Synthesis of copper nanoparticles via green synthesis and analyzing potential effect of copper nanoparticles on *Drosophila melanogaster*

Submitted in fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

IN

BIOTECHNOLOGY

By:

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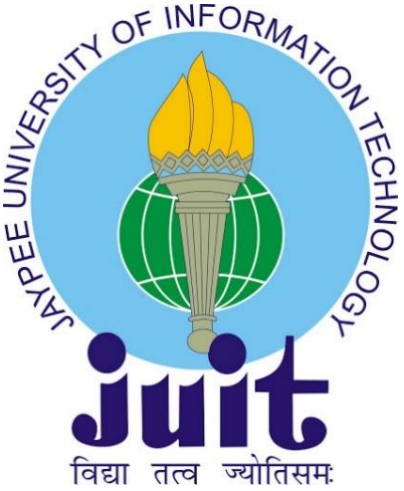

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DECLARATION BY STUDENT

We hereby declare that the project work entitled "Synthesis of copper nanoparticles via green synthesis and analyzing potential effect of copper nanoparticles on *Drosophila melanogaster*" submitted to the Department of Biotechnology and Bioinformatics, Jaypee University Of Information Technology Solan (H.P), is a bonafide record of original work done by me . The work was carried out under the supervision of Dr. Udayabanu Malairaman and co-supervision of Dr. Abhishek Chaudhary.

Harshita Shringi

This is to certify that the above statement made by the student is true to the best of my knowledge.

Date :

SUPERVISOR'S CERTIFICATE

This is to certify that the work titled "Synthesis of copper nanoparticles via green synthesis and analyzing potential effect of copper nanoparticles on *Drosophila melanogaster*" by Harshita Shringi during the end semester in June 2022 in fulfilment for the award of degree of Masters of Science in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision .This work can be sent totally or partially to any other university or college to obtain any degree or recognition.

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Signature of Co - Supervisor
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M. Sc. Biotechnology

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LIST OF ABBREVIATION

Abbreviation	Full form
Cu	Copper
NP	Nanoparticles
CuNP	Copper nanoparticles
µg/ mcg	Microgram
nm	Nanometre
mL	Milliliter
PE	Plant extract

ABSTRACT

Copper nanoparticles have became center of attraction in last decades. With their broad range of application in pharmaceuticals, medicine and drugs, we can't neglect the effect copper nanoparticles on human and environment. Their way to biosphere should be highly regulated and tested. The established physico-chemical methods came with lots of limitations and disadvantages when it comes to their usage in synthesizing particles that can be used in biological or pharmaceutical applications. Thus, we need a method which can be sustainable, safe and eco-friendly. This paved the way for term Green nanobiotechnology, where biological entities are used in producing nanoparticles. Our study aims to synthesize copper nanoparticles using extract of plant *Myrica esculenta* and also test the effect of copper nanoparticles on widely used animal model, *Drosophila melanogaster.* We were able to synthesis copper nanoparticles by using equal amount of copper sulfate pentahydrate and plant extract. Other factor affecting the synthesis of copper nanoparticles like pH, incubation time period and temperature were also studied. Also we tested the copper nanoparticles in three different concentration on *Drosophila*, where we found significant result in control versus the treated media of flies.

Keywords - Copper nanoparticles, Green synthesis, *Myrica esculenta*, Toxicity, *Drosophila melanogaster*

CHAPTER -1
INTRODUCTION

1.1 Background

The emergence of nanoscience and advancement in nanotechnology has opened the new gates in the field of science. The idea for nanotechnology was originated from the famous lecture at California Institute of Technology given by Nobel laureate Richard Feyman on 29th December,1959 titled "There is plenty of room at the bottom", this lead to the discussion of idea of nanomaterials [1]. In the last two decades major researches on nanomaterials have been carried out, scientists have explored various pathways to exploit maximum out of nanoparticles. Nanoparticles are not something new to human creations, they are obviously existed in nature from a long time. With the long history of nanoparticle's creation, their synthesis is not an absolute result of contemporary research nor it is restricted to man made substances [2]. The naturally occurring nanoparticles are prepared via natural process like weathering, redox and precipitation, physical fragmentation, gas solid, nucleation in the atmosphere, volcano eruption, wildfires or microbial process [3]. They occur in organic (proteins, polysaccharide, virus, etc.) as well as inorganic (iron oxyhydroxides, aluminosilicates, metals, etc.) form [2].

1.2 Copper Nanoparticles

Recent progress has grabbed the attention of researchers towards the generating of copper nanoparticles. With the diverse applications in wound healing and biocidal properties, being used as antibacterial, antifungal and molluscicidal agent, copper has become an attraction center. As copper is also an essential element of human body processes, therefore its amount of uptake in body must be highly regulated. Copper is large amount will show its toxic nature in environment, and for living beings. This restricts the direct usage of copper and its compound. To find a substitute to be used copper can be utilized in form of nanoparticles [4].

With their wide range of properties like high electrical conductivity, high melting conductivity, excellent solderability, low material cost, copper nanoparticles provides a cheap substitute to noble metal nanoparticles, which required large amount of resources as well as economic help [5]. Copper nanoparticles are highly oxidant and with the issues like stability, oxidation and aggregation, they remain much less investigated. The another advantage of easily oxidation property of copper is, it

oxidized to form copper oxide (CuO) nanoparticles, oxide nanoparticles easily get mixed with polymers/ macromolecules and are comparatively more stable in terms of physical and chemical properties of nanoparticles [6].

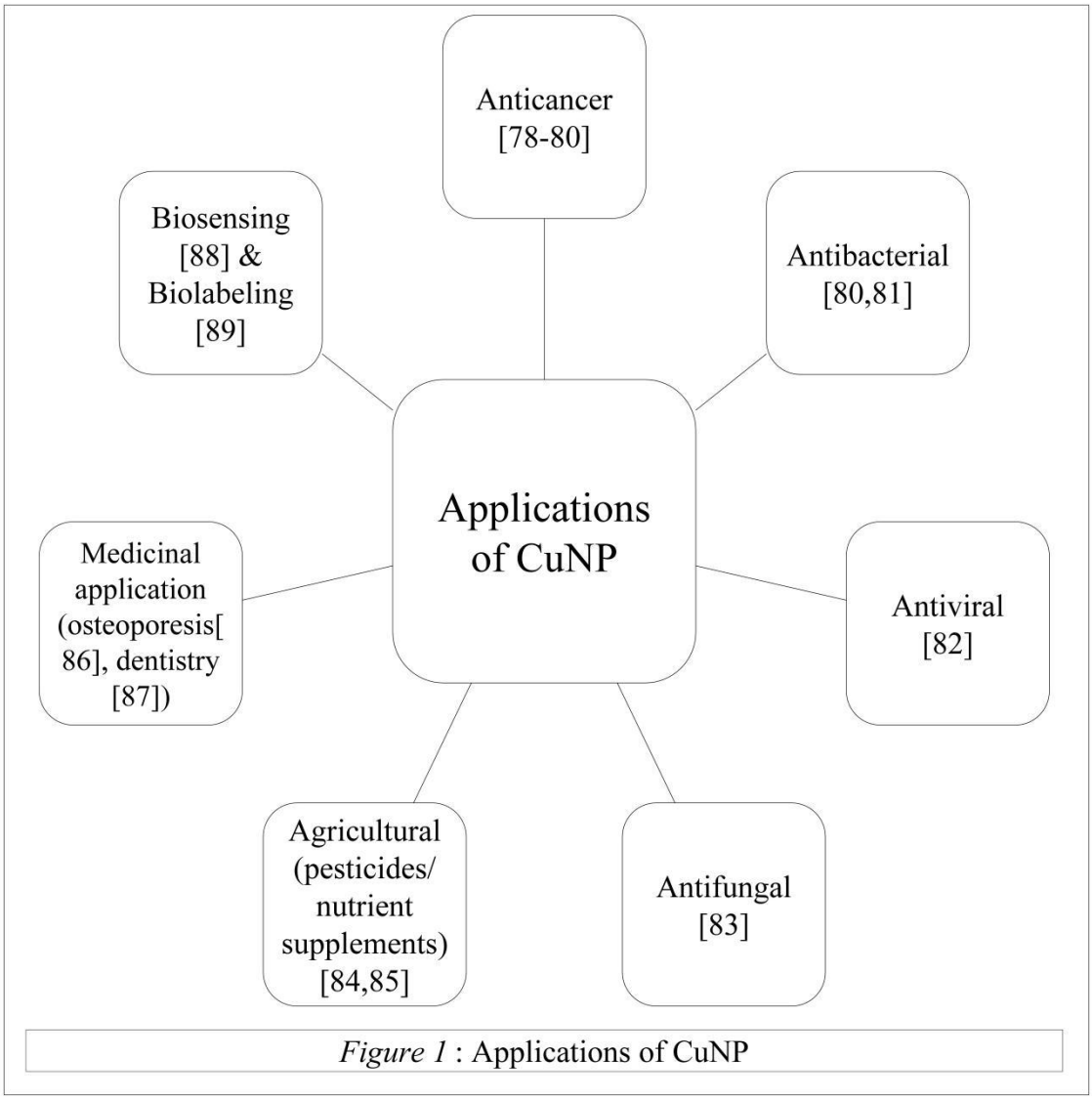


Figure 1 : Applications of CuNP

1.3 Green Nanobiotechnology

The generation of copper nanoparticles through old and conventional physical and chemical methods leads in formation of some toxic compounds as byproducts, which are environmentally hazardous. The nanoparticles synthesized from these conventional methods are not safe for medicinal purposes. The issue gave the concept of green technology or green nanobiotechnology. The term Green nanobiotechnology is defined as the generation of nanoparticles, via biological entities such as bacteria,fungi, plats and enzymes or their byproducts with the help of various biotech tools. The biological based synthesis of nanoparticles is categorized under the bottom

up methodology. The biggest advantages of exploiting plant in generating of nanoparticles is the absence of pathogenicity and toxicity of certain chemicals and physical methods. Biological synthesis is an effort to create an eco-friendly, easy and rapid methodology [7]. Our study focuses on using plants as biological route for the synthesis of copper nanoparticles. Plant extracts contains flavonoids as alkaloids, phenolic acids, polyphenol, proteins, sugar or terpenoids, these help in reducing the metal ions and then stabilizes them [8]. We have used the bark extract of *Myrica esculenta* (Figure 2b).

