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IMPROVEMENT OF 'INJERA' SHELF LIFE THROUGH THE USE OF SILVER (AG) AND ZINC OXIDE (ZNO) NANOPARTICLES

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN FOOD SCIENCE AND NUTRITION

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BY

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November, 2020

The Board of Examiners for Approval

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DECLARATION

I declare that this thesis is my original work and has not been submitted as a partial requirement for a degree in any university

Signature _____ Date_____

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November, 2020

The thesis has been submitted for examination with our approval as university advisors.

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BIOGRAPHY OF THE AUTHOR

The author was born in November, 1987 (G.C) in Gidami Woreda, Kelem Wollega Zone, Oromia Regional State. He attended his primary school at Abono Komatir primary school (grade 1-5), Mekanisa primary School (grade 6) and Amerti Kumbo Primary School (gade 7 and 8). Then he continued his secondary education at Biftu Gidami Secondary School (grade 9 and 10) and Kelem secondary and Prepatory School (grade 11 and 12), respectively. After completion of the Ethiopian School Leaving Certificate Exam (ESLCE), he joined Hawassa University in 2008 and graduated with BSc degree in Food Science and Post Harvest Technology in July, 2013. After his graduation, he was employed at Cool Ice Food Complex (CIFC PLC.) in 2014. In the same year he was employed at Ethiopian Institute Agricultural Research (EIAR) , Debre Zeit Center. After four years of research work experience, he joined the school of Graduate Studies at Addis Ababa University in 2019 (G.C) to pursue his MSc study.

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CONTENTS

LISTS OF ABREVIATED WORDS/PHRASES

ANOVA – Analysis of Variance

CAP – Controlled Atmospheric Packaging

CFU – Colony Forming Unit

CRD – Completely Randomized Design1

DNA – Deoxy Nucleic Acid

GRAS - Generally Regarded as Safe

LDPE – Low Density Polyethylene

FWHM – Full Width at Half Maximum

MAP – Modified Atmospheric Packaging

Mc – Moisture Content

M_{final} – final moisture content

MP-AES - Microwave Plasma - Atomic Emission Spectroscopy

MIC – Minimum Inhibitory Concentration

$M_{initial}$ – Initial Moisture content

mM – millimollar

nM - nanomollar

nm - nanometer

NP - Nano Particle

PDA - Potato Dextrose Agar

PE - Poly Ethylene

PV - Poly Vinyl

RDI – Recommended Daily Intake

ROS - Reactive Oxygen Species

SEM - Scanning Electron Microscopy

SPAB – Surface Plasmon Absorption Band

SP - Species

SPSS - Statistical Package for Social Sciences

UV-VIS - Ultra Violet Visible Spectroscopy

XRD - X-Ray Diffracto meter

ZOI - Zone of Inhibition

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ABSTRACT

About two-third of Ethiopian diet consists of Injera, a *thin, fermented traditional baked bread, made from the most popular grain of teff*(Eragrostis tef (Zucc) *Trotter). Even though it is a nutritious food, the shelf life of injera does not usually exceed 3 days due to mould spoilage.* ***This research was conducted with the aim to evaluate the effect of biosynthesized silver Nano particles (AgNPs) and Zinc oxide nanoparticles (ZnONPs) in improving*** *injera shelf life. AgNPs and ZnONPs are synthesized from Eucalyptus globulus and* Calpurnia aurea (Ait.) Benth *leaf extracts, respectively. Formation of nanoparticle was confirmed by color change and by characterization techniques (UV-vis spectroscopy, scanning electron microscopy (SEM) and X-ray diffraction (XRD). Antimicrobial test on* Aspergillus, Pencillinium, and Rhizopus spp. *was conducted using disc diffusion method. Then both nano particles were dip-coated on plastic zipper bags at different percentages after disinfection with 70% alcohol and dried in an oven at* 60^0*c and used for injera storage. The shelf life was determined as one day before mold growth appearance. Moisture content and pH was determined by oven and pH meter, respectively. Internal temperature of the package during storage was measured by infrared spectroscopy whereas pour plate method was used for mold and yeast count. Finally, migration level was determined by Microwave Plasma - Atomic Emission Spectroscopy (MP-AES). The results from Uvi-vis spectroscopy shows that characteristic peaks observed at 420 and 300 to 400 nm for AgNPs and ZnONPs, respectively. The XRD data also shows that both synthesized nano particles were below 100nm. The SEM image shows that AgNPs was mostly irregular whereas ZnONPs was mostly rod shaped. The result has shown that both of them have significant antimicrobial effect against the studied fungus. ZnONPs has induced greater inhibition zone than AgNPs, which is caused by difference in crystallite sizes (ZnONPs = 64.25nm, AgNPs = 84.07nm).The shelf life of the stored injera samples were increased significantly based on their concentrations. For both Ag and ZnONPs , the optimum concentration was 50% in which injera shelf life increased for 10 days to 15 days, respectively. Both NPs have no significant effect on moisture, pH and internal temperature of the package during storage. The Cfu/g of molds and yeast decreased as the percentage of these nanoparticles coated on plastic bags increased. At 50% concentration the migration was 1.34 and 375mg/Kg for both Ag and ZnONPs, respectively. The migration of both Ag and ZnONPs was relatively high, which was increased with concentration and time of storage. Because, it needs further study and improvements to use these nano particles for injera storage. Thus, it would be advisable if these nano particles will be incorporated as ingredients of plastics to decrease this migration problem.*

Key words: Injera, *Nano particles, moulds and yeasts, shelf life, dip coating, storage*

1. INTRODUCTION

1.1. Background

The shelf life of a food is described as the period for which it remains safe and suitable for consumption. For baked food products, shelf life is limited by different factors like water activity and microbiological spoilage (Kyzlink, 2001). Foods with pH value less than 4.5 are more susceptible to mold spoilage because of their tolerance to acid conditions than bacteria (Smith, 1993). Injera is one of baked products with pH value less than this value.In addition to this, it has high moisture content that makes it more conducive for mold growth. As a result, shelf life of injera does not usually exceed 3 days being suitable for consumption at normal temperature(Ashagrie and Abate, 2012). This limited shelf life caused by mould growth is the economic loss due to wastage (Skirdal and Eklund, 1993).

About two-third of Ethiopian diet (Ball et al., 1996) consists of injera, a thin, fermented traditional baked bread, made from the most popular grain of teff (Eragrostis tef (Zucc) Trotter). Fellow (1997) reported that normal injera is round, soft, spongy, and about 6 mm thick, 60 cm in diameter with uniformly spaced honeycomb-like 'eyes' on the top.Its major quality is determined by its slightly sour taste, (Zegeye, 1997). Different studies show that injera has a high moisture content. Injera has a good nutritional value, as it is rich in calcium and iron.

Mould spoilage is a common problem that affects the shelf life of injera (Ashagrie and Abate, 2012).The three fungal species found to be responsible for injera spoilage is Penicillium sp. and Rhizopus sp and Aspergillus niger (Ashagrie and Abate, 2012).

Different preservation methods like reduction of available water (dehydration, additives etc), and chemical preservative are commonly used to elongate shelf life of bakery foods. According to Ashagrie and Abate (2012), chemical preservatives (0.1% of sodium benzoates and benzoic acids) have prolonged the shelf life of injera for up to 12 days. However, they are applied as additives, which are not convenient for injera preparation because not still practically used. Reduction of available water also does not work for injera as its high moisture content is the nature of adapted injera quality. Because, another special preservation method needs to be searched for injera.

Another recently growing food preservation method is the development of nano-enabled food packaging that have antimicrobial action. Ag and ZnO NPs are some of metal's nano particles used in packaging materials as antimicrobial agents having both fungi-static and fungicidal action (Carbone et al., 2015). They can be hosted in different polymers (Toker *et al.,* 2013, Espitia *et al,* 2012). Such metal nano particles can be coated, absorbed, or directly incorporated in the synthesis processes of packaging materials (Martinez-Abad *et al.,* 2012). This is a type of ''active packaging", where the nanoparticles interact directly with the packaged food to allow a better protection and shelf life extension.

In addition to shelf life extension, the urgency of preventing foodborne diseases required acceleration in the development of antimicrobial food packaging. This antimicrobial action may be obtained from the packaging by releasing the biocide directly into the food or in the space around the food (Vermeiren et al., 2002). This can be exerted by both organic and inorganic materials (Malhotra *et al.,* 2015). The former ones are mostly organic acids and enzymes whereas the latter ones are nanoparticles of metals or metal oxides. The organic antimicrobial materials are less stable at high temperatures compared to inorganic ones, whereas metal and metal oxide nanoparticles withstand a harsher processing condition (Metak and Ajaal, 2013, Metak, 2015) which is additional advantage of using inorganic materials.

Migration of nano particles from packaging materials to food is considered to be a risky of using metal nano particles. However, EU and USA food safety authorities regulate the application of AgNPs in food packaging and their use is evaluated in terms of Ag^{+} migration into the packed food. European Food Safety Authority (ESFA, 2011) did provide upper limits of Ag migration to less than 0.05 mg/kg in food. European Food Safety Authority (ESFA, 2011) has also addressed that consumption ZnO NPs should not exceed 25mg/100g (250mg/kg) which RDI for Zn.

Different studies were conducted (Echegoyen and Nerin, 2013) concerning the migration of silver nano particle from different types of nanocomposites, (Low Density Poly Ethylene, DPE and polypropylene) into food simulants. However, in their study they found that the Ag migration is well below the limits stated by the European Union legislation. For example, Cushen *et al., (2014*) studied the effect of time and temperature on the migration of silver from

polyethylene (PE) nanocomposites to boneless chicken breasts and found that migration of silver NPs was in a range from 0.003 to 0.005 mg/L, which is below the limit. Metak (2015) also found that bread samples showed the lowest migration (<0.05mg/L) level of AgNPs than other food samples. Since *injera* is somewhat similar products with bread, this type of antimicrobial packaging can also perform for it.

1.2. Statement of the Problem

Injera is nutritionally high value, and the most favorite food with nearly two-third of Ethiopian nutrition covers of *injera* (Guesh and Anteneh, 2017). In addition to internal consumption, Ethiopia is benefitting from the sharp improvement of '*injera'* export in which over 15 exporters are operational (Mengisteab, 2019).

However, because of fungal spoilage, the shelf life of *injera* is limited to only 3-4 days (Ashagre and Abate, 2012). As a result, *injera* preparation cycles every 3 or 4 days. This leads to a significant energy (power) consumption, time wastage, and burden (especially on women) in preparing *injera*. A consequence of mould growth is economic loss due to wastage (Skirdal and Eklund, 1993)

Even though *injera* has good nutritional value and being used as export commodity, it lacks preservation mechanisms to reduce this fungal spoilage. The traditional preservation methods (drying, cooling, salting, oiling etc.) are not generally applicable to *injera*. In addition, chemical preservatives, though widely used in bakery products, are not still convenient to use for *injera*. For example, it was proved that Chemical preservatives can extend shelf life of *injera* up to 8 to 12 days (Ashagrie and Abate, 2012). Nevertheless, population does not adapt to its applicability.

It is widely reported that nano-enhanced coatings are the most interesting innovations in food packaging in reducing microbial spoilage. For instance, Noshirvania *et al*., (2017) has reported that the shelf life of bread was increased up to 15 to 35 days when packed with nano-enabled packages. Metak (2015) has also reported that significantly lower rate of

deterioration was observed on bread and other products tested up to day 10, due to the antibacterial activity of Ag and TiO_2 nanoparticles.

There is no any research conducted on storage of *injera* with nano-enabled package. Therefore, the present study envisaged to establish nanomaterials coatings based on polymeric plastic package. Thus, can we impart plastic polymer with Ag and ZnONPs as antifungal in the form of dip-coated plastic packages for *injera* preservation application? is the key question to be addressed in this research.

1.3. **Objectives of the Study**

This project was designed with general and different specific objectives to achieve the expected results.

1.3.1. General objective

- Synthesis and characterization of Ag and ZnO nanoparticles from plant extracts and using them for nano-enabled coatings on plastic package to improve the shelf life of *injera*

1.3.2. Specific objectives

The specific objectives of this study are listed as follows;

- Green synthesis of Ag and ZnO NPs using plant derivatives, and characterization of these NPs using XRD, UV-Vis, and SEM techniques,
- Determination of antimicrobial activities both biosynthesized NPs (Ag and ZnONPs) by PDA agar plates test on pure culture of *Penicillium sp, Rhizopus sp and Aspergillus niger* fungal colonies,
- Evaluation of performance and related effects of plastic packages coated with Ag and ZnO through shelf life test on *injera*
- Examination of the nanoparticles migration from the nano-coated plastic package materials to *injera*.

2. LITERATURE REVIEW

2.1. Injera

2.1.1. Definition and nutritional importance of *injera*

Injera is fermented Ethiopian traditional bread made from flour, water and starter (*ersho*) (Ashagrie and Abate, 2012). *injera* is made from teff (*Eragrostis teff*) (Zuccagni) but other cereals may also be used in combination with teff. *Injera* from white teff is more preferable to red teff *injera* due to its color. However, many literature shows that the red teff contains a high amount of iron which is needed for improvement of anemia.

