toxicity tests that can be used for hydrophobic chemicals that may accumulate in the fatty tissue of aquatic organisms. Toxicants with low solubility in water generally can be stored in the fatty tissue due to the high lipid content in this tissue. The storage of these toxicants within the organism may lead to cumulative toxicity [44]. Bioaccumulation tests use bio concentration factors (BCF) to predict concentrations of hydrophobic contaminants in organisms. The BCF is the ratio of the average concentration of test chemical accumulated in the tissue of the test organism (under steady state conditions) to the average measured concentration in the water. Freshwater tests and saltwater tests have different standard methods, especially as set by the regulatory agencies. However, these tests generally include a control (negative and/or positive), a geometric dilution series or other appropriate logarithmic dilution series, test chambers and equal numbers of replicates, and a test organism [45]. Exact exposure time and test duration will depend on type of test (acute vs. chronic) and organism type. Temperature, water quality parameters and light will depend on regulatory requirements and organism type.

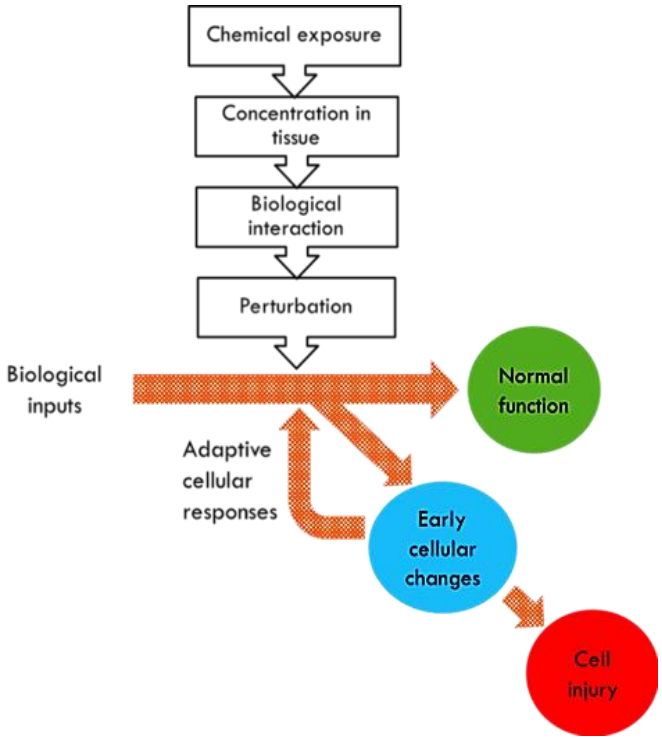

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Fig 5: A toxicity pathway depicting how chemical exposure could lead to cell injury

In some countries wastewater dischargers (e.g., factories, power plants, refineries, mines, and municipal sewage treatment plants) are required to conduct periodic whole effluent toxicity (WET) tests. For facilities discharging to freshwater, effluent is used to perform static-acute multi-concentration toxicity tests with *Ceriodaphnia dubia* (water flea) and *Pimephales promelas* (fathead minnow), among other species [46].

The test organisms are exposed for 48 hours under static conditions with five concentrations of the effluent. The major deviation in the short-term chronic effluent toxicity tests and the acute effluent toxicity tests is that the short-term chronic test lasts for seven days and the acute test lasts for 48 hours. For discharges to marine and estuarine waters, the test species used are sheepshead minnow (*Cyprinodon variegatus*), inland silverside (*Menidia beryllina*), *Americamysis bahia* and purple sea urchin (*Strongylocentrotus purpuratus*).

Sediment tests: Sediment toxicity testing has become a fundamental component of regulatory frameworks for assessing the risks posed by contaminated sediments and for development of chemical sediment quality guidelines [47]. Over the past few decades, sediment toxicity testing methods have advanced considerably, with tests now providing greater environmental relevance. There is better understanding of species sensitivity to contaminants, organism behaviour, exposure pathways, and design considerations necessary to provide relevant exposure conditions.

At some point most chemicals originating from both anthropogenic and natural sources accumulate in sediment. For this reason, sediment toxicity can play a major role in the adverse biological effects seen in aquatic organisms, especially those inhabiting benthic habitats [48]. A recommended approach for sediment testing is to apply the Sediment Quality Triad (SQT) which involves simultaneously examining sediment chemistry, toxicity, and field alterations so that more complete information can be gathered. Collection, handling, and storage of sediment can have an effect on bioavailability and for this reason standard methods have been developed to suit this purpose.

Advances in our understanding of how chemical bioavailability influences toxicity, and the increased range of sub lethal endpoints used for assessment, have resulted in the need for improved guidance on how to design tests, what to measure and monitor, and how to interpret results to achieve sound test outcomes.