1.3.1 Plant extract - *Myrica esculenta*

Myrica esculenta (Table 1)was used as biological source in production of copper nanoparticles. *M. esculenta* is the only species of *Myrica* found in India in the regions of Meghalaya and sub-Himalayas (Figure 2a). They are commonly called as Boxberry, Kaiphal and Kathphala [9]. The plant has vitamin C in an abundant amount and also contains phytochemicals like tannins, flavonoid, phenols and flavonols [10]. Bark extract was chosen as the plant part from M. esculenta. Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenoy)-4-chromonone) is an important chemical constituent found in the bark extracts of plant which belongs to phytochemical class - flavonoid. It act as an antioxidant and has various health benefits [11]. Bark extract is acting as reducer and stabilizer throughout the production process of copper nanoparticles.

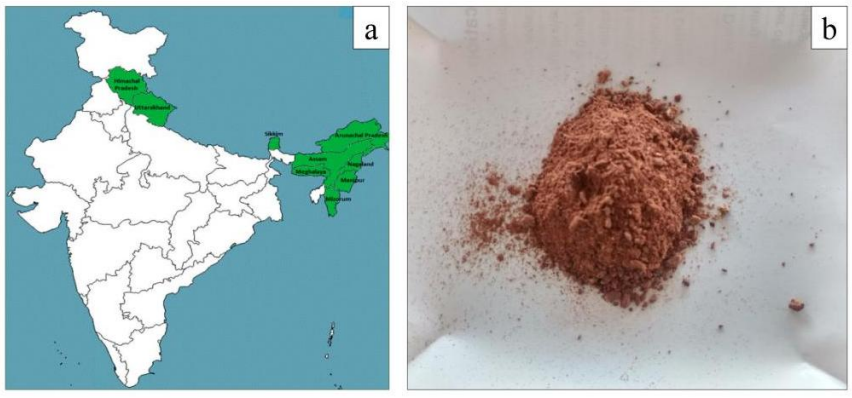


Figure 2 :(a) Natural distribution of *Myrica esculenta* [9] (b) Bark extract

Table No - 1: *Scientific classification of Myrica esculenta*	
Kingdom	Plantae
Clade	Tracheophyte
Clade	Angiosperms
Clade	Eudicots
Clade	Rosids
Order	Fagales
Family	Myricaceae
Genus	Myrica
Species	Esculenta

1.4 Animal model - *Drosophila melanogaster*

Since 100 years ago, *Drosophila melanogaster* are widely used as in-vivo model organism in order to understand the basic concepts of genetics and development. With their short life cycle, the well-defined life stages and most important the known sequence of genome and physiological similarity of *Drosophila* with human beings made them an excellent model for the experimentation. Recently, *Drosophila* has grabbed an attention to analyze the toxicity of nanomaterials. The most direct approach to analyse the toxicity of nanoparticles in *Drosophila* is by determining the survivorship after exposing nanoparticles. Nanoparticles can enter the human body through numerous pathways such as oral, dermal and inhalation routes, it is important to evaluated these entry routes in *Drosophila* model. One of the most common possible route to expose nanoparticles in *Drosophila* is through ingestion. For example, adding of nanoparticles with various concentration in the standard food medium of *Drosophila* [12].

1.5 Problem identification

With the increase exploitation of copper nanoparticles in various application of pharmaceutical science, medicine and biology, we can't undermine the amount of toxicity which can reach in our environment through nanoparticles. Once nanoparticles reaches inside the biome system it is unachievable to eliminate them from the system. Therefore, it is critical to regulate the proportion of nanoparticles

that reaches the environmental system. Our study aims to analyze the effect of nanoparticles on *Drosophila*. Also we need an eco-friendly method for the synthesis of copper nanoparticles, this leads us to our first objective of study, to synthesis nanoparticles using biogenic method.

1.6 Objectives of the study

1. To synthesis copper nanoparticles by using biological method (via plant extract).
2. To test the potential effect of copper nanoparticles on animal model - *Drosophila melanogaster*.

CHAPTER - 2

REVIEW OF LITERATURE

2.1 *Drosophila melanogaster*

Drosophila is the most common experimental model used in laboratories to study to various aspects of biology. The organism has contribution in many biological discipline - gene biology, cell biology, developmental biology and population genetics.

2.1.1 History of *Drosophila* in research

Undoubtedly *Drosophila* is a versatile model that is in use from more than 100 years. The first documented work on Drosophila in laboratory was done by Dr. Castle in 1901. Dr. Castle was working on the genetics of coat color in mice and pigs. Since it got difficult to work with these models, he choose *Drosophila*. In 1906, Morgan's work came providing evidence for the Chromosome theory of inheritance. Later on 1915, discovery of Notch pathway by John S. Dextor was done. It is a conserved cell signaling pathway which plays crucial role in cancer development and transformation. In 1946, Hermann J. Muller got Nobel prize for his work on mutation induced by ionizing radiation in fruit flies. Between 1960s to 70s, Seymour Benzer and his student Ronald Konopka contributed in understanding the circadian rhythms in organism. During mid 1970s, Christiane Nüsslein-Volhard, Edward B. Lewis and Eric F. Wieschaus helped in understanding the embryonic development of nervous system. Studying on *Drosophila* becomes more easy from 2000, as during that year genome project of *Drosophila* was completed. From 1990s till today, *Drosophila* is extensively used as the model organism for various diseases [13-17].

2.1.2 Life cycle of *Drosophila*

A fertile mating *Drosophila* pair can reproduce hundreds of genetically indistinguishable progeny within roughly time period of 10-12 days at 25 °C. *Drosophila* is a model organism which is well-defined by its developmental stage: the embryo, larva, pupa, and adult (Figure 3).

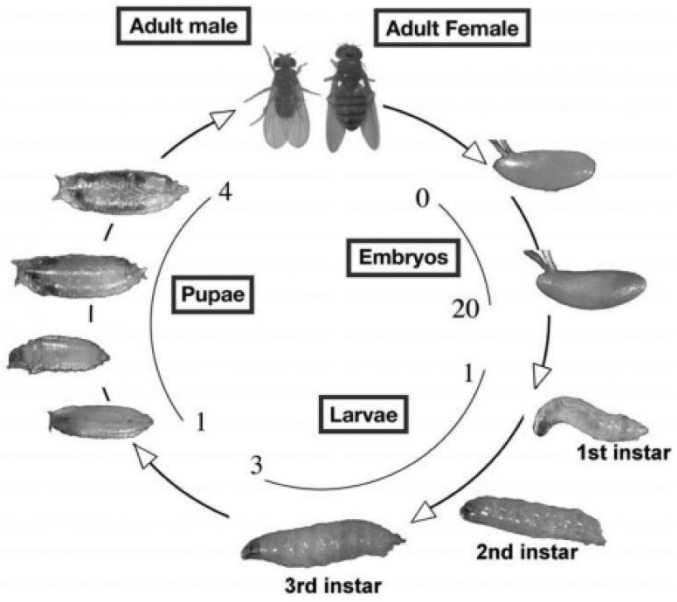


Figure 3 : Life cycle of *Drosophila*

Source - Fernández-Moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). Drosophila melanogaster as a model system to study mitochondrial biology. In Mitochondria (pp. 33-49). Humana Press.

Features for determining the sex of adult fruit fly [19]:

1. Size of adult fly - The female flies are generally much larger than the male flies.
2. Shape of abdomen - The abdomen tip is rounded in male and elongated in female.
3. Markings on abdomen - Females have alternate dark and light bands on entire rear portion whereas in males last few segments are fused together.
4. External genitalia on abdomen - The ovipositor of female is pointed and located at the tip of abdomen. The males have claspers which are dark pigmented which are arranged in circular form and located at ventral side of the tip.

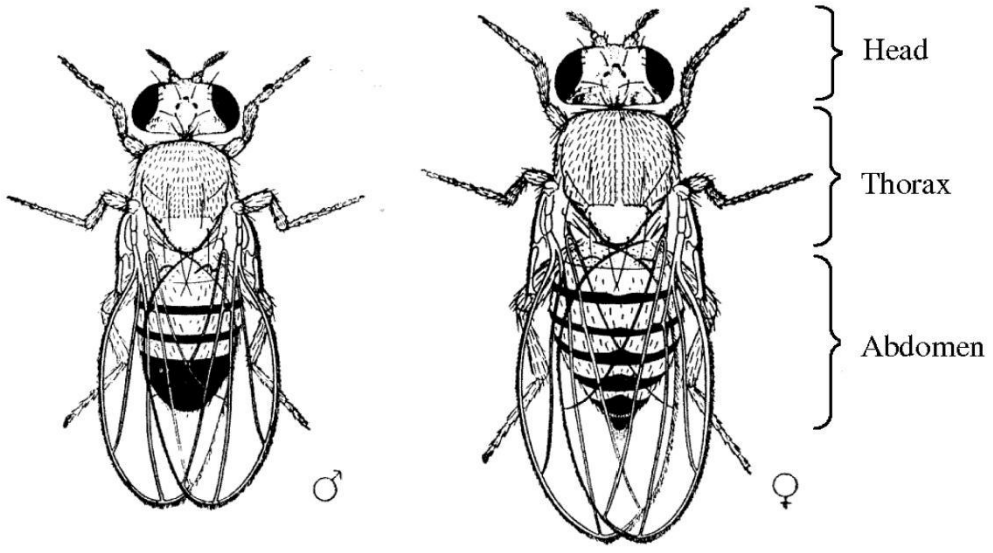


Figure 4 :Difference between male and female *Drosophila*
https://www.mun.ca/biology/scarr/4241_Devo_DrosophilaSex.htmltim [20]

2.1.3 Importance of *Drosophila* as animal model

Drosophila provides certain advantages over vertebrate animal models which makes them the most celebrated non-mammalian model organism.