Teff is a minor cereal crop worldwide, whereas in Ethiopia, it is a major food grain (Tadesse, 1993; Gebremariam *et al.,* 2012). It is an ancient tropical cereal that has its center of origin and diversity in the northern Ethiopian highlands (Ketema 1997; Demissie 2001). The amount of teff produced in the world is increasing rapidly due to the plant's popularity as an especial nutritious grain. Teff grain does not contain gluten and is an increasingly important dietary component for individuals who suffer from gluten intolerance (Bemihiretu Boka *et al.,* 2013).

In Ethiopia, cereal crops predominate grain production with 80% area coverage and 87% of the total harvest (CSA, 2015). From these main cereal crops grown in the country, teff takes first rank with a production area of 2,866,052.99 ha (CSA, 2015). There is a growing interest in teff (whole grain) usage because of its nutritional merits. About 2/3 of Ethiopian diet consists of *injera* and it accounts for about two-third of the daily protein intake (Arogundade, 2006).

Teff is a dual-purpose cereal, valued for both grain and forage production in dry areas with short rainy season. Teff grain is rich in protein, carbohydrates, and fiber and is mainly used for human food, particularly in Ethiopia where it used for the production of the bread (*injera*) and beer (*tela*). Since the late 1990s, the recognition of teff as a gluten-free cereal of good nutritional value has resulted in newfound interest (Baye, 2015).

The major quality attribute of a good *injera* is its slightly sour flavor (Zegeye, 1997). Fellow (1997) reported that normal and typical i*njera* is round, soft, spongy, and about 6 mm thick, 60 cm in diameter with uniformly spaced honeycomb-like 'eyes' on the top *injera* has a very high value, as it is rich in calcium and iron.

2.1.2. Processing of *injera*

Injera is made from teff flour, water and ersho (starter culture). Teff flour is mixed with clean water in the ratio 1:2 (w/w) and 10 % of starter (*ersho*) by the weight of the flour and kneaded by hand. The resultant dough is allowed to ferment for about 72hrs. After this primary fermentation, the surface water is discarded. For every 1 kg of original flour, 200 mL of the fermented mixture is mixed with 400 mL of distilled water and boiled (known as '*absit*' making). It is cooled to about 45 ^{0}C and added into the main dough. The main dough is thinned by adding water equal to the original weight of the flour and stirred for 15 minutes. The batter is left covered for 2 hours for secondary fermentation. Then the *absit* is added to the thinned dough and mixed (called batter making). The batter is left for about 30 min to rise. More water is added to thin down and form the right consistency. Finally, about half a liter of batter is poured onto the hot clay griddle in a circular motion from the outside, working towards the Centre. After 2-3 minutes of cooking using traditional baking equipment (*metad*), the *injera* is removed and stored in a traditional basket container *mesob* (Ashagrie and Abate, 2012).

2.1.3. Shelf life of *injera*

The microbial shelf life is defined as the period in days during which the spoilage caused by microorganisms was first observed. The shelf life is expressed in relation to the corresponding control (Katsinis *et al.,* 2008). The shelf life of *injera* is very limited due to microbial (mostly fungus growth). The moisture contents and pH (acidic) of *injera* is very conducive for their growth that limits its shelf life to 3 to 4 days. Mould spoilage is a common problem that affects the shelf life of *injera* (Ashagrie and Abate, 2012).

2.1.4. Energy economics of *injera*

In developing countries like Ethiopia, residential sector takes the dominant share of the total national energy consumption. The majority of the residential energy is used for cooking or preparation of food, and the energy carrier is mostly traditional biomass; however, the usage of modern fuels like electricity is increasing.

Injera baking is one of the main cooking activities undertaken in Ethiopia. For instance, 25–30 pieces of *injera* will be baked per session for a single household in Ethiopia and thus, the average amount of energy required to bake a single session *injera* become 14.6 MJ. If we are

be able to extend the shelf life of *injera* by 8 days without losing its quality than we can reduce the energy consumption by at least 50%. In addition, the emission of greenhouse gases are also expected to diminish significantly if the households utilize biomass as source of energy.

2.1.5. Microorganisms in *injera*

The most common microorganisms found in *injera* are fungus type (yeasts and moulds).

2.1.5.1. Moulds

Moulds, those dusty little spots found spreading over different foods, cause the loss of millions of dollars to our economy every year (Malloch, 1981).There is an increasing knowledge and understanding of the role played by moulds in food spoilage. Especially the discovery of mycotoxin production in foods has highlighted the importance of moulds in food quality. It is, however, only within the last 5–10 years that major progresses have been made towards the prevention of spoilage caused by moulds. This is due to recent international agreements on taxonomy and analytical methods for food borne moulds, which has led to the discovery, that a specific, very limited fungus (mycobiota) is responsible for the spoilage of each kind of food. This is called the associated or critical fungi and has been shown to consist of less than ten species (Filtenborg *et al.*, 1996).

The microbial spoilage of foods may be viewed simply as an attempt by the food biota to carry out what appears to be their primary role in nature. In spite of their simplicity when compared to higher forms, microorganisms are capable of carrying out many complex chemical reactions essential to their perpetuation. To do this, they must obtain nutrients from organic matter, some of which constitutes our food supply (Jay, 2000).

Moulds are ubiquitous which can be found in a wide variety of environments. This broad occurrence can be explained by the fact that moulds can utilize a variety of substrates. Moulds are relatively tolerant to low pH, low water activity, and the presence of preservatives (Huis in't Veld, 1996).

In addition to visible spoilage, moulds can also accumulate toxins hazardous to health. It has now been established that more than 200 different types of moulds do form substances that are toxic to man if grow on certain foods. Although most research has been carried out on the

metabolites of *Aspergilus flavus*, it is obvious that in addition to the so-called aflatoxins, many other mycotoxins may be of great significance (Huis in't Veld, 1996). Some moulds are capable of producing toxic and carcinogenic metabolites. Multiplication of these organisms on foods must be regarded as a potential health problem. A further consequence of such mould growth is economic loss due to wastage (Skirdal and Eklund, 1993).

2.1.5.2. Characteristics of moulds

Molds are filamentous fungi that grow in the form of a tangled mass that spreads rapidly and may cover several inches of area in 2 to 3 days. The total of the mass or any large portion of it is referred to as mycelium. Mycelium is composed of branches or filaments referred to as hyphae **(**Heritage *et al., 1996*). Those of greatest importance in foods multiply by ascospores, zygospores, or conidia. The ascospores of some genera are notable for their extreme degrees of heat resistance. One group forms pycnidia or acervuli (small, flask-shaped, fruiting bodies lined with conidiophores). Arthrospores result from the fragmentation of hyphae in some groups.

2.2. Antimicrobial Detection Methods

Different detection methods are used in antimicrobial determination. It can be antifungal or antibacterial detection method. Each method are used based on feasibility and efficiency needed. For acidic foods, fungal detection is common because of acidic media conduciveness for mould and yeast. The pH of Injera is about 3.4 (Ashagrie and Abate, 2012). It is very conducive for fungus like *Aspergillus niger, pencillinum sp., Rhizopus sp*. and other fungi. Some of antimicrobial testing methods are listed in Table 1 below:

Table 1: Test methods for antimicrobials evaluation

Methods	Application	References
Disc diffusion method	Standard antibiotics is compared with test antimicrobial against pure culture and inhibited zone is measured	Banerjee and Nath (2015).
Agar/broth dilution method	Antibacterial samples are prepared in two fold dilution and applied onto Agar plate, then MIC is recorded	Jiang. L.(2011)
Food poisoned method	Cultured Fungal (5-7 days) is punched aseptically with a sterile cork borer. The fungal discs are then put on the gelled agar plate. The agar plates have been prepared by impregnating desired concentration of plant extract at a temperature of 45 - 50°C.	Das *et al*.,*(*2010)
Well diffusion	Antimicrobial sample is pour into well made by cork borer and inhibited zone is measured	Thomas *et al*., (2014)

2.3. Preservation of Foods

Food preservation is a method of maintaining food at desirable level of properties for their maximum benefits (Rahman, 2007). All foods begin to spoil as soon as they are harvested or slaughtered. Such microorganisms as bacteria and moulds cause some spoilage. Other spoilage resulted from chemical changes within the food itself due to natural processes, such as enzyme action or oxidation (Jay, 2000).

Major Food Preservation Methods

The major food preservation techniques that are employed are based on a relatively limited set of factors, so that their range is necessarily limited also (Gould, 1996; Jay, 2000). The major food preservation methods are summarized in Table 2.

Table 2**: Major food preservation methods**

Cold	Heat	Drying	Fermentation	Physical	Chemical
Freezing	Cooking	Tunnel	Alcoholic	Filtration	Sugar
Chilling	Pasteurization	Solar	Acetic	Separation	Salt
	Canning	Spray	Lactic	Distillation	Spice
		Vacuum		Irradiation	Acids nitrite, benzoate,
		Freeze drying		Concentration	Additives e.g. antioxidants
				(MAP/CAP)	

Sources: Huis in't Veld (1996)

2.4. Nanoparticles

Another newly emerging nanotechnology is metals' nanoparticles use in foods preservation. Nanoparticles are the particles having the size between 1-100 nm. They are having different properties than the bulk material of the same element due to variation in size, and shape.

Among all the noble metals nano silver is most important that it possess catalytic and anti-bacterial activity. Due to these properties, silver nanoparticles can be used as catalysts in chemical reactions, and in medical field (Elumalai *et al.,* 2010, Liangpeng *et al.,* 2014 Sheehy *et al.,* 20150,). The use of zinc oxide nanoparticles in antimicrobial food packaging has also been reported (Perez Espitia *et al.,* 2012). It is more preferable than others since it is used as component of foods even though its nano particle characteristics are not well studied yet.

Use of nanotechnology in the food industry quality control has significantly increased. It is widely employed in numerous fields such as diagnosis of microbial toxin of foods using nano biosensors, increasing food shelf life with the help of nano-clay and nano-silver packaging, as well as changing some of the unpleasant organoleptic properties such as taste, color, and smell. Because, usage of this technology is growing with an increasing pace (Ahari1**,** 2017).

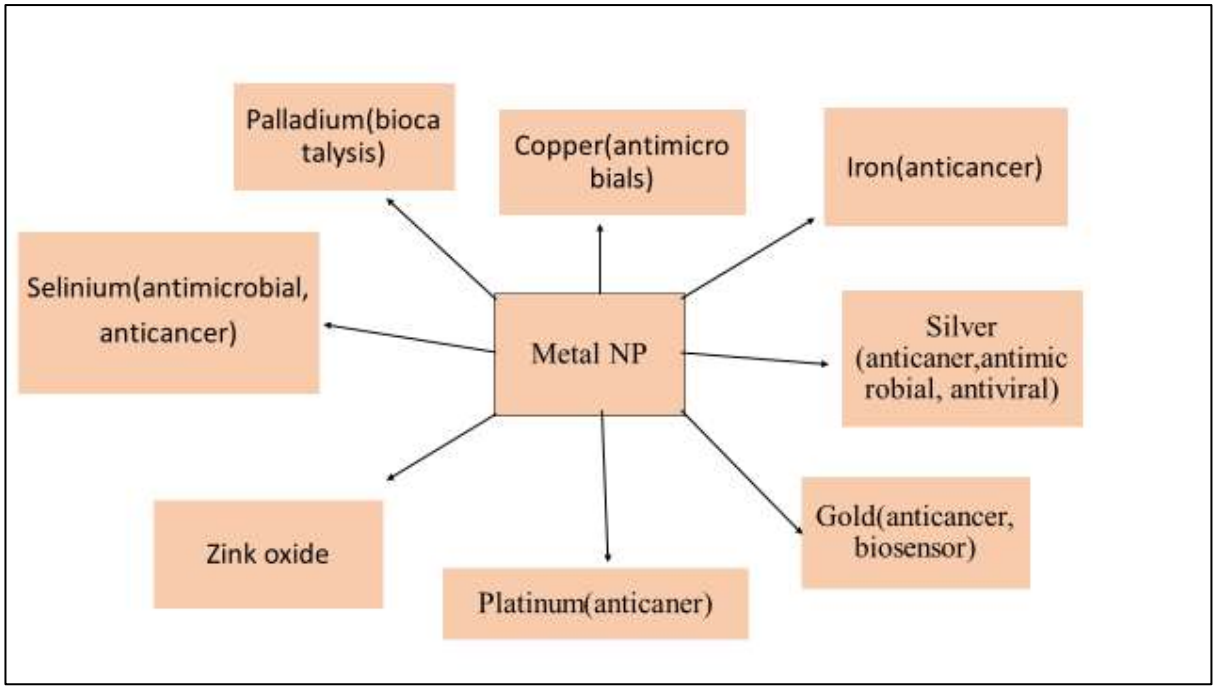


Figure 1: The Scope of Nanoparticles uses (Sources: Mittal et al., 2013)