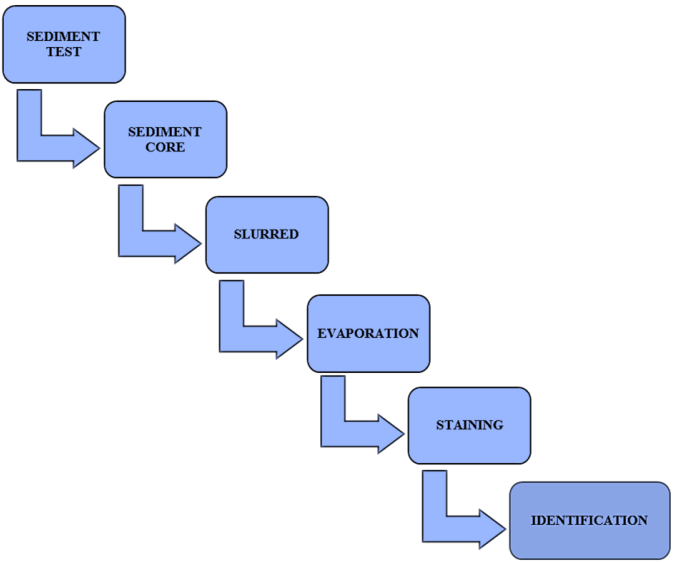

Fig 6: Flowchart of steps involved in sediment test

Sediment testing includes taking sediment samples from the sediment of an aquatic environment and quantifying the amount of microplastics in benthic regions.

Importance of aquatic toxicology resources

Aquatic toxicology resources help in developing international voluntary standard methods for aquatic toxicity testing [49]. "Ecotox" A database maintained by Environmental Protection Agency provides single chemical toxicity information for both aquatic and terrestrial purposes [50]. United States Environmental Protection Agency publishes manuals on aquatic toxicity test procedures. Some of the available resources are as follows:

Several worldwide, non-profitable scientific research centres help us to understand regarding the environmental stressors, environmental education, and the use of science in environmental policy.

Conclusion

This chapter concludes with an idea about aquatic toxicology, test involved in identifying the source and the importance of available resources. Aquatic toxicology is one of the key problems faced in our modern world. Though there have been massive technological advancement in these recent days, it has also invited negative effects to the environment. It is the duty of every individual to save our mother nature.

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Chapter - 6
Human Genome Research and Its Implication in Understanding Human Biological Categories

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Chapter - 6

Human Genome Research and Its Implication in Understanding Human Biological Categories

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Abstract

The post HGP period has witnessed a revolutionary progress in human genome research, particularly due to rapid development in genomic technology. Human genome research carried out so far has revealed that 99.9 percent genetic sequence of all human beings is identical. This 0.1 percent genetic variation which is due to single base pair difference in the nucleotide of the DNA is responsible for the differences among us. New findings of human genome research have reignited the debate on the existence of biological categories among the humans. As part of this there has been much discussion whether the Human Genome Project supports concepts of race or undermines them.The mapping of total genetic sequences has found that different genes for physical traits such as skin and hair colour can be identified between individuals, but, no consistent patterns of gene across the human genome exist to distinguish one race from another. The HGP found that 99.9 percent genetic sequence of all human beings are identical and hence their does not exist any question of biological categorization of human population. The biological concept of race is now believed to be untenable. In this way mapping of human genome could be pivotal in promoting the concept of one race, the human race. There is no existence of any 'particular race' having distinct, unifying genetic identities. In contrast, there is ample variation within the same race.

Keywords: DNA, SNP, genome, human genome project, HapMap, biological categories, race

Introduction

Biological anthropology as a discipline has traversed a long chapter starting from the notes and diaries of the explorers and travelers to the most exciting field of genomic studies. DNA is found inside the nucleus in form of coiled thin thread like structures called chromosomes. In addition to