1. For analyzing the function of human disease gene *Drosophila* is said to be the most effective tools. With mentioning the fact that 75% of human disease related genes are said to have functional homology in fruit fly [21]. These genes include those that are important for the development of fly and also for regulating the neurological disorders, tumor , diseases related to circulatory system, metabolic and storage as well as genes essential for functioning of visual, auditory and immune system [22].
2. Many important biological mechanisms and molecular pathways such as physiological and neurological pathways are conserved between *Drosophila* and vertebrates [23].
3. *Drosophila* required less of care and culturing is very simple, inexpensive.
4. The generation time (12days) of *Drosophila* provides another advantage to *Drosophila* being used as animal model.

5. In terms of base pairs, *Drosophila* has a very compact genetic makeup and also the arrangement of chromosomes are very low in number, 4pairs (one X/Y pair and three autosomal pairs) [24].

6. The organs and tissues of *Drosophila* are very much easy to identify, collect and study [25].

7. *Drosophila* also provides an advantage of bypassing some bio-ethical concerns of biomedical research on vertebrate organism [26].

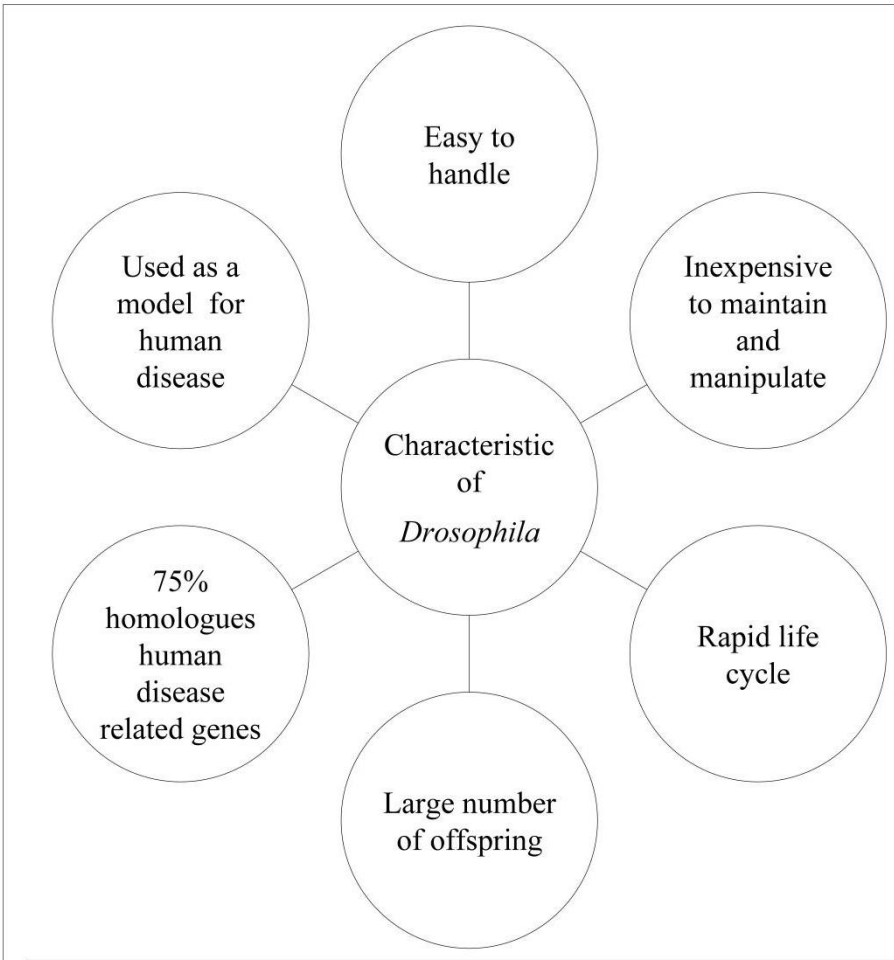


Figure 5 : Characteristics of *Drosophila* which makes them a versatile study model

2.2 Copper

Copper is the 8th abundant metallic element of the Earth's crust [27] and it belongs to the Block D, Period 4 element. Copper is a reddish metal with a face-centered cubic crystalline structure. The reddish color is due to its band structure, Copper reflects red and orange light and also absorb other frequencies of visible spectrum. Copper is also a crucial trace element for humans, plants, and animals [28].

Table No.-2 : ***About copper***	
Atomic number	29
Atomic mass	64.54 g/mol
Melting point	1083℃
Boiling point	2595℃
Isotopes	^{63}Cu ^{65}Cu
Oxidation state [29]	Cu^{0} Cu^{+1} Cu^{+2}
Properties [30]	Good ductility Malleability High corrosion resistance Low chemical reactivity Cheap antibiotic/ antifungal agent

2.2.1 Importance of Copper in human body -

Biological role - Copper helps in sustaining the strength of the skin, blood vessels, epithelial and connective tissue throughout the body. It has a major role in the generating the hemoglobin, myelin, melanin and it also helps in normal functioning of thyroid gland. Copper also in-act as both antioxidant and pro-oxidant [31-35]

Copper act as an enzyme - A copper containing metalloenzyme, cytochrome c oxidase plays an important role in electron transport. Copper is a constituent of lysyl oxidase, an enzyme that engage in the synthesis of collagen and elastin, two important structural proteins found in bone and connective tissue. It is also essential in producing the thyroid hormone, thyroxine. A copper- containing enzyme tyrosinase, help in transforming the tyrosine to melanin which is important in synthesing the phospholipids that are found in myelin sheath of peripheral nerves [35-36]

Disease related to copper - Excessive copper leads to Wilson's disease [37] whereas deficiency of copper causes Menke disease [38]. Copper also leads to neurodegenerative disorders such as Huntington's disease [39], Parkinson's disease [40] and Alzheimer's disease[41].

2.2.2 Recommended intakes for copper

The Estimated Average Requirement (EAR) for copper intake is chiefly estimated by combination of indicators which includes plasma copper and ceruloplasmic concentration, erythrocyte superoxide dismutase activity and platelet copper concentration in controlled human depletion/ repletion studies. Table (3) shows the amount of copper intake recommended by Recommended Dietary Allowance (RDA). The Tolerable Upper Intake Level (UL) is 10,000mcg [42].

Table No -3 : *Recommended Daily Intake (RDI) for copper*

Age	**Male**	**Female**
Birth to 6 months	200mcg	200mcg
7-12 months	200mcg	200mcg
1-3 years	340mcg	340mcg
4-8 years	440mcg	440mcg
9-13 years	700mcg	700mcg
14-18 years	890mcg	890mcg
19+ years	900mcg	900mcg
Pregnancy		1000mcg
Lactation		1300mcg

2.3 Nanoparticles

The term 'Nanoparticles' is derived from a Greek word 'nano' which means small/dwarf. Also the term can be used as prefix to denote the size 10^{-9}. The term Nanobiotechnology is a conglomeration between two fields nanotechnology and biotechnology which benefits in generation and implementation of many tools in understanding the study of living beings. Nanotechnology deals with the aspect of generating materials and devices on the scale of 1-100nm whereas Biotechnology deals with the biological processes of biological entities such as plants, animals or microbes. Both the fields together creates an expansive applications to benefit the human society [43].

2.3.1 Applications of Nanobiotechnology

Table No - 4 : ***Application of nanobiotechnology in various fields***

	DIAGNOSTIC APPLICATION		**Reference**
1.	Detection	Semiconductor nanocrystals (Quantum dots) are used to create extremely small probes that can significantly used as probes in immunoassay, immuno hischemical staining and life cell imaging.	[44]
2.	Individual target probes	Nanosphere [Northbrook, Illinosis] is a company which generate techniques that let doctors in visual detection of genetic composition of biological entities.	[45, 46]
3.	Protein chips	These are the devices which are treated with some chemical groups or smaller protein components, which particularly binds with proteins that contain biochemical motif. Also they can be used to detection any type of interaction at molecular cell such as cell adhesion.	[47]
		Two companies that are involves in engineering protein chips are Agilent, Inc and NanoInk, Inc.	[48, 49]
4.	Imaging	Quantum dots or synthetic chromophores	[50]

		such as fluorescent proteins are used to label target molecules, this had ease in direct analysis of intracellular signaling and imaging along with the aid of optical techniques like confocal fluorescence microscopy.	
	THERAPEUTIC APPLICATIONS		
1.	Drug delivery	Nanodrug are in construction which will facilitate the release of drug only at targeted molecules or only when external triggers are given to the system	[51]
		Encapsulation of drug in a nanosized substances (such as dendrimers, polymer capsule nanoshells) aids in controlled release of drug in a precise way.	[52]
		Nanoformulation research gives a promise in protecting the from vulnerability of degradation or denaturation when they are subjected to extreme conditions like pH. Also these forumlations expands the half life of drug.	[53]
		Nanomaterials can also cant as delivery agents for vaccination. This will decreased the usage of pathogenic agents being used as vector to transport the targeted molecules.	[54]
2.	Gene delivery	For the transportation of target molecules Non viral vectors based on nanoparticles can be used with the scale size of 50-500nm. For replacement or repairing of defective human genes nanosize carrier can act as a great substitute viral vectors.	[55]

3.	Liposomes	As liposomes are made of lipid bilayer they provide an advantage in gene therapy. They help in passing the therapeutic agents easily through lipid bilayers or the cell membrane and reach the target location. They are also advantageous in local delivery of molecules and establish targeted therapy.	[56]
4.	Orthopedic applications	Nanosized organic and minerals substances are used as a functional composition of bones. With the usage of nanomaterials, nanopolymers, carbon nanofibres, nanotubes and ceramic nanocomposites, high deposition of calcium containing minerals on implant has been done productively.	[57]
	FOOD SECTOR		
1.	Food packaging	With efficient thermal, mechanical and gas barrier properties, Bionanocomposites material have been in research to be used as food packaging material. They are the hybrid nanostructured material which protects the food from contamination, increases the shelf life, improves the quality of packaging and moreover they are environment friendly.	[58]
2.	Nanosensors	They can be used for detection of food spoilage. The nanosensor aims to reduce the time for the detection of harmful microbe from days to hours to even minutes.	[59]
		Nanosensor can be placed inside the food packaging material, where they act as electronic tongue or noses, thus helping in detection of any chemical released during	

		food contamination.	
		Nanosensor based on the principle of microfluidics devices are used in detection of pathogen in real time and they are highly sensitive.	
		Nanocantilever are used in detecting the contamination caused by chemicals, toxins and any antibiotic residues in food products. Nanocantilever are based on the principle of detection of biological binding interactions via physical and or electrochemical signaling (such as interaction between enzyme and substrate/ cofactor, antigen and antibody and receptor and ligand)	[60]
	AGRICULTURAL SECTOR		
1.	Nanofertilizers	They are till the date mostly seen in the literature studies. Nanofertilizers promises to increase the Nutrient use efficiency (NUE) by three times and also have great stress tolerance capacity.	[61]
		Nanotech leads to the extensive use of biosource and they are ecofriendly. They also increases the uptake of carbon and help in improving the soil aggregation.	
		Nanofertilizers have their content like nutrients factor and growth promoters encapsulated inside the nanoscale polymers. This helps in the slow and specific release of the materials inside the encapsulated nanofertilizers	
2.	Nanosensors	They are spread all throughout the field where are they used to detector for the soil conditions and growth of crops.	[62]

3.	Encapsulation control	Nanotechnology are used to influence the properties of outershell of capsule which provides better control in releasing the substances into the targeted area or field.	[63]
		'Controlled release' are highly used technique in medicinal field. These nano formulations are patented by agro-industry Monsanto, Syngenta and Kraft, where they are use the formulation the be delivered at a specific targets.	