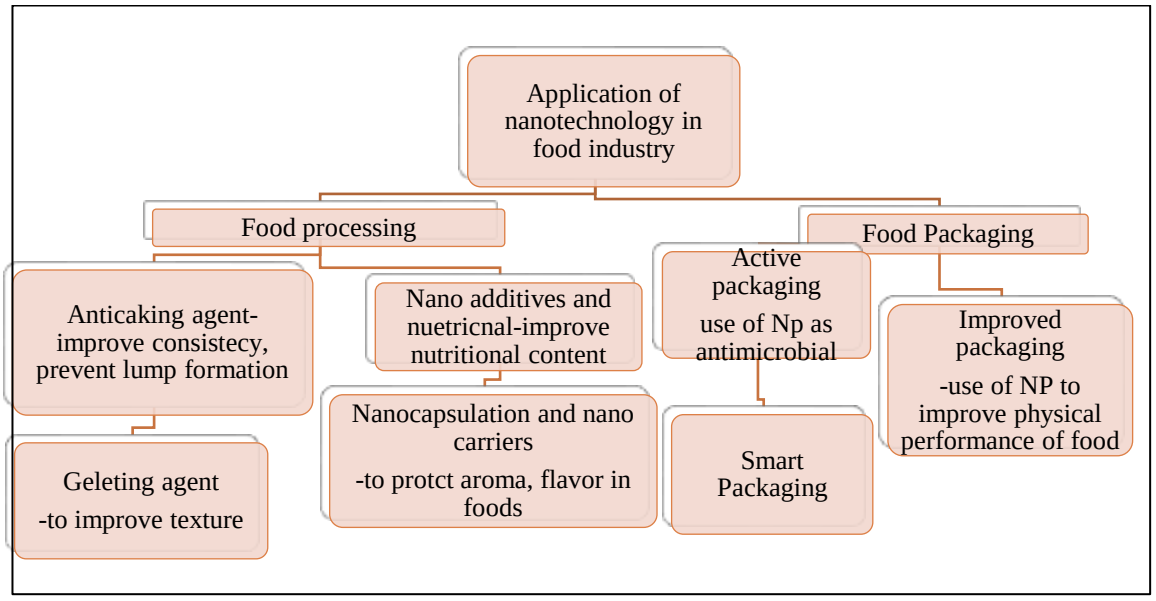


Figure 2: Role of nanotechnology in different aspects of food sectors (Source: Singh *et al.*,2017).

2.4.1. Biosynthesis of nanoparticles

Nanoparticles can be synthesized by chemical methods from plants, bacteria and fungus. Synthesis of nanoparticles from plants is being important since it is environmentally friendly.

The shape and effectiveness of these nanoparticles are based on their source. The following Table 4 and 5 show the plant species used for biosynthesis of AgNPs.

The synthesis of metal oxide nanoparticles with the use of plant extract is a promising alternative to the conventional chemical method (Ogunyemi *et al***.,** 2019). The need for biosynthesis of nanoparticles rose as the physical and chemical processes were costly. Therefore, in the search of for cheaper pathways for nanoparticle synthesis, scientists used microorganisms and then plant extracts for synthesis. Nature has devised various processes for the synthesis of nano- micro- length scaled inorganic materials that have contributed to the development of relatively new, and largely unexplored area of research based on the biosynthesis of nanomaterials (Mohanpuria, 2008).

Biosynthesis of nanoparticles is a kind of bottom up approach where the main reaction occurring is reduction/oxidation. The microbial enzymes or the plant phytochemicals with anti-oxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles. The three main steps in the preparation of nanoparticles that should be evaluated from a green chemistry perspective are the choice of the solvent medium used for the synthesis, the choice of an environmentally benign reducing agent and the choice of a nontoxic material for the stabilization of the nanoparticles. Most of the synthetic methods reported to date rely heavily on organic solvents. This is mainly due to the hydrophobicity of the capping agents used (Raveendran, 2003)

Synthesis using bio-organisms is compatible with the green chemistry principles: the bio-organism is (i) eco-friendly as are (ii) the reducing agent employed and (iii) the capping agent in the reaction (Li *et al.,* 2007). Often chemical synthesis methods lead to the presence of some toxic chemical species adsorbed on the surface that may have adverse effects in applications (Parashara and Srivastava, 2009).

2.4.2. Shapes of biosynthesized Ag and Zno nanoparticles

Biosynthesized nanoparticles have different shapes and sizes based their precursor and capping agents**.** The same type of nanoparticles can have different shapes when synthesized from different plant sources. Also the surface plasmon absorption band of the same metal nano

particle may be different if plant sources are different. The size of nanoparticles is a determinant factor in their antibacterial effectiveness.The nanoparticles with smaller size

are preferred for microbial inhibition. Table 3 and Table 4 show shapes and sizes of Ag and ZnONPs biosynthesized from different plant species.

Table 3: Plant species used for synthesis of metal oxide nanoparticles

Plants species	NPs	Size of NP (nm)	Morphology	Application	Reference
Pongamia pinnata	ZnO	30-41	spherical	Antimicrobial, anticancer	Malaikozhundan *et al*., 2017
Rhipicephalus microplus	ZnO	20 -65	Spherical, hexagonal	Acaricidal	Banumathi *et al.*, 2016
Camellia senensis	ZnO	19	Rod	Pharmaceatical active compounds removal	Hassan *et al.*, 2016

Deborah *et al.,* (2018)

Table 4: Plant species used for agnps synthesis

Plant species	Size of NPs	Morphology	Application	Reference
Annona Muricata	22	sphare	Vector control	Santhosh *et al.*, 2015
Arachis hypogea	10-50	Sphare, oval	antifungal	Velmurugan *et al.*, 2015
Baccarea ramiflora Lour.	10-15	sphare	biosensor	Alam *et al.*, 2015
Barleria prionitis	10-20	sphare	environmental	Ghosh *et al*.,2016
Centella Asiatica	30-50	sphare	Oxidative stress and antidiabetic activity	Wilson *et al.*, 2015
Couroupita guianensis	5-45	cubic	Vector control	Vimala *et al.*, 2015
Dracaena cinnabari	4-50	sphare	antiradiation	Hasan *et al.*, 2015
Erigeron bonariensis	13	sphare	cataylst	Kumar *et al*., 2016
Morinda tinctoria	80-100	Sphare, rod	Water treatment	Vennila *et al*.,2015
Quisqualisindica	1-30	sphare	Vector control	Govindarajan *et al.*, 2016
Simarouba glauca	33-50	sphare	antibacterial	Kanchana *et al.*, 2016
Styraxbenzoin	12-46	Sphare	Antimicrobial, anticancer	Du *et al.*, 2016
Zornia diphlla	28-61	Sphare, triangle, truncated triangle	Vector control	Govindarajan *et al.*, 2016

Deborah *et al.*, (2018)

Table 5: **Shapes of biosynthesized Ag and ZnONPs**

Plant Name	Type of NPs	Shape	UV-vis absorption (nm)	Reference
Sonneratia apetala	Ag	Spherical	425 to 475	Nagababu and Rao, 2017
S. asoca	Ag	-	410 to 440	Banerjee and Nath, 2015
Conocarpus erectus and Nerium indicum)	Ag	Irregular, spherical	-	Ahmed *et al*., (2016),
Azadirachta indica	Ag	Spherical	436 to 446	Ahmed, 2015
Acalypha indica	Ag	Spherical		Krishnaraj *et al.*, (2010)
Micrococca mercurialis(L.)Benth.	ZnO	-	305, 299	Manokari *et al.*, (2016)
Abrus precatorius seeds	ZnO	Spherical, rod shape	386-450	Vishwakarma, 2010

2.4.3. Nano food packaging

One of the main products of nano-technology in the field of food industry is the use of nano packaging covers including nano-silver, nano clay, and nano-cupper. Each one of these covers is classified based on the main metal used in them and its property (antibacterial, resisting the penetration of oxygen and moisture into the food product). One of the chief types of these covers is the nano-silver packaging cover (Moaddab *et al.*, 2011).

Bionanotechnology is the integration between biotechnology and nanotechnology for developing biosynthetic and environmental friendly technology for the synthesis of nanomaterials *(*Sobha, 2010). Nano scale particles have emerged as novel antimicrobial agents owing to the high surface area to volume ratio. These interests are due to the growing microbial resistances against metal ions, and antibiotics (Chan and Ming-HsunTsai, 2008).

Table 6: Summary of antimicrobial packaging with different nanoparticles

Nanoparticles	Polymer matrix	Tested microorganisms	Reference
Ag/Chitosan	PLA1	Staphylococcus aureus (ATCC 6538)	Turalija *et al.,,* (2016)
Ag	Agar banana powder	Escherichia coli Lysteria monocytogenes	Orsuwan *et al*., (2016)
ZnO/Ag/Cu	PLA1/PEG3	Lysteria monocytogenes , Salmonella typhimurium	Ahmed *et al*., (2016)
Ag	PE4	Escherichia coli	Eslami *et al.,* (2016)
Ag/TiO_2	PE	Aspergillus flavus	Li *et al.,* (2017)
Ag	Starch/PVA5	Aspergillus niger; Penicillium expansum	Cano *et al*.,(2016)
Ag	PHBV37	Salmonella entericaLysteria monocytogenes	Castro-Mayorga *et al*., (2017)
ZnO	LDPE	Bacillus subtilis; Enterobacter aerogenes	Esmailzadeh *et al*., (2016)
ZnO	MC9	Staphylococcusaureus;Lysteria monocytogenes	Espitia *et al.,* (2012)

Radusin *et al***.,** (2016) - Review article

Biomolecules present in plant extracts can be used to reduce metal ions to nanoparticles in a single-step green synthesis process. This biogenic reduction of metal ion to base metal is quite rapid, readily conducted at room temperature and pressure, and easily scaled up. Synthesis mediated by plant extracts is environmentally friendly. The reducing agents involved include the various water-soluble plant metabolites (e.g. alkaloids, phenolic compounds, terpenoids)

and co-enzymes. Silver (Ag) and gold (Au) nanoparticles have been the particular focus of plant-based syntheses. Extracts of a diverse range of plant species have been successfully used in making nanoparticles (Mittal *et al.,* 2013, Nair *et al.,* 2010, ;Perez-de-Luque and Rubiales, 2009). The use of zinc oxide nanoparticles in antimicrobial food packaging has also been reported (Perez Espitia *et al.,* 2012). Several types of nanoparticles have been shown to reduce the microbial loads in treated wastewater effluent (Duran *et al.,* 2007). Table 6 shows antimicrobial packaging systems with nanoparticles

2.4.4. Mechanism of action of nanoparticles on microbial growth

Silver nano particle is one type of metal used as antimicrobial agent. Silver ion is highly toxic to most microorganisms (Jung *et al., 2008*) and at least one mode of antimicrobial action of nanoparticles is through a slow release of silver ions via oxidation within or outside the cell. Silver nanoparticles are known to affect the permeability of membranes of microbial and other cells (Li *et al.,* 2010).

Zinc oxide nano particle can also be used as antimicrobial agent on bacteria and fungus. It has got lots of attention for their antimicrobial activities, and good stability. The recent growth in the field of nano-metric materials prepared by non-conventional processes has stimulated the search of new applications of ZnONPs (Amekura, 2006). Intensive studies have been made for applications in foods. ZnONPs offer broad-spectrum antimicrobial activity because they damage cell membranes and generate ROS, and these properties allow ZnONPs to inhibit the growth of microorganisms. Furthermore, adding ZnO NPs to food packaging materials offered improvements to the mechanical and barrier properties of the material (Noshirvani *et al.* 2017).