nucleus, a small amount of DNA is also found in the mitochondria of the cell that we called as mitochondrial DNA. The complete set of DNA present in the chromosomes (23 pairs in the somatic cell and 23 numbers in the germ cells) and mitochondria is called as human genome. The DNA sets present in the nucleus are called as nuclear genome, whereas DNA found in the mitochondria are called mitochondrial genome. DNA is polynucleotide which is made up of deoxyribose sugar, nitrogenous base and phosphate group. Of these, the deoxyribose sugar and the phosphate forms the two strands of DNA, while the nitrogenous base binds the two strands together through weak hydrogen bonds. The nitrogenous bases of DNA are of four different types i.e. adenine (A), cytosine (C), guanine (G) and thymine (T).These four bases contribute in forming the helical structure of the DNA. These bases are called base pair because they are complementary to each other i.e. adenine is always paired with thymine and guanine paired with the cytosine by hydrogen bonds. The genetic code of DNA is determined by the order of the sequence of these four nucleotide bases. The size of an organism entire genome is often measured in base pairs. The haploid human genome (23 chromosomes) has around 3.2 billion base pairs with around 20000-25000 distinct protein-coding genes, while a somatic cell has twice this amount (6 billion base pair). Since male has a single Y chromosome which is smaller in size than the X chromosome of the female, they have fewer base pairs than the female. It is estimated that around 57 million base pairs is present inside the Y chromosome as compared to 156 million base pair in the X chromosome of the female. These base pair are arranged systematically in sequences in DNA. But these sequences are sometimes disturbed and thus a number of variations occurred in the sequences of the bases. This type of genetic variation which occurs as a result of difference in a single nucleotide is called single nucleotide polymorphisms (SNPs). For example, thymine of a base pair (T-A) is replaced by a cytosine in a certain stretch of DNA. Such an anomaly in a single nucleotide is called as SNP. Single Nucleotide Polymorphisms occurs throughout the human DNA with an average of one in every 1000 nucleotides. The main goal of the human genome research is to prepare a complete sequence of human genome to determine the sequence of individual's genes, larger genetic regions, full chromosomes, or entire genomes of any organism.

Scientists have been trying to sequence the base pair of human genome for a long period of time since 1977 when Sanger tried to determine the order of nucleotides of DNA. In the same year, the first human gene was isolated and sequenced. In 1987, with new techniques developed, sequencing of two genes was carried out. Further development of new methods and

technology has led to the rapid discovery and mapping of human genes, but complete mapping of human genome was still a distant dream. Finally in 1990, the Human Genome Project (HGP) was launched by the National Institute of Health and the U.S. Department of Energy with an objective of complete mapping of human genome and sequencing of 3 billion base pairs of human DNA. The first priority of the project was to determine the exact order of these base pairs in the DNA, which we termed as 'sequencing of base pair'. Besides US, several countries like Britain, France, Canada, China, Germany and Japan have made important contributions to this international collaborative research project. More than 28000 researchers from different countries were directly involved in the project. The DNA samples required for the project were collected from the volunteers belonging to diverse population groups. The collected DNA samples were used to create 'DNA libraries' from which samples were used for DNA sequencing.

The post HGP period has witnessed a revolutionary progress in human genome research, particularly due to rapid development in genomic technology. The various genomic studies carried out in different parts of the world have yielded important knowledge and information about human genome along with pattern of human migration and admixture of population groups of different geographic origins and ethnic origins. Like HGP, another mega international collaborative project on human genome research called International HapMap project was launched in 2002. The aim of International HapMap project was to develop a haplotype of the human genome to describe the common pattern of human variation. Haplotype may be defined as the sets of SNPs on the same chromosome which are inherited together in blocks. A SNP block may have a large number of SNPs, but a few SNPs are enough to identify the haplotypes in a block. After completion of the HGP and HapMap project, scientists from China, Germany, the U.K and the USA have started another international collaborative project called the 1000 Genomes Project in 2008. The aim of 1000 Genome project was sequencing of at least 1.000 volunteers from multiple population groups worldwide in order to understand the genetic contribution to human health and diseases. The project goal was to prepare a detailed catalogue of human genetic variation and genotype data of the major world population groups with frequencies of at least 1 percent in the population studied. The findings and the outcome of these human genome research projects are discussed in the following part of this chapter.

Findings of human genome research studies

Human genome research carried out so far has revealed that 99.9 percent genetic sequence of all human beings is identical. This 0.1 percent genetic

variation which is due to single base pair difference in the nucleotide of the DNA is responsible for the differences among us. As of 2015, the typical difference between the genomes of two individuals was estimated at 20 million base pairs which is equivalent to 0.6 percent of the total base pairs (Auton A *et al*. 2015). These variations may be unique or may common in many individuals and may act as genetic markers to locate genes that are associated with various types of diseases. The number of SNPs discovered till 2017 was 324 million (NCBI, 8 May 2017).