2.3.2 Synthesis of Nanoparticles

Synthesis of nanoparticles are categorized under two different types of mechanisms- (1) Top down method and (2) Bottom up method (Figure 6). In top down method, bulky sized particles are scaled down to nanosized dimension particles by using techniques like cutting, grinding or etching, nanomaterials are synthesized without any atomic control, whereas in bottom up method, nanoparticles are generated from the smallest atoms, molecules or clusters, nanomaterials are synthesized from the atomic level of substances [64].

Further, top down method includes physical methods and bottom down method is classified into two more types chemical methods and biological methods. (Table 5) defines the advantages and disadvantages of different types of physical and chemical methods.

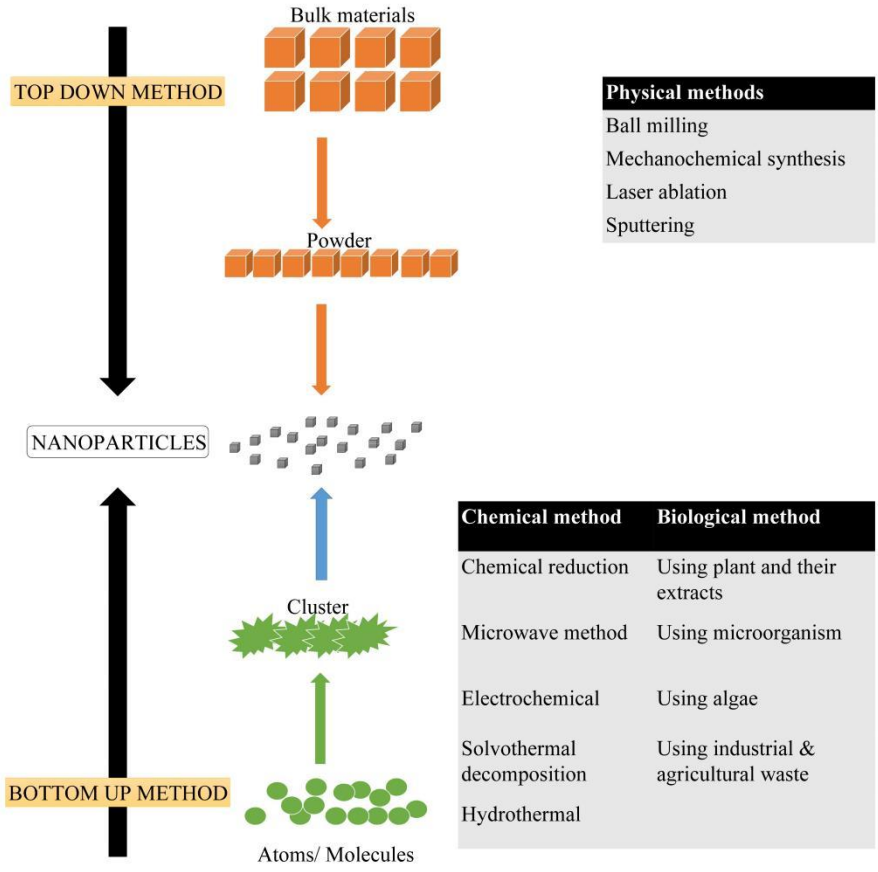


Figure 6 : Types of method for generation of nanoparticles

Table No - 5 : ***Advantages and disadvantages of various physico-chemical methods*** [65]

PHYSICAL METHODS			
	Technique	**Advantages**	**Disadvantages**
1.	Ball milling	1. Used for producing large scale NPs of high purity. 2. NPs are generated are of superior physical properties. 3. Improved grain size and material composition of	1. Requires high amount of energy. 2. Needs longer time period for milling. 3. Contamination can occur due to the usage of steel balls. 4. Sensitive

		generated NPs.	microstructure can be grinded during the milling.
2.	Mechanochemical synthesis	1. Easy and productive method for generating NP.	1. Unwanted contamination from milling media can occur in NP formed. 2. For smaller NP milling time period is longer.
3.	Laser ablation	1. Easy and productive method for generating NP in larger amount and smaller in size in form of suspension. 2. By selecting parameters of laser and nature of liquid, NP properties can be influenced.	1. Since high amount of NP are generated in colloidal solution, this causes the blockage of laser pathway and also lessen the ablation rate.
4.	Ion sputtering	1. Economical method and less amount of impurities are generated. 2. Easy to synthesize alloy NP due to managed control over composition. 3. Simple technique to generate ionic NPs with spacious size and composition. 4. Technique provides an easy control over various parameter - composition, size and charges of ion.	1. Nature of sputtering gases (He, Ne, Ar, Kr and Xe) produces an effect on different properties (morphology, texture, optical properties and composition) of nanocrystalline materials.

CHEMICAL METHODS			
1.	Chemical reduction	1. Simplest and easiest method for producing metal NPs.	1. Toxic and expensive method. 2. Poor reducing ability. 3. Generate impurities.
2.	Microwave assisted NP	1. Highly effective technique. 2. Simple and fast volumetric heating. 3. Increases the rate of reaction. 4. Uniform heating throughout the process helps in increases the rate of reaction.	1. Time for crystallization is shorter. 2. Due to uniform heating homogeneous nucleation occurs.
3.	Hydrothermal method	1. Easy to generate NP of desired size and shape. 2. Simple to form crystallized powder. 3. Nanocrystals with higher crystallinity are produced.	1. Process are laborious to control. 2. Limitation of dependability and reproducibility.
4.	Solvothermal method	1. High quality of monodispersed nanocrystals are prepared. 2. Method is useful for generating smaller size and high crystallized nanocrystals.	
5.	Electrochemical deposition	1. Easy, rapid and low cost method. 2. NPs of controlled size	

		and morphology are generated. 3. The biggest advantage with the method is NP gets attached to the substrate directly.	

2.3.3 Limitations of physico-chemical synthesis of nanoparticles -

1. Usage of harsh and toxic chemicals.
2. Requires large amount of capital for chemicals and equipments.
3. Chemical method involves the usage of more than one chemicals in one synthesis process, this can increase the particle reactivity and toxicity.
4. Due to toxic effects these methods can't be used in biomedical and pharmaceutical applications.
5. Both methods produce potential and hazardous toxic wastes.
6. Physicals methods require large amount of energy.
7. For scaling up, longer time period is needed.
8. Physical methods leads to contamination caused by their instrument parts.
9. Many time physical processes are difficult to control.
10. Large amount of impurities are generated as by product [66].

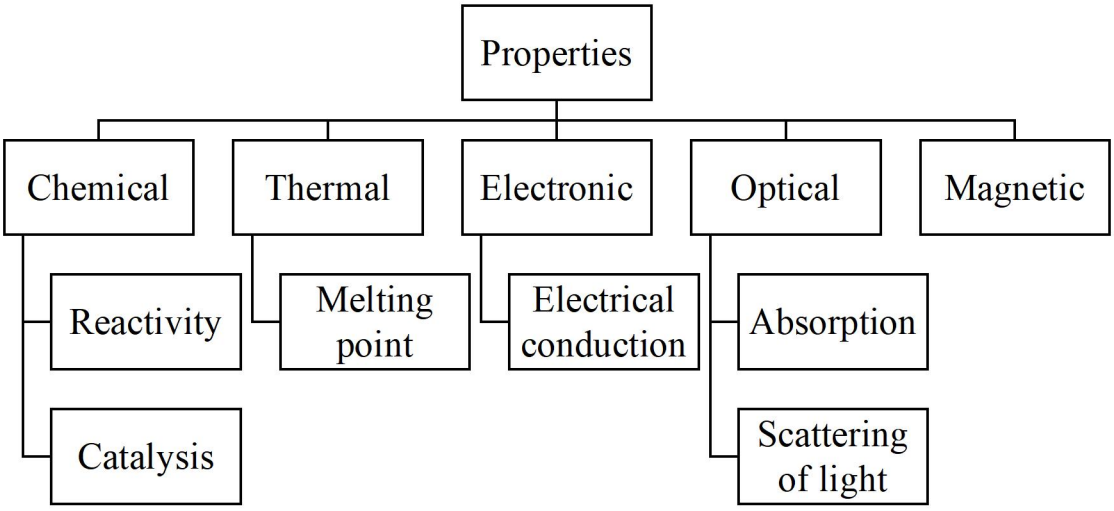
To overcome these limitations , a demand raised in for a sustainable method. That's when biological/ green or biogenic synthesis of nanoparticles became center of attraction.

2.3.4 Characterization of nanoparticles -

In order to understand the nature of particle and its size, and to know about its application, we need techniques to characterize the nanoparticles. The common techniques used to characterize the nanoparticles are - SEM, TEM, AFM, FTIR, XRD and UV vis spectroscopy. Various properties of nanoparticles like size and structure of particles, its topology or its morphology, crystallinity or surface roughness or texture etc are studies with the help of these techniques (Table 6).