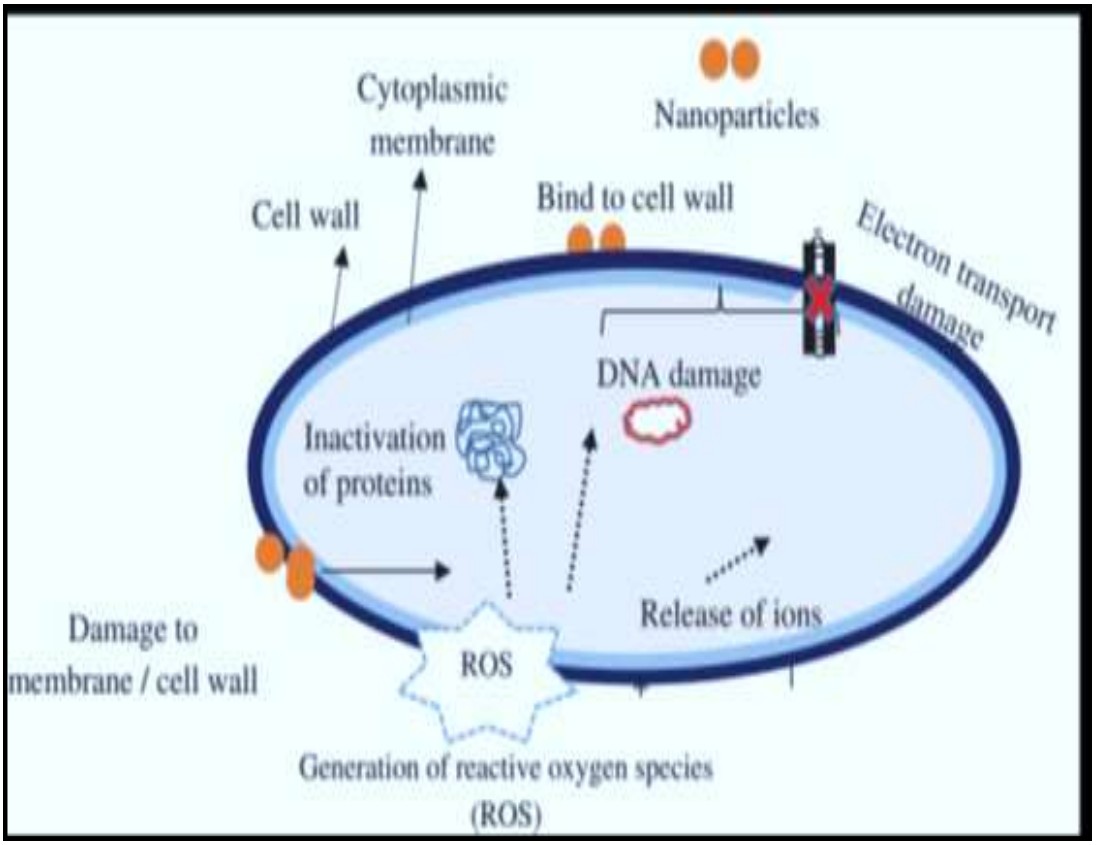


Figure 3::**Antimicrobial mechanisms of inorganic nanoparticles.(Huang *et al.,*2018).**

The antimicrobial activity of ZnO NPs falls into two major pathways. These are: interfering with the cell membrane by damaging the membrane integrity and permeability and increasing oxidative stress through ROS generation. The antimicrobial activity of ZnO NPs can be realized by either the nanomaterials themselves or free Zn^{2+} dissociated from the nano materials. At low concentrations, the antimicrobial effect is induced by nanomaterials themselves through physical absorption or electronic interaction–induced membrane damage (Yong *et al.* 2013), whereas excessive ROS production has been noted when Zn^{2+} concentrations reach 50 mg/L. ZnO NPs possess positive charges in water, whereas bacterial membranes composed of acidic phospholipids and lipopolysaccharides present negative charges. This provides the basis for electrostatic interaction between ZnO NPs and bacterial cells (Sarwar *et al.* 2016). The ZnO NPs first bind to the hydrophilic site on the lipopolysaccharide and then deform the outer membrane. About 1 mg/L ZnO NPs showed no significant effect on the membrane integrity of bacteria during a 45-day incubation. However, when the concentration reached 10 mg/L, the membrane integrity of bacteria was destroyed.

2.4.5 Silver nanoparticles (AgNPs)

Among the antimicrobials used in active packaging technology, silver takes the attention mostly because of its broad antimicrobial spectrum (Diblan and Kaya, 2017). There is historic source reported that silver spoon used in milk to prolong shelf life (Duncan, 2011). Starting from the past to current year, the silver usage has reached a wide range area of applications.

2.4.5.1. Antimicrobial activity of silver nanoparticles

Silver has been preferred as antimicrobial agent used in water which will be consumed by astronauts and since very long time storage needed. Moreover, in 2009, FDA has permitted direct addition of silver nitrate to water if not to exceed 17 µg/kg (Duncan, 2011). The study by Choudhary *et al.,* (2014) has shown that the AgNPs (100 µL) has an antimicrobial effect on both bacteria and fungus. Table 7 and 8 below show antibacterial and antifungal activity of AgNPs.

Table 7: Antibacterial and antifungal Activity of silver Nanoparticles (100 µL)

Name of Plant	Anti-bacterial activity (ZOI)	Antifungal activity (ZOI)
Ocimum sanctum	7 mm	5 mm
Cassia auriculata	10 mm	5 mm
Datura metel	6 mm	---
Carica papaya	5 mm	---

Source: Choudhary *et al.,* (2014)

Table 8: Comparison of antimicrobial activity of AgNPs (1 mg/mL) with standard drugs

Name of the bacterial species	Amount of silver nanoparticles 1 mg/mL	Zone of inhibition of Silver nanoparticles (mm)	Zone of inhibition of Standard drug (mm)
E. coli	10 µL	5	20
	25 µL	7.2	
	50 µL	8	
Bacillus subtilis	10 µL	11	19
	25 µL	14.22	
	50 µL	15	
S. aureus	10 µL	14	18
	25 µL	17.12	
	50 µL	17.11	
P. aeruginosa	10 µL	4.2	22
	25 µL	7	
	50 µL	7.12	

Banerjee and Nath (2015)

2.4.5.2. Antifungal assay

The potato dextrose agar plates were prepared and fungal cultures of A. niger was swabbed on these plates (Velavan, 2012). Silver nanoparticles solution (100 µl) placed in the agar plate and kept for incubation at 37 °C for 24 hrs. In bacteriological incubator. Ethanol and antibiotic chloramphenicol was used as a positive control and zone of inhibition was measured after incubation period.

2.4.6. Zinc oxide nanoparticles (ZnO NPs)

ZnO belongs to the class of metal oxides, which is characterized by photo catalytic and photo-oxidizing capacity against chemical and biological species (Sharma *et al.*, 2010). Zinc oxide nanoparticles (ZnO NPs) are extensively studied material due to their low toxicity and

photochemical stability (Zelechowska, 2014). Zinc nitrate was used as precursor material to synthesis zinc oxide nanoparticles from various biological sources (Shekhawat *et al.,* 2015).

Antimicrobial activity of ZnONPs

The powder of ZnO is widely used as an additive into numerous materials and products including plastics and foods (source of Zn nutrient). The antagonist activity of ZnO NPs against Xoo strain GZ 0003 was determined by measuring the inhibition zone. The inhibitory effect of the ZnO NPs increased with increasing of its concentration and its maximum inhibition is at 16.0 mg/mL for all the biologically synthesized zinc oxide nanoparticles (Ogunyemiet *et al.,* 2019). The Minimum Inhibitory Concentration (MIC) for the five Salmonella strains was 312.5 – 625 μg ZnO/mL and the relative sensitivity of Campylobacter to ZnO NPs was 25 – 50 μg/mL (Duffy *et al., 2018*). Also Xie *et al.* (2011) reported that ZnO NPs exhibited remarkable antibacterial activity against C. jejuni, even at low concentrations.

Table 9: The antibacterial activity of the prepared Carboxy methyl cellulose(CMC)/poly vinyl Alcohol (PVA)/ZnO nanocomposites

Inhibition zone (mm)			
Aspergillus Niger	Candida Albicans	CMC:PVA ratio	ZnONPs (%)
0	0	50:50	0
0	20	50:50	2
9	22	50:50	4
10	25	50:50	6
0	0	60:40	0
0	18	60:40	2
8	19	60:40	4
12	20	60:40	6

Ahmed *et al*., (2017)

2.5. Risks Associated With Nanotechnology

Besides many advantages of nanotechnology to the food industry, safety issues associated with the nanomaterial cannot be neglected. Many researchers discussed safety concerns associated with nanomaterial giving emphasis on the possibility of nanoparticles migrate from the packaging material into the food and their impact on consumer's health (Bradley *et al.,* 2011*;*

Jain *et al.,* 2016). Although the material being considered as GRAS (generally regarded as safe) substance, additional studies must be acquired to examine the risk of its nano counterparts because the physiochemical properties in nano states are completely different from that are in macro state. Moreover, the small size of these nanomaterials may increase the risk for bioaccumulation within body organs and tissues (Savolainen *et al.,* 2010). For example, silica nanoparticles, which are used as anti-caking agents, can be cytotoxic in human lung cells when subjected to exposure (Athinarayanan *et al., 2014*). Many factors affect dissolution including surface morphology of the particles, concentration, aggregation, and adsorption. A model to study the migration of particles from food packaging has been developed by Cushen *et al.*, (2014). They studied the migration of silver and copper from nanocomposites and observed that the percentage of nano filler in the nanocomposites was one of the most crucial parameters driving migration, more so than particle size, temperature, or contact time. Since every nanomaterial has its individual property, therefore, toxicity will likely be established on a case-by-case basis (Mahler *et al.,* 2012). Further, regulatory authorities must develop some standards for commercial products to ensure product quality, health and safety, and environmental regulations.

The few regulatory safety assessments of nano particulates in food contact materials take a cautious approach in which no migration of nanoparticles is permissible. Since there are still limitations with measuring NPs in food/food simulants from migration experiments, this essentially means elemental constituent migration must be lower than the detection limit (Drew and Hagen, 2016).

Drew and Hagen (2015) have concluded their work with suggestion based on few studies review results. The data reviewed for the report indicate for most of the studied nanomaterials in food packaging, migration of intact nanoparticles into food simulants is negligible; implying consumer exposure to these materials is likely to be low. It suggests that there is low potential for safety issues related to the 'nano-ness' of the materials incorporated into food packaging. If they were to migrate in nano particulate form, it would be anticipated at the resulting low concentration in food that many of the metal oxide nano particulates would likely dissolve into their ionic forms upon contact with acid foods or stomach acid. These conclusions are tempered by the relatively few studies, which have investigated the migration of nanoparticles from food

packaging materials and the uncertainties in current analytical techniques for measuring possible migrated nanoparticles in foods/simulants.

For zinc oxide NPs in polyolefin, however, EFSA (2015) took a different approach. Although no direct evidence was available on the physical form of the released zinc in the migration experiments that were conducted, the agency concluded any zinc present in particulate form would be expected to dissolve immediately into ionic zinc on contact with acid foods or stomach acid. This is in line with the suggested findings from the migration experiments with nano silver. The agency concluded the substances do not migrate in nano form; therefore, they focused their safety evaluation on soluble ionic zinc.

EFSA (2015) has assessed the safety of zinc oxide NPs (uncoated and coated with 3-(methacryloxy) propyl trimethoxysilane) intended for use as transparent UV absorbers in polyolefin for food packaging. The substance is used as a powder in nano-form (average particle size ~44 nm). In the final polymer (LDPE) the NPs are still present but largely aggregated (~120 - 205 nm; 10-35 % <100 nm).

Zinc oxide in bulk form is authorized in Europe as an additive for plastic materials and materials in contact with food with a standard migration limit (SML) of 25 mg/kg food (as zinc) (Drew and Hagen, 2016). Standard migration tests were carried out with the nanocomposite LDPE films, containing the maximum use level of uncoated (2 %) and coated (3 %) zinc oxide. Food simulants used were 3 % acetic acid, 10 % ethanol, and 50 % ethanol; exposures were for 10 days at 60 °C. Zinc was measured in food simulant solutions by ICP-MS and ICP-AA. Migration of zinc into 3 % acetic acid was up to 7.6 mg/kg (for 2 % uncoated ZnO) and 17.3 mg/kg (for 3 % coated ZnO). Migration into ethanol was much lower, up to 80 µg/kg.

EFSA (2015) indicate the zinc detected in the other simulants is likely ionic zinc because of solublization of zinc oxide. They noted, however, no direct evidence is available on the physical form of the released zinc. Nevertheless, EFSA (2015) commented if the zinc was in particulate form, it might be dissolved immediately into ionic zinc on contact with acid foods or stomach acid. The agency concluded that the substances do not migrate in nano form; therefore, they focused their safety evaluation on soluble ionic zinc. Although the migration

data comply with the current standard migration limit (SML), i.e. for zinc (25 mg/Kg), with dietary exposure from other sources, the upper limit intake of 25 mg/person/day could be exceeded. EFSA (2015) recommended that the Commission reconsider the standard migration limit of 25 mg/kg for zinc, taking into account that consumers exposed to zinc from sources other than food contact materials.

The majority of the migration studies found for nano silver food packaging composites have shown levels of migration of ionic silver into foods and food simulants below the European standard migration limit of 0.05 mg/Kg food, suggesting low risk of consumer exposure and subsequently low risk of adverse effects. However, there are also several studies, in which migration exceeded this limit. This indicates that for new food packaging products containing nano silver, it is still necessary to conduct migration experiments on a case-by-case basis (Drew and Hagen 2016).

3. MATERIALS AND METHODS

3.1. Materials and Equipments

$AgNO_3$ and $Zn(NO_3)_2.6H_2O$ were used as primary precursors. *Eucalyptus globulus* and Digita (*Calpurnia aurea (Ait.) Benth.)* Leaves were used for the synthesis of nanoparticles.These plants were randomly selected as exogeneous and endogenious species, respectively. LDPE polymers (18 x 21 cm zipper bag) was used for preparation of nano-enabled food contact packaging material. Potato Dextrose Agar (PDA), Mancozeb, chramphenicol, filter paper were purchased from super market.