The initial draft version of the Human genome sequence was completed in 2000 and the report was published in the journal *Nature* in February 2001. The final version of HGP with complete human genome sequence was finished in April 2003 two years ahead of the scheduled time. The difference between the draft and finished versions is defined by coverage, the number of gaps and the error rate. The draft version covers almost 90 percent of the entire DNA sequences of the human genome. The final version covers around 99.99 percent of the human genome with an accuracy of 99.99 percent. The final form of the human genome contained 2.85 billion nucleotides, with a predicted error rate of 1 event per 100,000 bases sequenced. Furthermore, the number of gaps has been reduced to only 341 gaps from 147,821 gaps remain in draft form. The number of protein-coding genes has been estimated in between 20,000 to 25,000 genes compared to 40,000 protein-coding genes predicted by earlier draft publications. In addition to accomplishments of the projected goals of HGP, the researcher has able to identify 3.7 million human genetic variations called *Single Nucleotide Polymorphisms* (SNPs) and generation of full length complimentary DNA (cDNAs) for more than 15000 (70%) of known human genes. Complimentary DNA is a synthetic type of DNA generated by the scientist by using mRNA as a template through use of enzymatic reactions to convert its information back into cDNA and then clone it, creating a collection of cDNAs, or a cDNA library.

The result of first phase of HapMap project was published on October 27, 2005. In its first phase, the haplotype data were collected from a total of 270 individuals belonging to four different ethnic groups, viz. Yoruba from Nigeria, European descent group from Utah, Han Chinese from China and Japanese from Japan. In this phase, a total of 1.1 million SNPs were genotyped. The phase II result was published in 2007 where a total of 3.1 million SNPs were studied in the same set of individuals of phase I. However, in phase III, the number of the sample has increased to 1184 individuals belonging to 11 different population groups. In addition to the

four earlier population groups, samples were collected from another seven new population groups. These population groups were Masai in Kinyara from Kenya, Luhya in Webuye from Kenya, Chinese of Metropolitan Denver, USA, Gujrati Indians in Houstand, USA, Toscani in Italy, African ancestry in Southwest USA and Mexican ancestry in Los Angeles. The total number of SNPs genotyped in this phase was however confined to 1.67 million. In addition to genotyping of SNPs, the phase III has sequenced ten segments of 100000 bases from the well-characterized region of the human genome. The researcher found that most variants are relatively uncommon, but they also found large number of rare variants. The International HapMap Project has thus provided a high-resolution catalog of the allele frequencies of hundreds of thousands of SNPs distributed throughout the genome, across human populations.

The 1000 genome project which ran between 2008 and 2015 and collected samples from 2504 individuals belonging to 26 population groups of West Africa, Europe, East and South Asia and the America. In its first phase, samples from 160 individuals were collected from the four original population of HapMap. In this initial phase, more than 9 million new SNPs, many indels and structural variations have been identified. The project finally discovered 88 million variants, including 84.7 million SNPs, 2.6 million short insertions and deletions and 60,000 structural variations. Some of the other findings of the project were that a typical human genome differs at 4-5 million sites, of which 99 per cent of these variants are SNPs and indels (insertions /deletions).This variation is found to be highest among the individuals of African ancestry; More than 2000 variants per genome is found to be associated with complex traits and 24-30 variants per genome implicated in rare diseases. The 1000 Genomes Project has shown, for example, that populations with African ancestry have the highest proportion of genetic variants, reflecting the greater diversity of African populations.

New findings of human genome research have reignited the debate on the existence of biological categories among the humans. In biological anthropology, these biological categories are often referred as ‘race’. The debate over race for long time is based on two opposing constructions, one is in support of the opinion that race is ‘culturally constructed’ favoured by most sociologists and the other is ‘natural and biological determined category’ supported mostly by biological scientist. In biological anthropology, the classification of race is based chiefly on some physical heritable characteristics called as ‘racial criteria’, such as, skin colour, stature, eyes, hair, etc. that are specific to a particular group of population.

According to the traditional concept of race, it is defined as a classificatory device, the members of which have relatively uniform genetic identity that differs from the other group. On the other hand, many scholars consider race as a 'social construct without biological foundation'. Many scholars are of opinion that scientific study of race as a biological category is scientifically obsolete. In this era, they are influenced by genetic research but are also shaped by discussion and debate that takes place far from laboratories. One of the newer influences on these concepts were from the outcome of the Human Genome Project. The findings of the project have created a new set of debates about possible links between genetics and human behaviour. As part of this there has been much discussion whether the Human Genome Project supports concepts of race or undermines them. It was also expected that the HGP would definitely provide answers to the enduring questions concerning the scientific validity of race as a biological category.