Table No - 6 : *Characterization techniques for analyzing nanoparticles*	
Techniques for characterization of nanoparticles	**Parameters analyzed by techniques**
SEM (Scanning electron microscopy)	Topology, Size, Morphology, Crystallography and Structure
TEM (Transmission electron microscopy)	Topology, Size, Morphology and Crystallography
AFM (Atomic force microscopy)	Size, Morphology, Surface roughness and Texture
FTIR (Fourier transformed infrared) spectroscopy	Functional group and Chemical bonding
XRD (X-ray diffraction)	Structure type and Crystallinity
UV-visible spectroscopy	Shape, Size, Composition and Concentration

2.3.5 Properties of Nanoparticles -



1) Chemical properties [67]-

On the basis of surface area to volume effect, nanoparticles exhibits -

i. Enlargement in their total surface area.

ii. Accumulation of number of atoms on the surface.

iii. Atoms with larger surface area shows increment in catalytic activity.

iv. By altering the shape, size and composition of nanomaterials, properties of surface catalysis can vary

Nanoparticle based catalysts can improve the reaction rate, selectivity and effectiveness various chemical reactions (Figure 7).

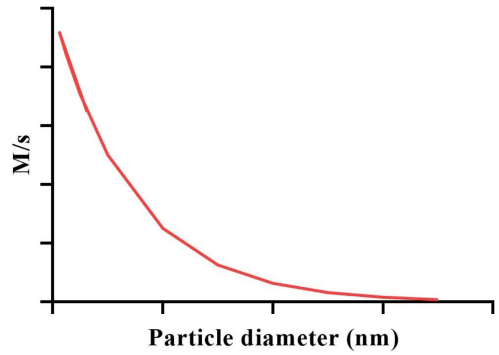


Figure 7 : Effect of particle size on rate of reaction

2) Thermal properties [68]-

The melting point of nanomaterial is directly linked with the bond strength of material. The melting temperature of nanoparticles is directly proportional to the size particles, thus temperature decreased with the smaller particle size (in diameter)(Figure8).

The reason is that, as surface atoms have liberation to move around so they are not confined to any direction of surface plane.

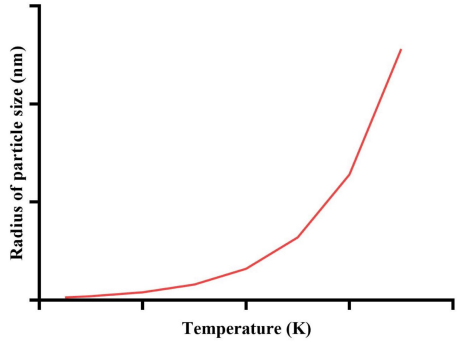


Figure 8 : Effect of particle size on temperature

3) Electronic properties [69]-

In bulky nanomaterials, electrons are able to move freely around all the direction, so the conduction of electrons is delocalized.

As said in case of zero dimensional materials, all the dimensions are at nanoscale, thus electrons are bounded in 3-D space. This causes in no delocalization of electrons.

In case of one dimensional materials, two dimensions are at nanoscale, so electrons are confined to 2-D space and delocalization occurs along the axis of nanowires or nanotubes/ nanorods.

Like this in two dimensional materials, electrons delocalized in 1-D space, where as in three dimensional space materials, electrons will have lots of space to move around as no dimension is at nanoscale. This property of electron confinement causes nanomaterial to behave either as insulators or semi conductors.

4) Optical properties [70]-

By controlling the dimensions of crystal of nanomaterials, linear and non-linear optical properties can be modulated.

The emission peak shifts toward the shorter wavelength, for nanomaterials smaller in size.

5) Magnetic properties [71]-

The size of nanomaterials highly impact the value of magnetization. The value of magnetization remarkably increases for grain size below 20nm. Thus, by increasing the particle size of nanomaterials, it is feasible to enhance the quality of value of magnetization (Figure9).

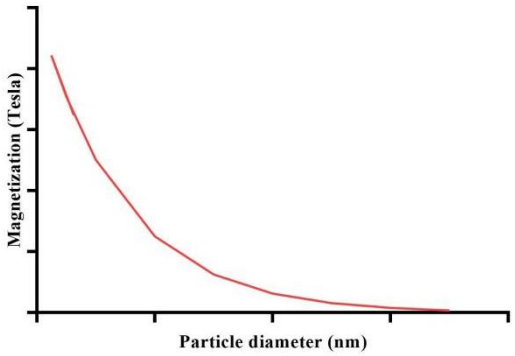


Figure 9 : Effect of particle size on magnetism

2.4 Green synthesis of Nanoparticles

Green synthesis works on the principle of utilization of biological entities along with green chemistry principle. Green synthesis provides an alternative route for the synthesis of nanoparticles, it is an eco-friendly and more sustainable technology (Figure10) .

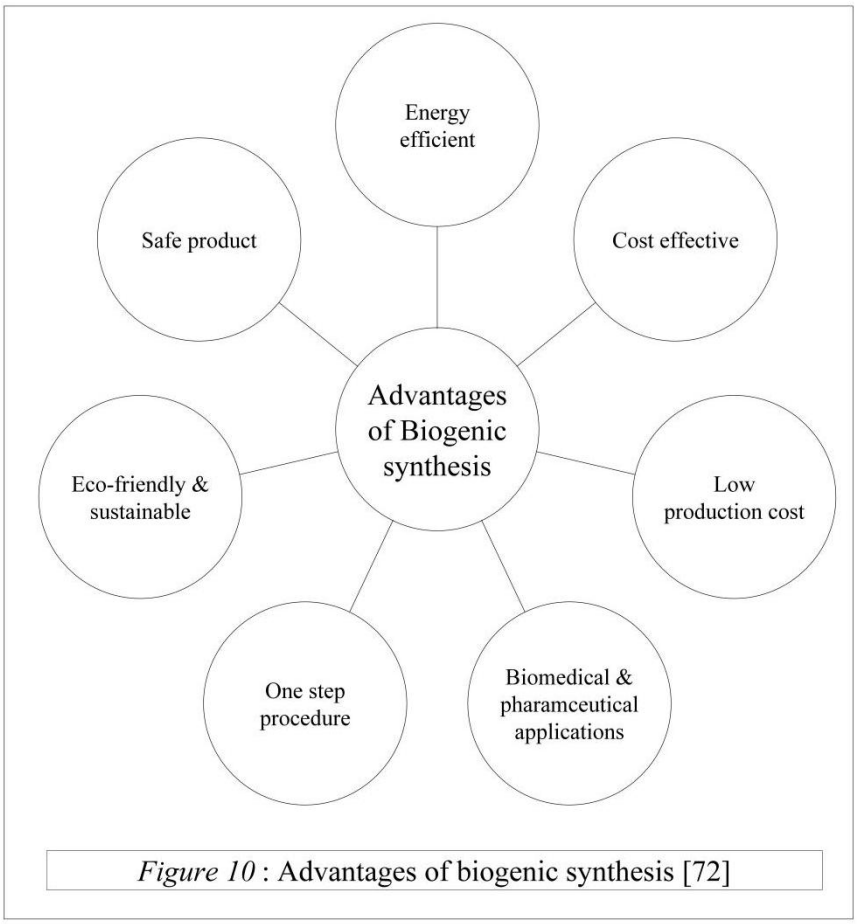
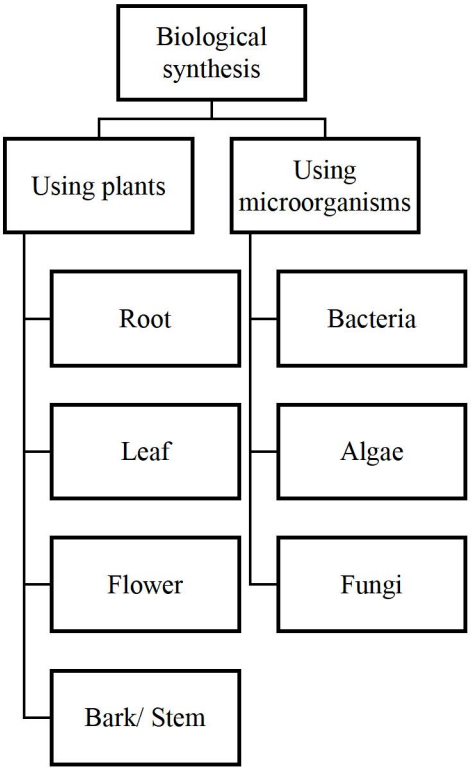


Figure 10 : Advantages of biogenic synthesis [72]

2.4.1 Types of Biological synthesis -

Biological synthesis can be classified into two ways on the basis of biological entities used - (1) using plants and (2) using microbes.



1. Using plant extracts -

Plants are said to be the chemical factories of nature which are cost effective and are very low maintenance. It is also been said by the researchers that plants have capability in detoxification of heavy metals, also they are able to remove the accumulation of small traces of heavy metals, this helps to overcome the environmental pollutant problem. Plant parts contains phytochemicals(flavonoid) in their extracts which has a potential stabilizers and reducing agents. Flavonoids have diverse classes - isoflavonoids, flavonols, chalcones, flavones and flavanones, they actively chelate and reduces metal ions into NPs. Flavonoids have functional groups that are able to work as reducing agent and aid the formation of NPs. These groups chelate the metal ions like Fe^{+2}, Fe^{+3}, Cu^{+2}, Zn^{+2}, Al^{+3}, Cr^{+3}, Pb^{+2} and Co^{+2}. Flavonoids transforms their enol- form to keto- form, releases reactive hydrogen atom that have capability to reduce metal ions and benefits the formation of NPs [73, 74] (Figure11).