Equipments used in this study were analytical balance, pH meter, refrigerator, incubator, oven, magnetic stirrer, centrifuge, miller, vortex, muffle furnace, X-ray diffractometer(XRD), UVi-Vis Spectroscopy, Scanning Electron Microscopy(SEM) and microscope.

3.2. Experimental Design

Completely Randomized Design (CRD) was used in this study. All the treatments were triplicated. For anti-microbial determination, both AgNPs and ZnONPs were used at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 % concentrations. Extract +AgNPs(Ext+AgNPs) was also taken as one treatment. Totally, 12 treatments were compared including control sample (Mancozeb). For coating dose determination through shelf life test, both types were taken at concentrations of 10, 30, 50, 70, 90, and 100 % whereas uncoated zipper bag was used as control sample. Totally 7 samples including the control sample were used for the treatments.

However, for other parameters (mold and yeast cfu/g, moisture content, pH, migration), both treatments (ZnONPs and AgNPs) were used at 10, 50 and 90 % concentrations. Extract of Eucalyptus +AgNPs (Ext+AgNPs) and Extract of Digita + ZnONPs (Ext+ZnONPs) were used as treatments. Untreated plastic zipper bag was used as a control. Totally 5 treatments were analyzed separately including untreated (control) sample

3.3. Plant Extract Preparation

Fresh and healthy *Eucalyptus globulus* and Digita (*Calpurnia aurea (Ait.) Benth.* Leaves were used for biosynthesis of Ag and ZnO nanoparticles, respectively. Both plant types were collected from fields and washed well with pure water to remove dusts and other

contaminants.Then they were air-dried and ground to fine powder. About 100 g of powder and 800 mL of distilled water were allowed to mix in 1000 mL beaker (Rose and Priya, 2017) and heated on magnetic stirrer. Afterwards, the extract was allowed to cool to room temperature and filtered using only Whatman® No.1 Filter paper to decrease size effect from the plant extracts. The extracts were kept in separate beakers at 4 ^{0}C (Manokari *et al.*, 2016) for further reaction with $AgNO_3$ and $ZnNO_3$, respectively.

3. 4. Synthesis, Optimization and Characterization of Zno and Ag Nanomaterials

3.4.1. Synthesis of Ag and ZnO nanoparticles

The synthesis of both nanoparticles was carried out as follows: aqueous solutions of silver nitrate ($AgNO_3$) andzinc nitrate $Zn(NO_3)_2$ were prepared with distilled water. About 100 mL of leaf extract was added to 300 mL of 15 mM (0.7641 g into 300 mL distilled water) aqueous solution of silver nitrate and 1136.4 mM zinc nitrate (15 g of zinc nitrate into 300 mL distilled water). Then, the mixture was heated at 82^{o}C for 45 minutes (Banerjee, and Nath, 2015) at 1500 rpm on magnetic stirrer (JANKEKUNKEL GMBH and CO.KG, IKA®- Labor technick-Achung: Netzstecher Ziehen!).

The change of color of the aqueous silver nitrate and zinc nitrate solutions after addition of plant extract was from deep red to dark brown and dark red to light yellow, respectively, indicating the synthesis of silver and zinc oxide nanoparticles. Both solutions were kept at room temperature for 48 hours. After complete settlement of nanoparticles, the mixtures were centrifuged at 12,000 rpm for 10 minutes (Thomas *et al.*, 2014) using centrifuge (Thermo Electron LED GmbH, Zwelgn Lederlassung Osterode, Am Kalkberg, 37520 Osterode am Harz, Germany). The supernatant was discarded and the pellet was collected and washed well with distilled water. For silver nanoparticles, the collected pellet was air dried and ground using mortar and pestle then finally silver nano particle obtained. But, the pellet from zinc nitrate solution was oven dried at 105 ^{0}C for overnight and finally incinerated at 450 ^{0}C for 3 hours (Haritha Meruvu, 2011) in Muffle Furnace (Parsons Lane, Hop Valley, S33 6RB, England)). The prepared nanoparticles were stored at 4 °C for characterization and further use

Table 10: Methods used for Ag and ZnONPs preparation

Nanoparticles	Amount in gm	Molarity (Mm)	Distilled water (mL)	Leaf extract (mL)	NP Formation indicator	Centrifugation	Drying and collection
Ag	0.7641	15	300	100	Dark brown	12,000 rpm for 10 minutes	Air dry and ground, then collected
ZnO	15	1136.4	300 mL	100	Light yellow	12, 000 rpm for 10 minutes	Oven dry and changed to ash at 450 ^{o}Cin furnace

3.4.2. Optimization of synthesis method

The optimization for the synthesis of the nanoparticles was carried out by varying the concentration of the precursor and mass to volume ratio of precursor to extract. The optimum concentration was selected based on the actual yield of the nanoparticles obtained after centrifugation (which is represented by * in the Table 11 and 12).

Table 11: Optimization of AgNPs

Mass of $AgNO_3$	Volume of Distilled Water (mL)	Molarity (mM)	Ratio of $AgNO_3$ solution to Extaract	Indicator
0.5094	300	10	1:1	deep red to slight brown
0.6113	300	12	3:2	deep red to brown
0.7641*	300	15	3:1*	deep red to dark brown

Table 12: Optimization of ZnONPs

Mass of $Zn(NO_3)_2$	Volume of Distilled Water (mL)	Molarity (mM)	Ratio of $Zn(NO_3)_2$ to Extract	Indicator
10	300	852.3	1:1	dark red to red
15*	300	1136.4	3:1*	dark red to light yellow
20	300	14773.2	3:2	dark red to light red

3.4.3. Characterization of the synthesized nanomaterials

The sample was investigated by X-ray Diffractometer (XRD) with Cu-Kα radiation of 1.541Å wavelength to study the formation, the crystallite size and the quality of the-synthesized nanoparticles. The surface morphology and particle size was investigated by using Scanning Electron Microscopy (SEM). An ultraviolet-visible spectrophotometer (UV-vis) was used to measure the absorption between 200 nm and 800 nm (Abdallaha *et al.*, 2019).

The average crystallite size D was calculated by the Debye Scherrer formula (Haritha Meruvu, 2011*),* degree is changed to radians *(=*β*) by* formula used by Metak, (2015)

$$D \text{ (crystallite size)} = K \lambda / \beta \cos\theta \text{----------eq2}$$

where K -is the sherrer constant(0.9)

ʎ - Is the X-ray wavelength (1.541Å)

β - is the peak width at half-maximum,

Theta (θ) - is the bragg diffraction angle.

$$\text{Degrees} = \text{radians} * \pi/180 \text{ ----------eq3}$$

3.5. Preparation of Nano-Enabled Coatings from Zno and Ag Nanomaterials

a) Preparation of plastic polymers containerfor nano-coating.

Dip coating was used to attach the nanoparticles onto the plastic bags packages. Zipped plastic bag containers (18 cm x 21 cm) were disinfected by 70 % ethanol and dried at 60 ^{o}C (Metak, 2015) for half an hour. The interior part of the plastic bags was exposed manually and dip-coated in different concentration level of (10, 20...100 %) Ag and ZnO nanoparticles solutions for 5 min and dried at 60 ^{o}C in an oven.

3.6. Performance Evaluation

3.6.1. Pure culture preparation

Sterile molten PDA with chloramphenicol (60 mg/L) was poured into petri dishes and allowed to solidify. Different colonies were taken from the mixed culture (*injera* stored for 5 days) using sterile needle and transferred to plates containing solidified PDA agar. Colonies were

selected based on their color (*Aspergillus Niger*-black, *Penicillium spp*-green, *Rhizopus*-dark brown). The plates were incubated at 25 ^{o}C for 72 h (Ravimannan, 2016). Growth and similar sub-culturing was done up to fourth generation to get pure culture. The structure of fungal isolates was identified by microscope(Helmmut Hund GmbH, D-6330 Wetzlar 21, Gemany) to confirm that the isolated cultures were pure culture.

3.6.2. Antifungal evaluation of AgNPs and znONPs

Disc diffusion method was used to assess the antifungal activity of different concentrations of Ag and ZnONPs against the test fungi as described by Clara *et al.,* (2013). Whattman filter paper (No:1) was used to prepare discs approximately 6 mm in diameter, which are placed in hot air for sterilization (Rose *and Pri*ya., 2017) . The suspension was adjusted to 0.5 mc Farland standard using cytometry method. The antifungal activity was evaluated against the final fungal concentration of 10^6 cfu/ml (Al-Zubaidi *et al.,* 2019). About 1 mL of spore suspension was uniformly spread on sterile Petri plates containing PDA medium

Sterile neutral discs (6 mm in diameter) were impregnated with different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 %) of Ag and ZnNPs and aseptically placed on the inoculated culture plates using a sterile pair of forceps. The plates were placed in an incubator (TYD: In 160, Memmer┼ GmbH + Co.KG, D.91126 Schwabach FRG, Germany) at 25 °C for 72 h (Ravimannan, 2016). At the end of the incubation period, antifungal activity was determined by measuring the zone of inhibition (mm) using digital caliper. The disc with Mancozeb (broad-spectrum antifungal) was used as a control.

3.6.3 *Injera* preparation

Injera was prepared as traditional method following the procedure used by Ashagrie and Abate (2012).

3.6.4 Coating dose determination

The interior side of zipper plastic bags were exposed and dip-coated with the nanoparticles at different concentration (10, 30, 50, 70, 90, and 100 %) and then dried in an oven at 60 ^{o}C (Metak, 2015) and used for *injera* storage. The conventional packages (without Ag or ZnONPs) were used as a control package.

3.6.5. Sampling method

From each of the baking cycles, samples were taken to determine pH and moisture content of *injera.* Sampling was conducted by taking pieces of *injera* from every quarter of the *injera* roll and blending it. For fungus colony growth and migration evaluation, the stored samples were sampled at 2, 4, 6, 8, and 10 days of storage and done as its procedure.

3.6.6 Determination of pH and moisture content of *injera*

The sampling of *injera* and pH test was done as procedure used by Ashagrie and Abate (2012).

3.6.6.1 Measurement of pH

An electronic pH meter (Jeneway, model 3510, serial No: 64243, Stone, staffs, UK.ST15 USA) was used to measure pH. After calibration using standard solutions at pH 4 and 7, each *injera* suspension (10 g of ground *injera* added to 100 mL distilled water and the dispersion homogenized using a shaker) was measured.

3.6.6.2 Moisture content determination

The electronic balance (KERN & SOHN GmbH, Ziegelel 1,723336 Balingen, Germany) was used to determine the moisture contents of the stored *injera* gravimetrically at different day intervals. About 5 g of the *injera* samples were measured and pre-dried at 65 ^{o}C (partial drying). Further drying will be done at temperature of 100 ^{0}C in an air oven (Lab TECH, Model: LDO-150F, S.NO; 20190442410, Gyeonggi-Do, Korea) until constant weight reached (AOAC, 2000). Moisture content was calculated as:

$$\text{Moisture Content (\%)} = \left[\frac{(M_{initial} - M_{final})}{M_{initial}}\right] x100\% \quad \text{.....(eq.4)}$$

3.6.7 Shelf life test

Shelf life is defined as the period in which visible mould and yeast growth not seen (Katsini, *et al*, 2008). The stored *injera* was monitored daily until mould growths occur. The shelf life was interpreted in relation to control sample. Effective dose (10, 50, and 90 %) for this test was taken from coating dose determination results.Temperature and relative humidity were recorded by data logger(Serial Number-36954746 www.Testo.com).

3.6.8. Internal temperature

Internal temperature was measured by Infrared testing method (Testo SE and Co. KGaA, SN; 43594093).

3.6.9. Mould and yeast

Pour plate method (Ravimannan, 2016) was used to analyze yeast and mould counts. The media used was Potato Dextrose Agar (PDA). It was prepared by weighing 9.75 g of the agar into a 500 mL conical flask and dissolving it with 250 mL distilled water. Then the medium in the flask was sterilized in an autoclave at 121 ^{0}C for 15 min. Then, chloramphenicol was added aseptically to molten PDA at 45 ^{0}C in order to retard bacterial growth.

In order to obtain a representative sample, 10 g of *injera* samples all quarter parts was taken aseptically and then, 90 mL of sterile water added and homogenized for 3 min (Azlin-Hasim, 2015) using Vortex (BARNSTEAD/THERMOLYNE, 2555 KERPER BOULEVARD DUBUQUE, 10 WA 52001, M63210-33, S/N: 632010435266, U.S.A).

Serial dilution was done as follows. 1 g of each sample was added into test tubes containing 10mL sterile water and these were used as stock solutions. 1mL was removed from each of the solution and added to another set of test tubes containing 9mL sterile water which made 10^{-1} dilution. The same procedure was repeated to make 10^{-5} dilution. Then, 1mL of the 10^2 and 10^5 dilutions added into sterile Petri dishes and sterile molten agar was poured into the +plates. The inoculated plates were allowed to set and incubated. The plates were kept in an inverted position to avoid the condensation of water vapor on the plate cover from dropping on the culture. The PDA plates (for fungal cultures) were incubated at 25 ^{0}C for 72 h. The number of colonies found on each media was counted. Count plates containing 10-150 colonies (Tournas *et al*., 2001). Determined as numerous to count (NTC) if above 150, few to count (FTC) if below 10..