The publication of draft version of HGP in 2000 and final version in 2003 has made significant revelations about human genetic make-up and has opened the door for better understanding about the concept of human biological categories. The mapping of total genetic sequences has found that different genes for physical traits such as skin and hair colour can be identified between individuals, but, no consistent patterns of gene across the human genome exist to distinguish one race from another. The geneticists started to put forwarded their opinion that such differences are primarily cosmetic and superficial that involve a very small number of genes which were selected historically in particular environment. The US Department of Energy, which is one of the coordinator of HGP, made a public statement on the existence of the racial categories among human population after the publication of the project report, According to the statement, *'DNA studies do not integrate that separate classifiable sub-species (race) exist within modern humans'*. The HGP found that 99.9 percent genetic sequence of all human beings are identical and hence their does not exist any question of biological categorization of human population. Craig Venter, the chief scientist of Celera Genomics, which was an associated partner in the HGP made a public statement after the publication of the draft report that *'Race is not a scientifically valid construct. It has no scientific or genetic basis'*. This scientific claim about the non-existence of the biological categories was reaffirmed by an another press release by Celera Genomics, authored by Culliton in 2001 which states that '…in the past, some scientist used observation of racial differences to support the racist doctrines, Racism, rooted in the erroneous concept of biological racial superiority. The biological concept of race is now believed to be untenable. In this way

mapping of human genome could be pivotal in promoting the concept of one race, the human race'. In 2004, Francis Collins, then head of the National Human Genome Research Institute called race as a 'flawed' and 'weak' concept and argued that science needed to move beyond race. S. Paablo a geneticist and the then director of the Max Planck Institute for Evolutionary Anthropology in Germany, who worked on the Neanderthal genome has stated that 'the study of complete genomes from different parts of the world has shown that even between Africa and Europe, for example, there is not a single absolute genetic difference, meaning no single variant where all Africans have one variant and all Europeans another one, even when recent migration is disregarded,'. To him, two persons from the same part of the world who look superficially alike are less related to each other than they are to persons from other parts of the world that may look very different. According to him, race although culturally important reflects just few continuous traits determined by few fractions of our genes'. A landmark study carried out by the scientist of Stanford University in 2002 on human variation by examining 4000 alleles across seven geographical regions also negates the genetic validity of the human biological categories. The study has found that only 1 percent of the studied population have region-specific gene, 7.4 percent of the alleles were specific to one particular geographical region and almost half of the alleles were present in all the seven regions. No trademark genes or alleles or any other specific genetic features that can be identified to a particular population group was found. The basic understanding about human biological categories on the basis of HGP can be summarized very briefly in following lines:

The HGP supports the fundamental unity of all human beings which share 99.9% of their DNA. The diversity of human beings at genetic level is only 0.1 percent. There is no existence of any 'particular race' having distinct, unifying genetic identities. In contrast, there is ample variation within the same race. There are 80 to 90 percent differences within these boundaries of the population and the difference between populations is only 10 to 12 percent. The differences in physical characteristic between racial groups are mostly cosmetic and superficial that involve a very small number of genes that were selected historically in particular environments. These superficial differences do not reflect any additional genetic distinctiveness.

The Human Genome research has provided new insights to look ourselves in a new way. The outcome of the Human Genome Project has played a significant role in the newly evolving concept of race. It has provided both natural and social scientists the opportunity to gain new

knowledge about the complex relationships between our socio-politically and biologically constructed definitions of race. Now, there is a consensus among the evolutionary biologists, geneticists and biological anthropologists that genetically identifiable biological categories do not exist among human population. There are no gene variants that are present in all individuals of one population group and not in individuals of another. No sharp genetic boundaries can be drawn between human population groups. The genetic variation that exists within a socially recognised population group is greater than the genetic variability between population groups. According to A. Angier, 'racial categories recognized by the society are not reflected in the genetic level'. The study of human genome has established that the standard labels used to distinguish people by race have little or no biological meaning. Scientists are of opinion that that the human species are evolutionarily so young, it has simply not had the chance to divide itself into separate biological groups or race. The Human Genome project has able to define the concept of race in a more scientifically credible and useful way. At the simplest level, each of us carries some set of genes that determines some of our physical traits, but they represent only a fraction of estimated 30000 total genes in our genomes. Human Genome Project has established that at base-pair level, all humans are 99.9 percent similar and that 0.1 difference is responsible for our differences in our physical traits like height, weight, skin colour, etc. The Human Genome Project has given us solid backings in understanding our genetics in a better way from where we have to move forward.

Conclusion

Despite emergence of such construction, race continues to be an evolving concept. Some researchers argue that self-identified race can be used as an indicator of geographic ancestry. They are of opinion that though the genetic differences among human groups are relatively small, these differences in certain genes can be used to categorize many individuals within broad, geographically based groupings. Computer analyses of such variations have proved the existence of genetic clustering that historically have occupied large continental and sub continental groupings. They argued that these continental clustering correspond to the division of human beings which provide biological basis for the use of traditional term race.

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