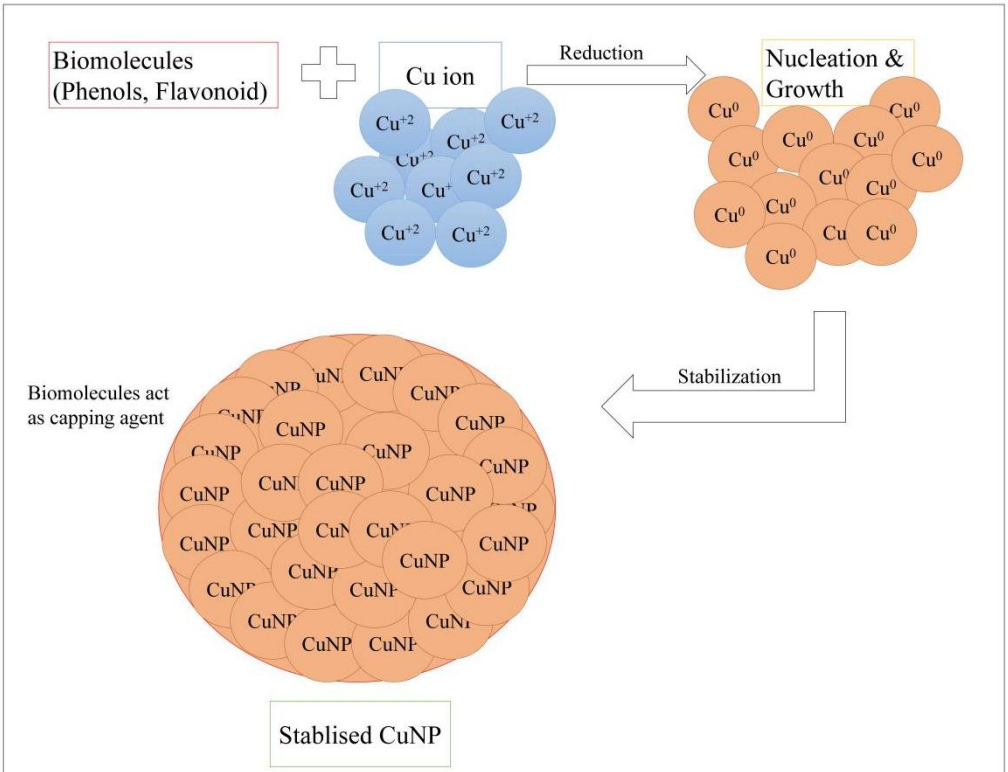


Figure 11 : Mechanism of formation of CuNP via plant extract

2. Using microorganisms - Microorganisms acts as nanofactories for accumulation and detoxification of heavy metals in the presence of reductase enzyme that helps in reducing the metal salts to metal nanoparticles. Mechanism of action for synthesizing the nanoparticles are different for distinct microorganisms. The most common process through which nanoparticles are generated is by the following way - firstly, metal ions are trapped either on the surface or inside the microbial cells. Then these trapped metal ions in the presence of certain enzymes are reduced to nanoparticles [75].

In addition, many metal resistant genes, peptide or proteins, enzymes, or reducing cofactors and certain organic materials have an important contribution by acting as reducing agents. Besides, these agents aid the synthesis by functioning as natural capping agent to nanoparticles. This prevents the aggregation of nanoparticles and makes them stable for a longer period of time [76, 77].

CHAPTER 3
METHODOLOGY

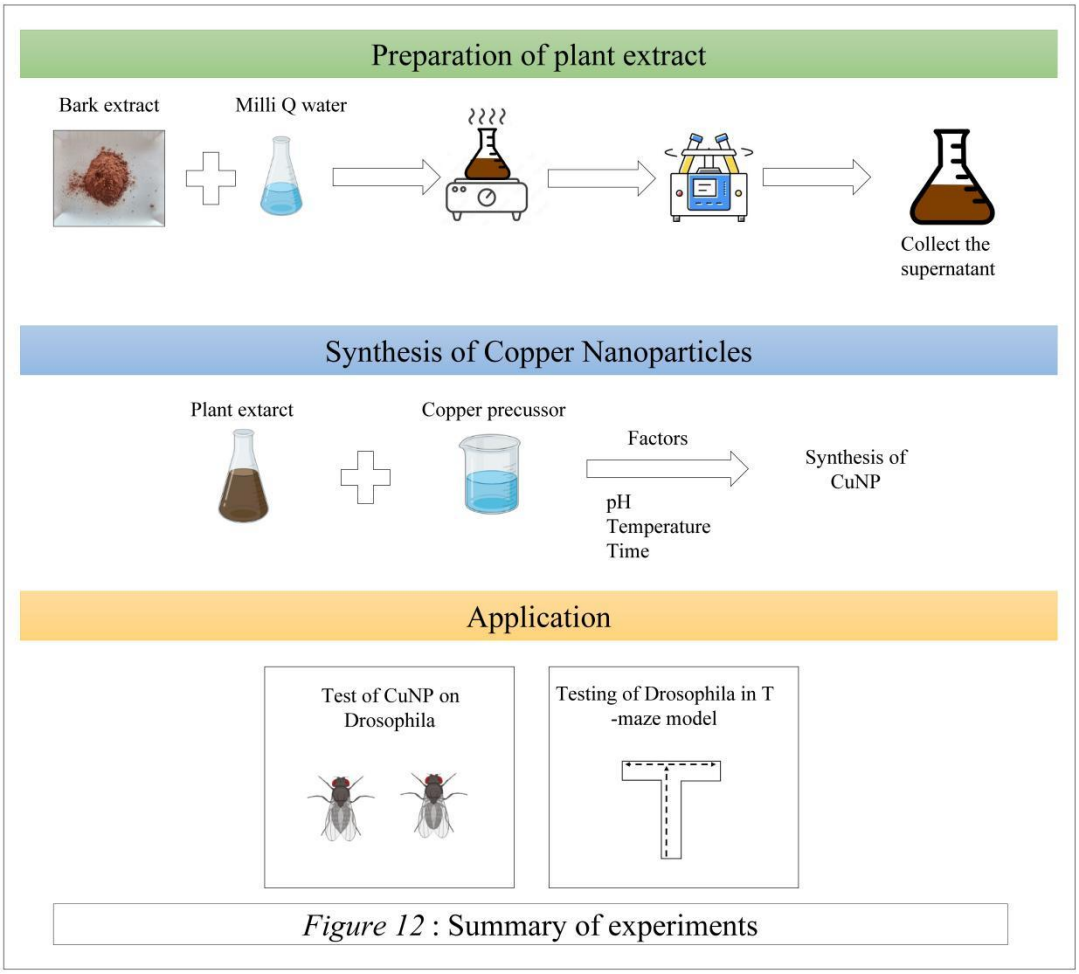


Figure 12 : Summary of experiments

3.1 Preparation of plant extract

In a 500mL flask, add 2gm of bark extract in 200mL of milliQ water. Prepared mixture was kept on magnetic stirrer with hot plate for 30min at 60°C. Allow the mixture to cool down and then centrifuge the mixture at 8000rpm for 10min. After centrifuge, collect the supernatant in different vessel and store them at 4°C for future use.

3.2 Synthesis of copper nanoparticles

Prepare 50mL, 5mM copper sulfate pentahydrate solution - Add copper sulfate pentahydrate in different concentration to prepare plant extracts Table (7). Optimize the volume ratio for the copper nanoparticles. Further, check the effect of different factors like pH, time and temperature of optimized ratio of plant extract and copper sulfate pentahydrate.

Table No -7 : *Volume ratio optimization*		
Concentration ratio	Plant extract amount	$CuSO_4.5H_2O$ amount
1:1	2	2
1:2	2	4
1:3	2	6
1:4	2	8
1:5	2	10

3.3 Characterization of copper nanoparticles

Characterization of copper nanoparticles was done using UV spectroscopy. The scanning range used for sample was 200nm - 800nm. All the record and numerical data were compiled using Graphpad Prism 9 software.

3.4 Testing the effect of copper nanoparticles on *Drosophila*

Three different concentration (10µg, 1µg and 100ng) of copper nanoparticles were used to test their effect on *Drosophila*.100mL of *Drosophila* media was prepared in three different flask for three concentrations. Different concentrations of copper nanoparticles were prepared using serial dilution (the amount of media used in serial dilution was 10mL). The diluted 10mL media with different concentration was spread on the top of initially prepared 100mL media. Before adding of flies to the media they were starved for 6hours. And then in each flask around 15-20 flies was added. All the flasks with different concentration (media treated with different concentration of copper nanoparticles) along with control flask were observed for 8days. After 8 days of observation, all the treated flask along with control were starved for 18 hours and then flies were observed under T-maze model.

Table No. - 8 : *Media for culturing Drosophila*	
Requirements	**Amount (100mL)**
Yeast extract	1.42gm
Maize powder	4.8gm
Jaggery	4.5gm
Agar	2gm
Methyl paraben	1gm

CHAPTER - 4

RESULTS & DISCUSSION

RESULTS

4.1 Synthesis of Copper nanoparticles

A peak was observed between the 350nm to 400nm indiciating the synthesis of copper oxide nanoparticles. Figure 14 shows the comparison between the peaks of formed nanoparticles, plant extract and copper sulfate pentahydrate solution.

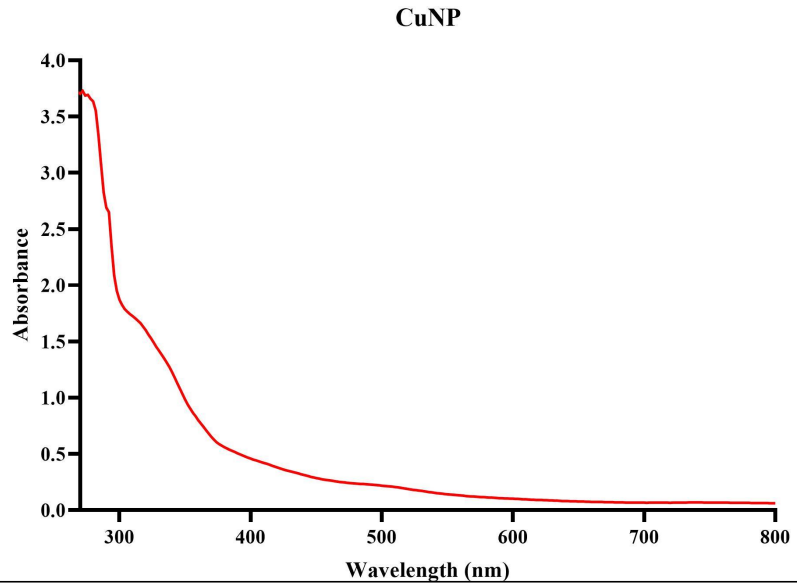


Figure 13 :UV visible spectrum of formed copper oxide nanoparticles ranging from 350nm to 400nm.