3.6.10. Migration test (Ag and ZnO)

Daily visual observation was performed on packaged *injera* stored in the antimicrobial containers and conventional containers at room temperatures for each type, over 10 days (Metak, 2015). Microwave plasma-atomic emission spectroscopy (MP-AES) was used to

determine the amount of Ag and Zn in the sample. The stored samples were sampled at 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} days of storage and dried. The dried samples were tested after acid digestion.

3.7. Statistical Analysis

A completely randomized design statistics were carried out with the analysis of variance (One-way ANOVA) procedure in SPSS software. Differences among average values were detected by Duncan's multiple range test *($P < 0.05$)*. The result was interpreted as mean ± standard error.

4. RESULTS AND DISCUSSION

4.1. Optimization of Ag and ZnO Nps synthesis

The optimum Ag and ZnO NPs synthesised by changing the concentration of precursor and ratio of mass of precursor to extract were indicated in Table 11 and 12 by astrix (*). Further experments were conducted based on the optimized nanoparticls.

4.2. Characterization of Ag And ZnO Nps

4.2.1. Characterization of Ag NPs

a) Observation and UV-vis spectroscopy

Reduction (Ag^{+} to Ag^{0}) of silver ions into silver nanoparticles during exposure to plant extracts was observed by the color change(deep red to dark brown) (Fig 4). The dark brown color is due to the Surface Plasmon Resonance Phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. This color change is due to excitation of surface Plasmon vibrations in silver nanoparticles (Veerasamy *et al*., 2011, Ahmad *et al*., 2015).

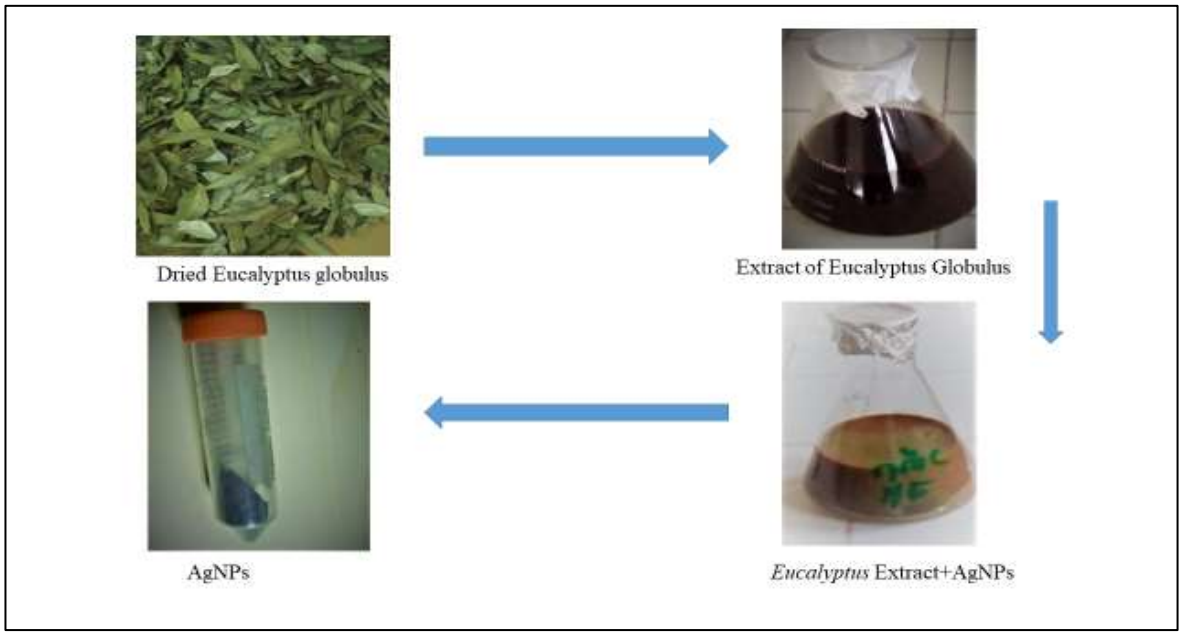


Figure 4: Production of AgNPs from Eucalyptus globulus Extract

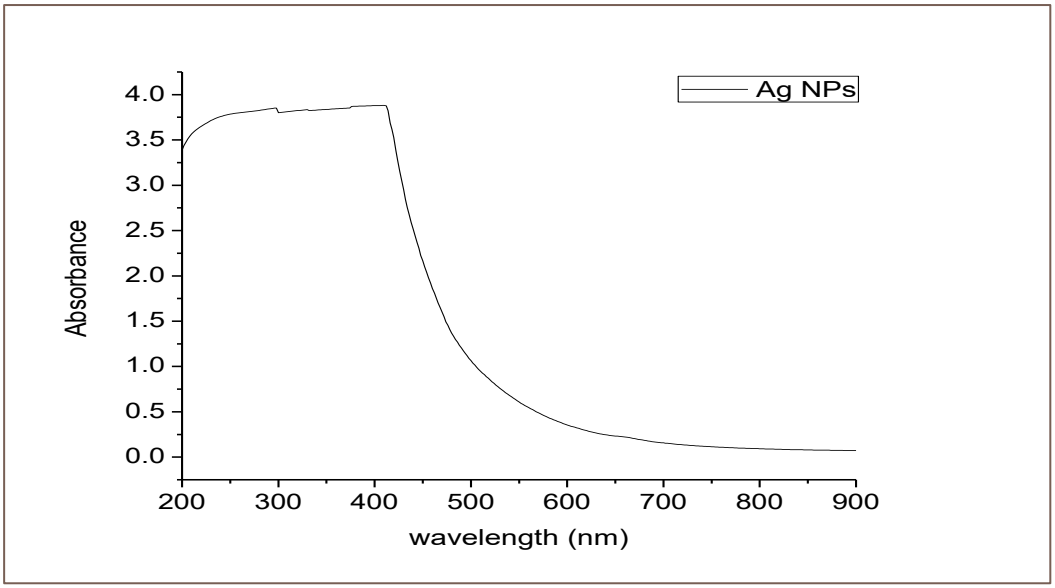


Figure 5: UV-vis Spectrum of AgNPs at 420nm obtained in present study

The UV-vis spectrum of the synthesized AgNPs is shown in Fig. 5. The peak centered at 420 nm, which is associated with the absorption of AgNPs. Synthesis of the AgNPss was confirmed by recording the absorption spectrum at a wavelength range of 200-800 nm. The characteristic surface Plasmon absorption bands (SPAB) was observed at 420 nm (highest peak). This is the characteristic of silver nanoparticles and clearly indicates the formation of nanoparticles in the solution. Somewhat similar results were found by other previous researches. Ahmed *et al*., (2015) has also reported that SPAB of AgNPs from different amount of leaf extract was in range of 436 to 446 nm. Rose *et al*., (2017) has also reported that SPAB of AgNPs was observed at 440 nm. Broadening of peak of silver nanoparticles formed in the reaction indicated that the particles are poly dispersed. It was similar with finding of Omara *et al.,* (2017) conducted on AgNPs.

b) **X-Ray diffractometer (XRD)**

The powdered form of silver nano particle was analyzed to confirm the presence of AgNPs. XRD analysis of sample was conducted in range of 2θ from 10 to 80 degree. The 2θ peaks (Abraham *et al*., 2014) were noted to confirm the presence of the nanoparticles.

Seven peaks at 2θ values of 27.72, 32.24, 46.34, 54.94, 57.5, 67.66, and 76.74 degrees were observed with miller indices (hkl) of (111), (200), (220), (311), (222), (400) and (420) respectively (see Table 13). Abraham *et al*., (2014), have reported the 2θ values of AgNPs were

observed at 2θ value of 31.002, 46.761, 56.700, which is very similar with current study result. Ahmed *et al.,* (2016) have reported that AgNPs has five peaks at 38.1, 44.5, 64.3, 75.9, and 81.7 with miller indices (hkl) of (111), (200), (220), (311), and (222), respectively. According to Priyadharshini *et al., (*2014), the spectra results indicated the presence of eight major distinct Bragg's reflection peaks generated at 2θ values of 27.83°, 32.28°, 38.25°, 46.28°, 54.83°, 57.37°, 64.56°, and 76.69°. They correspond to (210), (122),

(111), (231), (142), (241), (220), and (311) sets of lattice planes.This report is the same to results obtained currently.

The broadening of Bragg's peaks around their bases indicates the formation of small sized silver nanoparticles. According to Velavan and Amargeetha (2018), the broadening of peaks shows the small size formation of nanoparticles.

The average crystallite size estimated from sherrer equation (eq. 2) was 84.07nm. The average size calculated from the intense peaks at 2θ(=27.72, 32.24, 46.34) were 80.17, and 82.55 and 89.48 respectively. Somewhat similar results were reported by different researchers which ranges from 28 to 61 nm (Govindarajan *et al.,* 2016), 80-100 nm and 33-50 nm, (Kanchan and Zantye, 2016).

Table 13: Indexed consecutive peaks of AgNPs

2θ	θ	d=ʎ/2Sin θ	100*$\sin^2\theta$	1000*$\sin^2\theta$/19.71	Reflection (hkl)	Remarks
27.72	13.86	1.129	57.38	3	111	$1^2+1^2+1^2=3$
32.24	16.12	0.9783	77.09	4	200	$2^2+0^2+0^2=4$
46.34	23.17	0.6875	154.81	8	220	$2^2+2^2+0^2=8$
54.94	27.47	0.4454	212.7830	11	311	$3^2+1^2+1^2=11$
57.50	28.75	0.5624	231.35	12	222	$2^2+2^2+2^2=12$
67.66	33.83	0.2429	309.949	16	400	$4^2+0^2+0^2=16$
76.74	38.37	0.4358	385.3149	20	420	$4^2+2^2+0^2=20$

Table 14: AgNPs crystallite size calculated from intense peaks

2theta (2θ)	Theta(θ)	FWHM	D(crystallite size) in nm	Average in nm
27.72	13.86	0.0173	80.17	84.07
32.24	16.12	0.0168	82.55	
46.34	23.17	0.0155	89.48	

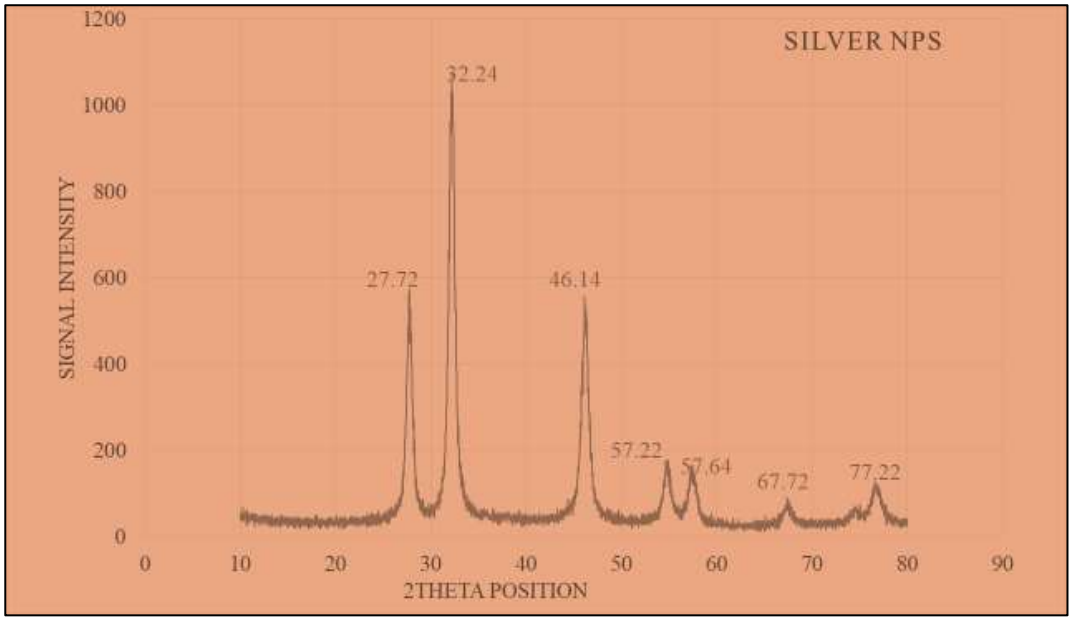


Figure 6: XRD spectra of AgNPs from Eucalyptus Extract

C) Scanning Electron Microscope (SEM) Image

The Scanning Electron Microscopy (SEM) analysis was used to determine the morphology of Ag Nanoparticles. The SEM image of silver nanoparticles synthesized from *Eucalyptus glubulus* leaf extract is shown in Fig. 7, which shows distinct and clear image of synthesized silver nanoparticles having irregular(mostly) and spherical shape. Vennila *et al*.,(2015) have found that biosynthesized AgNPs have both spherical and rod shape. Ahmed *et al*., (2016) have also reported that the shape of biosynthesized AgNPs were irregular and spherical. These reports were in agreement with current morphological appearance of biosynthesized AgNPs. The shape of AgNPs was found to be spherical in shape by different researchers (Nagababu

and Rao, 2017, Krishnaraj *et al.* 2010, Vishwakarma, 2010). The SEM image disclosed a number of discrete and other larger groups that shows poly-dispersion of the crystalline AgNPs).

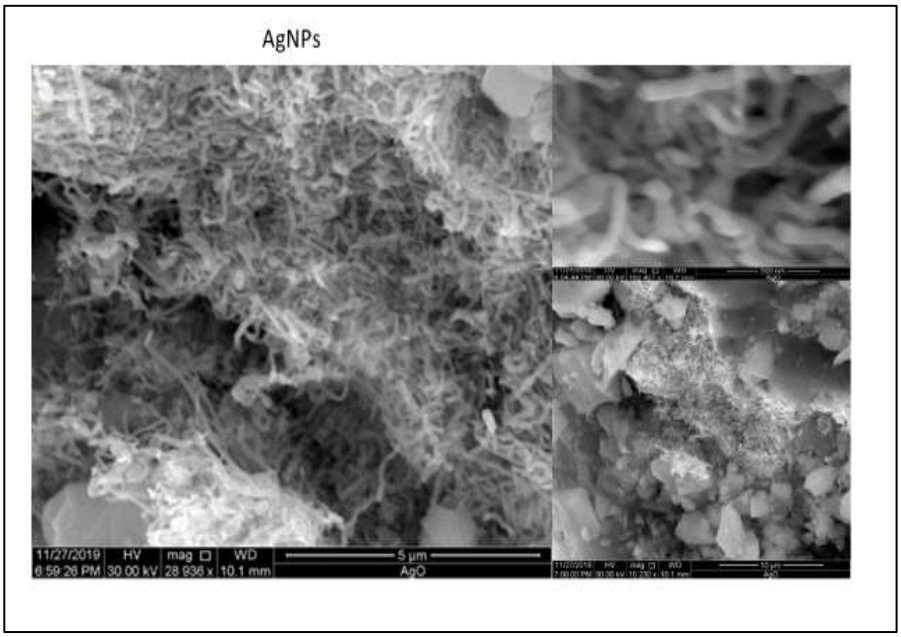


Figure 7: **SEM Image of AgNPs biosynthesized from Eucalyptus extract (at 500nm magnification**

4.1.2. Characterization of (ZnONPs)

a) Observation and Uv-visible spectroscopy

The reduction of ZnONPs biosynthesized with Digita (*Calpurnia aurea (Ait.) Benth.)* leaf extract was confirmed by a color change of deep red to light yellow indicating formation of ZnONPs with the reduction of Zn to Zn^{2+}. The light yellowish color is due to the Surface Plasmon Resonance phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. This color change is due to excitation of surface plasmon vibrations in silver nanoparticles (veerasamy *et al.*, 2011, Ahmad *et al.*, 2015).

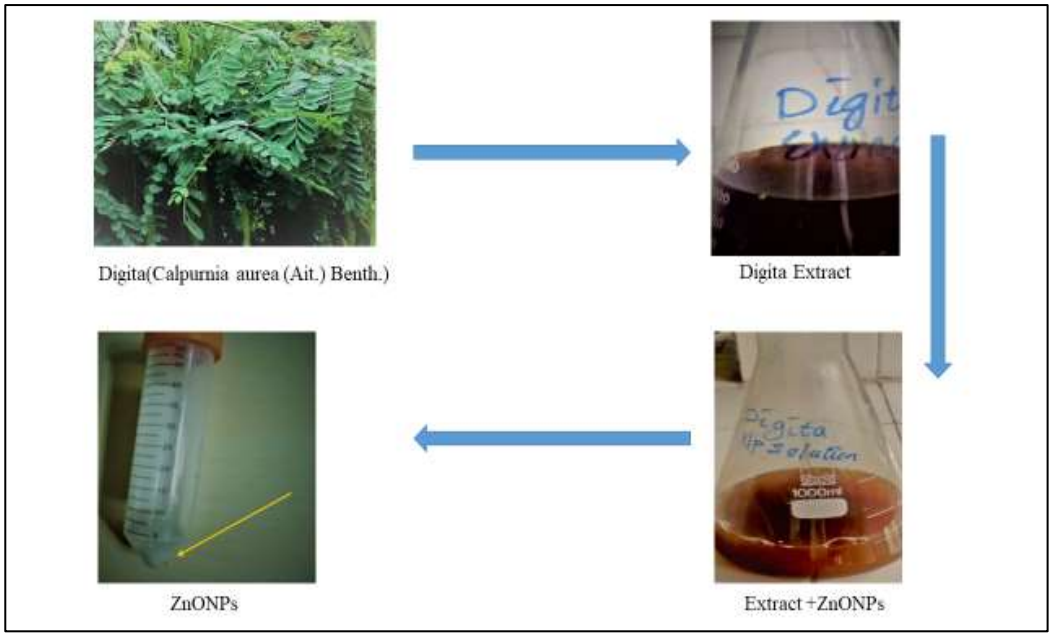


Figure 8: **Production of ZnONPs from Digita (Calpurnia aurea (Ait.) Benth):**

The zinc oxide nanoparticles were characterized for their maximum absorbance using UV-vis spectrophotometry. Biosynthesis of the ZnONPs was checked by the absorption spectrum at a wavelength range of 200-800 nm (Ogunyemi *et al.,* 2019). The characteristic surface Plasmon absorption bands were observed at 300 to 400 nm (Fig 9). This is characteristic of ZnONPs that shows the formation of ZnONPs. Ogunyemi *et al.,* (2019) have reported that ZnONPs biosynthesized from different plants have showed characteristic surface plasmon absorption around 400 nm which is very similar to present result. Maity *et al*., (2018) has reported that the sharp bands of zinc colloids was observed at 361 nm, which proves that the zinc ion is efficiently reduced by leaf extract. The minor difference between this report and the current result may be raised from plant source difference. Manokari *et al.,* (2016) have reported that the SPAB of ZnNPs was in range of 299 to 311 nm. Ogunyemi *et al.,* (2019) has revealed that synthesized zinc oxide nanoparticles were confirmed by the UV-Vis absorption spectra at the wavelength range of 380 to 386 nm, which is the characteristic wavelength range of zinc oxide nanoparticles. Pal *et al.,* (2020) have reported that a high UV-Vis spectrum of ZnONPs was observed at 350 nm.

The broadening of ZnONPs from digita extract was greater than that of AgNPs from Eucalyptus extract which indicates the smaller particles of ZnONPs. Broadening of peak of silver nanoparticles formed in the reaction indicated that the particles are poly dispersed. Manokari *et al*., (2016) found that the produced ZnONPs was poly-dispersed which means not aggregated in its nature. It was similar with finding of Omara *et al*., (2017) conducted on AgNPs.

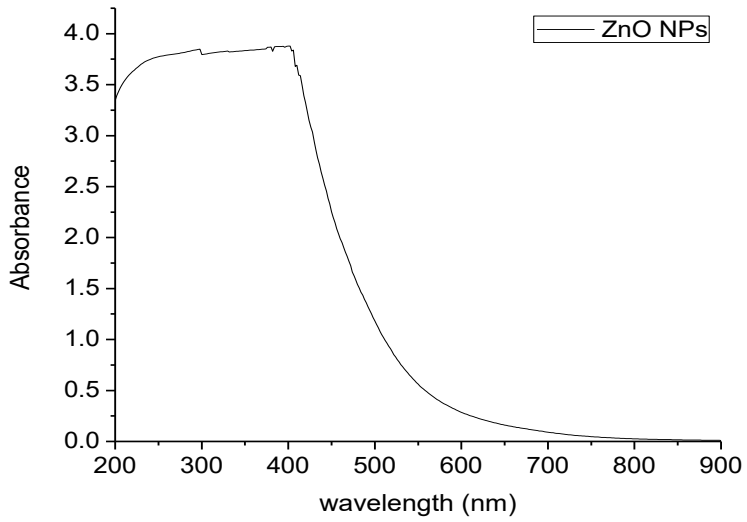


Figure 9: UV-vis spectrum of ZnO biosynthesized Nanoparticles

b) X-Ray diffractometer (XRD)

The major peaks at 2θ values of 28.88, 32.03, 34.69 36.54, 47.77, and 40.50 and 56.78 degrees were observed with miller indices (hkl) of (101), (202), (003), (301), (200) ,(201) and (112) respectively (Table 15). The report by Ogunyemi *et al*., (2018) has shown that peaks were formed at 2θ for ZnONPs at 31.8, 34.5, 36.3 47.6, 56.6, 62.9, 66.4, 68.0, 72.6, 77.0, 81.4, 89.7 that correspond to indexed peaks (hkl) of (100), (002), (101), (102), (110), (103), (200), (112), (004), (202), (104) and (203) respectively. Also, Pal *et al*., (2020) has reported that peak position(2θ) of ZnNPs are 31.91, 36.48, 56.71, 66.57, 68.09, and 69.36 with corresponding miller indeces (hkl) of (100), (101), m(110), (200), (112), and (201). These reports are very similar to miller indices obtained currently confirming that ZnONPs was synthesised.Table 15 shows the indexed peaks of ZnNPs according to formula used by (Bykkam *et al.*, 2015).

The crystallite size of ZnONPs was calculated from positions of the intense peaks. The average size obtained from the intense peaks at 2θ of 28.88, 32.03, 34.69, 36.54, and 56.78 were 70.40, 76.62, 70.40, 63.04, and 40.79 nm, respectively, whereas the average crystallite size of peaks was 64.25nm. Banumathi *et al.*, (2016) have found ZnNPs with size of 20 -65 nm whereas, Hassan *et al., (2016*) have found ZnONPs with about 19 nm. Malaikozhundan *et al.,* (2017) also found ZnONPs having 30-41nm in size. Ogunyemi *et al*, (2016) found ZnONPs with about 48.0 - 65.4 nm. The ZnNPs biosynthesized in the present study have smaller sizes when compared with the previous study, which is preferable to achieve better antimicrobial effects.

Table 15: Indexed consecutive peaks of ZnONPs

2θ	θ	d=ʎ/2Sin θ	1000*$\sin^2\theta$	1000*$\sin^2\theta$/36.86	Reflection(hkl)	Remarks
28.88	14.44	3.08	62.69	2	101	$1^2 + 0^2 + 1^2 = 2$
32.03	16.02	2.80	281.29	8	202	$2^2 + 0^2 + 2^2 = 8$
34.69	17.35	2.75	323.92	9	003	$0^2 + 0^2 + 3^2 = 9$
36.540	18.27	2.61	354.48	10	103	$1^2 + 0^2 + 3^2 = 10$
40.50	20.25	2.23	127.15	4	200	$2^2 + 0^2 + 0^2 = 4$
47.77	23.89	2.49	164.01	5	201	$2^2 + 0^2 + 1^2 = 5$
56.78	28.39	1.62	227.6	6	112	$1^2 + 1^2 + 2^2 = 6$

Table 16: ZnONPs Crystallite size calculated from intense peaks

(2θ)	(θ)	FWHM	D(crystallite size) in nm	Average in nm
28.88	15.95	0.0197	70.40	64.25
32.03	17.25	0.0181	76.62	
34.69	18.15	0.0197	70.40	
36.54	18.27	0.022	63.04	
56.87	28.39	0.034	40.79	

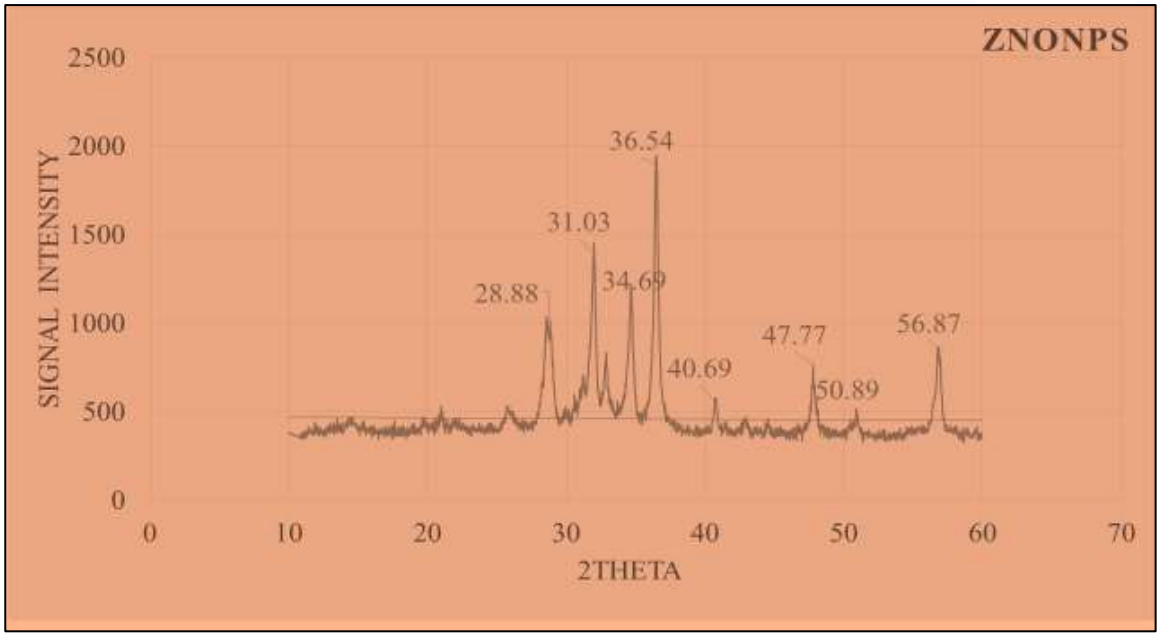


Figure 10: XRD graph of ZnONPs of present study

a) Scanning Electon Microscope (SEM)

The Scanning Electron Microscopy (SEM) analysis is used in determining the morphology of the Nanoparticles. The (SEM) image of ZnONPs synthesized from Digita leaf extract is shown in Fig.11. This image indicates that the ZnNPs are not aggregated. The SEM image (Fig. 11) shows a number of discrete ZnONPs as well as larger groups. The SEM image of ZnO NPs also revealed that rod(mostly) and irregular shaped nanoparticles were obtaind.