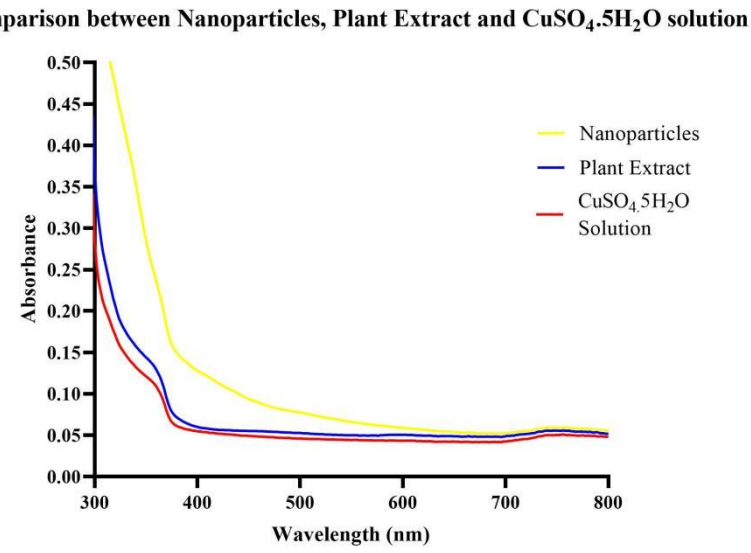


Figure 14 : UV visible spectral comparison between nanoparticles, plant extract and $CuSO_4.5H_2O$

Figure 15 : Digital image of Copper nanoparticles

4.1.1 Volume ratio optimization

Copper sulfate pentahydrate was mixed with plant extract solution in 5 different concentration [1:1, 1:2, 1:3, 1:4 and 1:5]. Initially the pH of all the solutions was at pH7.

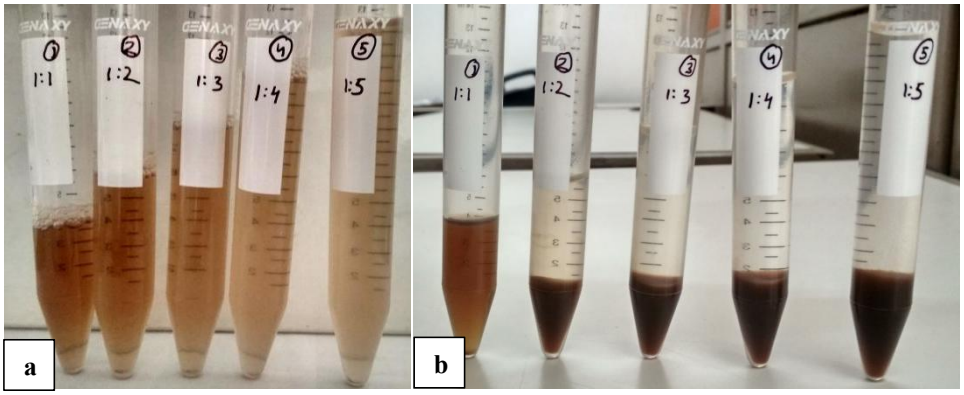


Figure 16 : (a) Solution before adjusting pH. (b) Solution after adjusting pH.

All the solution showed aggregation of particles after adjusting the pH except in the solution 1:1 [one part copper sulfate pentahydrate and one part plant extract]. This suggested to use 1:1 as an optimized volume ratio of copper sulfate pentahydrate and plant extract. Spectrum was obtained of all the solutions (Figure 17), where ratio 1:1 (with red line) showed a peak between 350nm to 450nm indicating the formation of copper oxide nanoparticles [90].

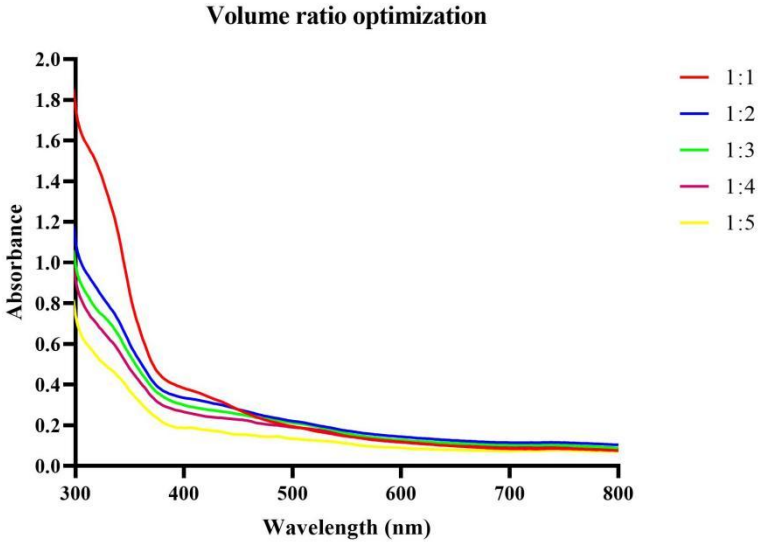


Figure 17 : UV visible spectrum of different ratio of $CuSO_4.5H_2O$ and plant extract.

4.1.2 Factors affecting the synthesis of copper nanoparticles

a) Effect of pH - The role of pH has been said in determining the size of particles, literature stated with the increase in pH the particle size of nanoparticles decreases significantly [91]. Also a visible color change is seen on increasing the pH of nanoparticles solution [92]. For our optimized ratio 1:1, we tested the solution with 7 different pH (3, 5, 7, 9, 11, 12 and 13). We observe a striking change in color of pH3, light brown to pH13, very dark brown color (Figure 18).Out of all the pH solution, pH 12 showed a significant increase in peak from 350nm to 400nm to 400nm to 500nm (Figure 19). On comparing pH 11 and pH 12, pH 12 showed better remarkable result (Figure 20). Also the particle aggregated in all the solution other than pH 11 and pH12.

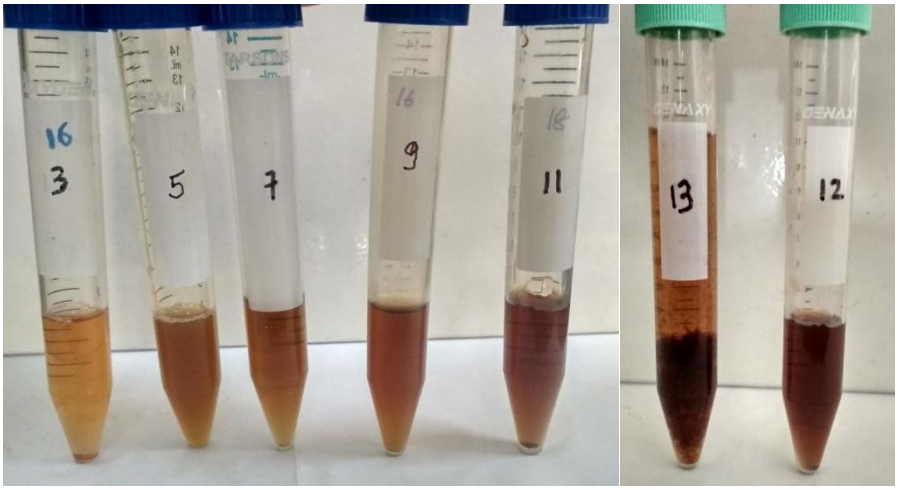


Figure 18: Digital picture of color change of solution as we increase the pH

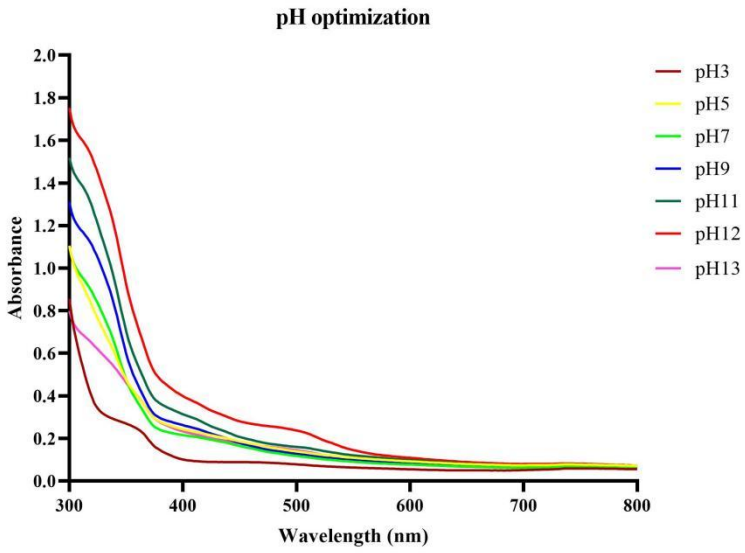


Figure 19 : UV visible spectrum of pH optimization of 1:1 ratio

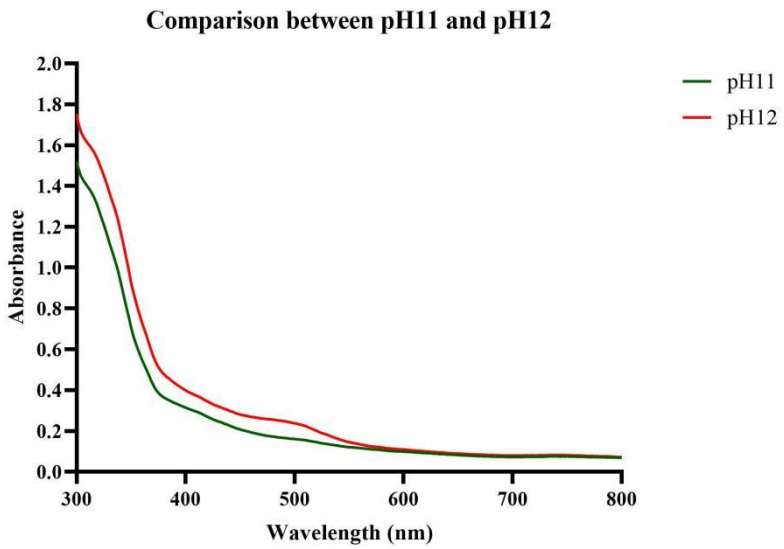


Figure 20 : UV visible spectral comparison between pH 11 and pH12

b) Effect of time on copper nanoparticles

After optimizing the pH, effect of time was seen for the ratio 1:1. After 7 to 8 hour there was a drop in peak at 9th hour. From this it was concluded the incubation time required for copper nanoparticles is 7 to 8 hour, after that there is a drop in peak of nanoparticles.

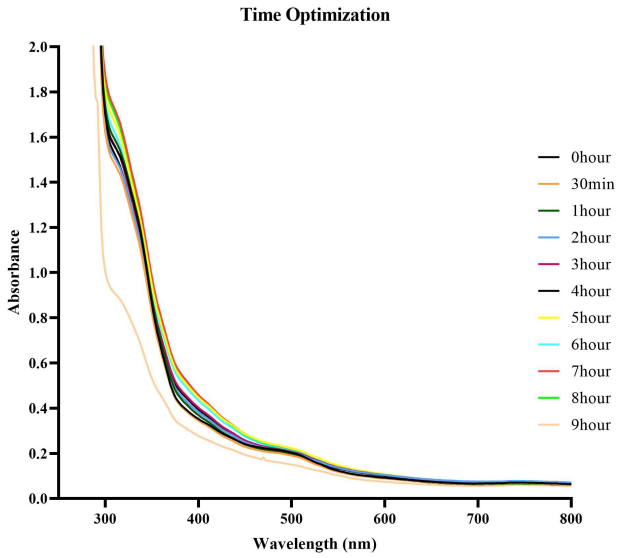


Figure 21 : UV visible spectrum of ratio 1:1, seeing the effect of time of incubation for 9hour period

c) Effect of temperature on copper nanoparticles

After knowing the optimized pH and incubation time period, effect of temperature was monitored. 1:1 ratio samples were kept at three different temperatures (20℃, 30℃ and 36℃) for 7-8hours. Any major variation in peak was not observed due to change in temperature.

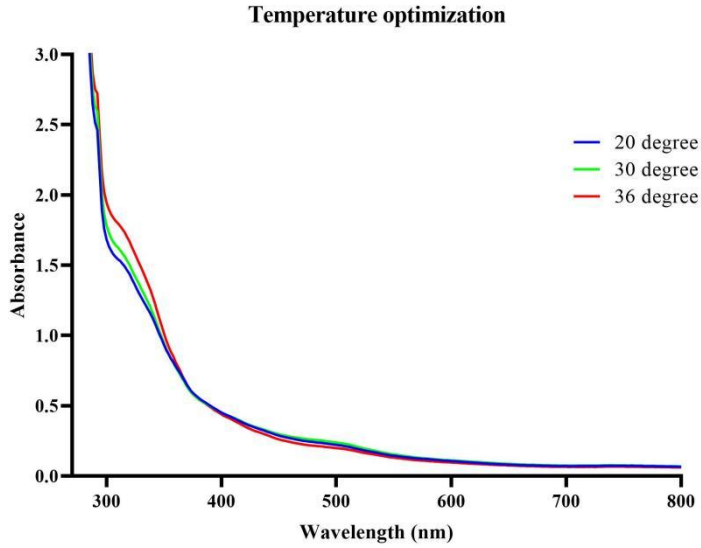


Figure 22 : UV visible spectrum of ratio 1:1 showing the effect of temperature

4.2 Testing of copper nanoparticles on *Drosophila*

Copper nanoparticles were prepared in three different concentrations (10µg, 1µg and 100ng) for testing. Before adding Drosophila to treated media, they were starved for 6 hours. And then they were observed for 8 days. Then they were tested on T-maze model (Figure23). Before subjecting them to T-maze model they were starved for 18hours. After this many flies died in treated flasks were as in control death rate of flies was very low.

In T-maze model, efficiency of flies to move towards the flies was tested and also if is there any difficulty in locomotion. It was seen that as compared to flies in control, flies in treated flask took long time to travel towards feed (Figure24). And also few flies in treated flask showed partial difficulty in movement.

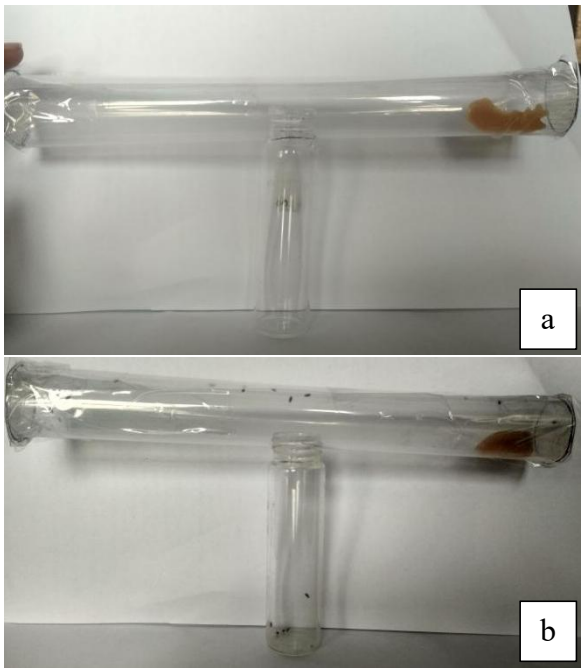


Figure 23 : (a) T-maze model with no flies (b) T-maze model with flies

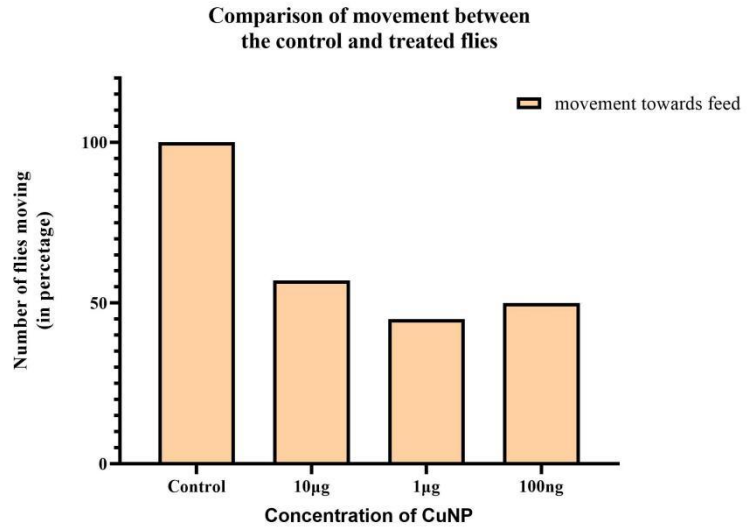


Figure 24 : Graphical representation of movement of flies towards the feed in control versus treated flies

NOTE - Experiments on flies need to be repeated they couldn't be conducted due to restriction in number of flies.

DISCUSSION

The toxic effects of copper nanoparticles still needs to be researched deeply. With wide range of application, copper nanoparticles also comes with warning sign. As much as copper is important to biological system, any carelessness with its uptake can harm the body. Copper nanoparticles are indirect way to supply copper to our body, but it required lots of regulation before reaching the ecosystem or human biological system. The old and conventional methods had their own challenges, what's when Green synthesis of nanoparticles came into research. This study had two major objectives - (1) Green synthesis of copper nanoparticles using plant extract- *Myrica esculenta* and (2) To study any toxic effect of copper nanoparticle using- *Drosophila melanogaster*.

We used bark extract of the plant in synthesizing the copper nanoparticle. We obtained 1:1 as any optimized ratio for generating our copper nanoparticle, where we used equal parts of copper sulfate pentahydrate and plant extract. Then we studied the effect of pH, time and temperature. We noticed significant and clear peak for pH 12 between 400nm to 500nm. In case of time of incubation 7 to 8hourswere found to be appropriate as after 8^{th} hour at 9^{th} hour, there was a noticeable decrement the peak of nanoparticles. As speaking of temperature there was not much variation observed between the three temperatures.

To study the toxic effect of copper nanoparticles we treated flies with three different concentration of copper nanoparticles (10µg, 1µg and 100ng). All the flies in three flask were starved for 18hours before putting them in T-maze model. After 18hours of starvation period it was found that number of flies that died were seen more in treated media flies as compared to control flies. Due to restriction in number of flies experiments need to be repeated. Although an assumption can be drawn on basis of current result, flies treated with copper nanoparticles took a longer period of time to reach to their feed in comparison to flies in control media. Also few flies showed difficulty in locomotion.

CHAPTER - 5

CONCLUSION

The old and traditional method for producing the nanoparticles came with lots of challenges and limitation like expensive, longer time period, toxic effect of chemical or contamination due to equipments. A demand raised for sustainable, eco-friendly and economical method and lead the researchers in direction of biological synthesis of nanoparticles. Thus the term Green Nanobiotechnology raised, which means generating the nanoparticles via biogenic methods or entities. Nanoparticles also came with harmful effects along with broad spectrum application. Our study aimed to synthesis copper nanoparticles by using biogenic method and also test the effect of copper nanoparticles on animal model -*Drosophila melanogaster*. *Drosophila* providing lots of advantages - shorter reproductive cycle, easy to care and maintain became our animal model for the study. Copper nanoparticles area cheap alternative to silver and gold nanoparticles. With not many researches on going on copper nanoparticles, we choose them to synthesis via biological method. We used bark extract from the plant *Myrica esculenta* commonly known as kaphal are dominantly found in the region of sub Himalayas and Meghalayas.

Copper nanoparticles were produced by using the equal parts of copper sulfate pentahydrate and plant extract (1:1 ratio). Also factors affecting the synthesis like pH, time of incubation and temperature were also analyzed. We observed pH12 to be most appropriate as in other pH solution aggregation of particles were observed. The time of incubation was found to be 7 to 8hours as after 8^{th} hour there was a significant downfall in the peak at 9^{th} hour. In regard to temperature no major difference was seen. The testing on Drosophila needs to be repeated as due to restriction in number of flies experiment could not be repeated. But on the ground work of current experiment done we can create a presumption. All the flies treated with different nanoparticle concentration and flies in control media were starved for 18hours and then putted in T-maze. We observed decline in number of flies with treated concentration after starvation in comparison to control flies, which leads us to a belief that treated flies might not be getting the required supplements for the growth. Also treated flies took longer time period to reach their feed inside the tube as compared to control flies and their was partial issues in the locomotion of flies in treated concentration. In future, the study can be conducted on larger number of flies and also other application like antibacterial or antioxidant effect of copper nanoparticles can be explored.

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