Scanning electron microscope was used to size, and shape of the zinc oxide nanoparticles Haritha Meruvu, (2011). These images demonstrated that produced zinc oxide nanoparticles were rectangular and rod shaped (mostly). The report by Vishwakarma, (2010) has revealed that the shape of biosynthesized ZnONPs was both spherical and rod shape. In addition, Hassan *et al*., (2016) have found rod shaped ZnONPs, which also mostly observed shape (Fig 11) in current study. This study is in agreement with present study, which mostly rod shaped ZnONPs. Banumathi *et al*., (2016) have found ZnONPs spherical and hexagonal shapeZnONPs. The difference may come from plant type used for reduction of the metal.

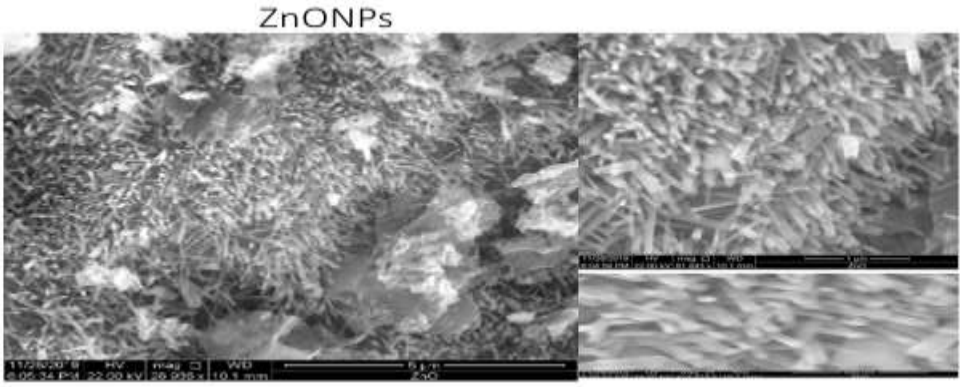


Figure 11: SEM image of ZnONPs biosynthesized from Digita leaf Exrract

4.2 Morphology of the Fungus

The microscopic structures of all the three fungus were identified by microscope. The color of *Aspergillus, PenIcillium* and *Rhizopus spp*. grown on PDA media were black, green and dark brown, respectively. These fungus species were also further identified by their structural difference.

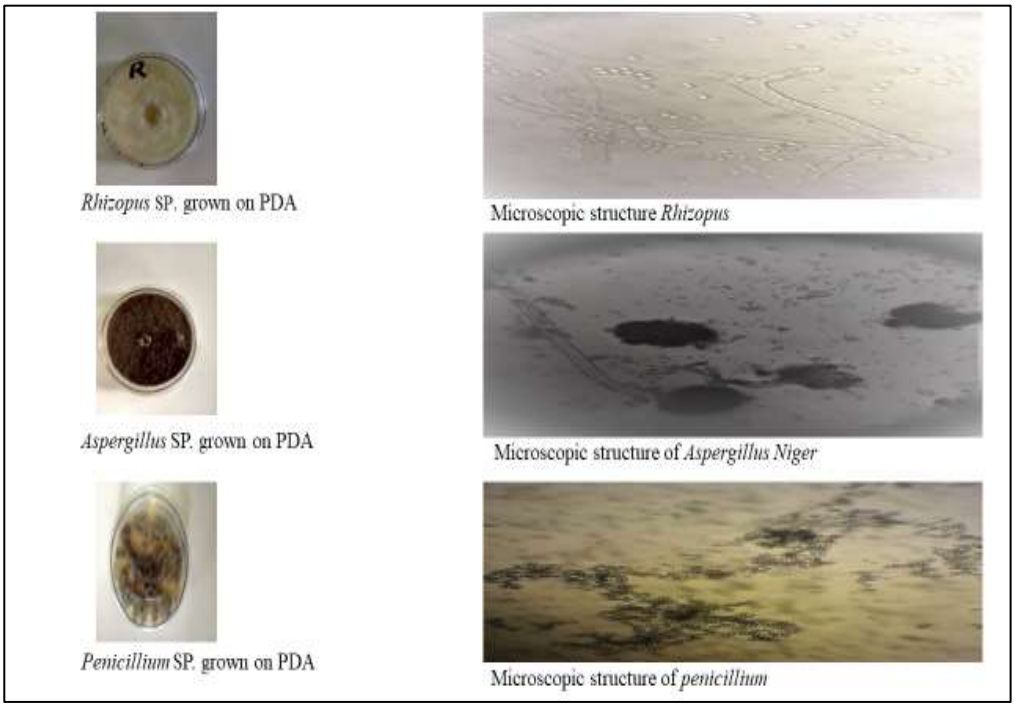
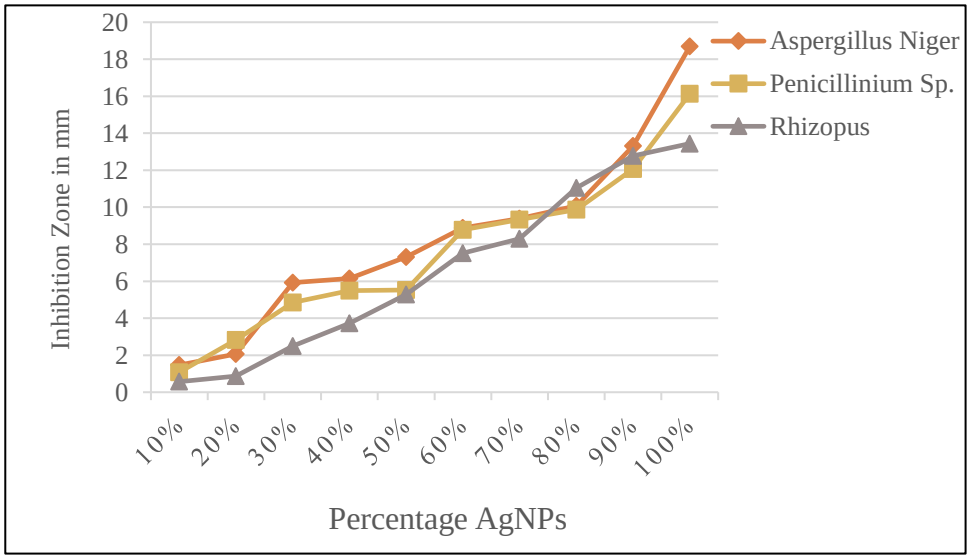


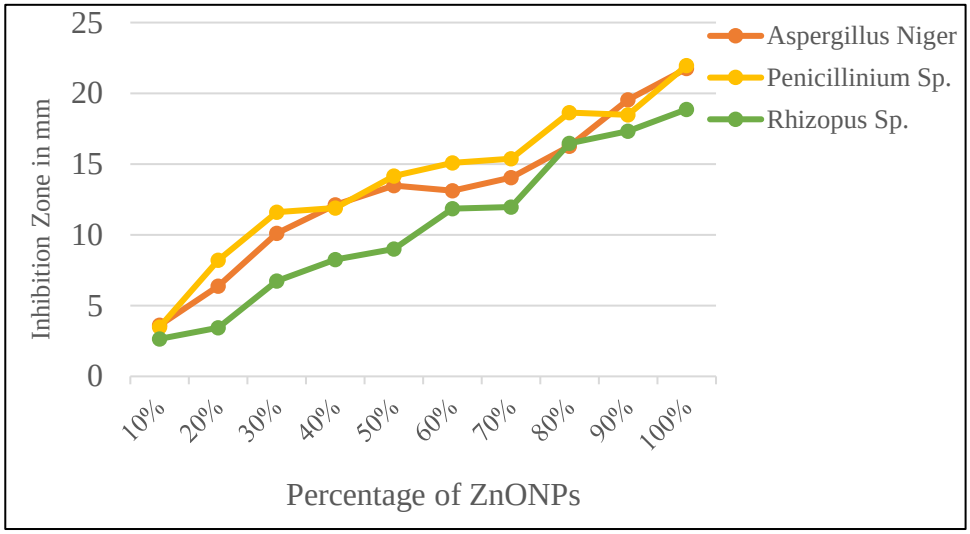
Figure 12*:* Physical and Microscopic structure of Rhizopua sp., Penicillium sp. and Aspergillus *SP.*

4.3. Antimicrobial Activity of Ag And Znonps

The results on the antimicrobial test of both Ag and ZnONPs at different percentages are shown in Fig 13. It shows the patterns of antimicrobial capacity of Ag and ZnONPs which was determined by applying them against *Aspegillus Niger*, *Penicillum spp* and *Rhizopus* fungal types.



a) Anticmicrobial ativity of AgNPs from *Eucalyptus globulus* extract



b) Antimicrobiaal activity of ZnONPs from Digita(*Calpurnia aurea (Ait.) Benth*.) Extract

Figure 13*:* Antimicrobial activity of both Ag and ZnONPs

Both nanoparticles (AgNPs and ZnONPs) had significantly ($p<0.05$) affected the growth of all fungus. The diameter of inhibited zone was increased as the concentration level of Ag and ZnONPs increased. It can be understood from the Table 13 that not all fungi were affected equally by the treatments.

There was a numerical difference between both types of treatments. The inhibition zone of ZnONPs against *Aspergillus niger*, *Penicillinium sp.* and *Rhizopus sp.* was higher than that of AgNPs at the same concentrations. For instant, the inhibition zone of 100% ZnONPs against *Aspergillus*, *Penicillinum* and *Rhizopus* were 18.69, 16.13, and 13.44mm whereas that of 100 %AgNPs was 21.75, 21.72, and 18.87mm, respectively. In addition, the inhibition zone of ZnONPs and AgNPs at 10 % of concentration level against *Aspergillus*, *Penicillinum* and *Rhizopus* were 1.47, 1.08, 0.57 and 3.61, 3.49 and 2.65mm, respectively. Both treatments were not significantly different from the control sample at 100 % concentration. For *penicillinium sp.*, 90 % of both treatments were also not significantly different from the control sample (Mancozeb).

This effect may come from attachment of the NPs to the cells of the fungi, which destroy their cellular integrity. This destruction of their fungal cells may also leads to inability to multiply to form more mycelium, which appeared physically on *injera* during storage. This growth inhibition may also be the results of enzyme inhibition through attachment of these small sized crystallite nano particles to their cells that affects way of their metabolization. Fungus cells are composed of other proteins in addition to enzymes that may be malfunctioned when these nano particles are attached to the protein structures that leads to denaturation of the protein. These factors may be considered in growth inhibition of these fungus types.

Silver ion is highly toxic to most microorganisms (Jung *et al., 2008*) and at least one mode of antimicrobial action of nanoparticles is through a slow release of silver ions via oxidation within or outside the cell. Silver nanoparticles are known to affect the permeability of membranes of microbial and other cells (Li *et al.,* 2010).

The antimicrobial activity of ZnO NPs falls into two major pathways. These are: interfering with the cell membrane by damaging the membrane integrity and permeability and increasing oxidative stress through reactive oxygen species (ROS) generation. At low concentrations, the

antimicrobial effect is induced by nanomaterials themselves through physical absorption or electronic interaction–induced membrane damage (Yong *et al.* 2013), whereas excessive ROS production has been noted when Zn^{2+} concentrations reach 50 mg/L. ZnO NPs possess positive charges in water, whereas bacterial membranes composed of acidic phospholipids and lipopolysaccharides present negative charges. This provides the basis for electrostatic interaction between ZnONPs and bacterial cells (Sarwar *et al.* 2016). The ZnO NPs first bind to the hydrophilic site on the lipopolysaccharide and then deform the outer membrane. This deformed membrane may not be able to multiply and continue the normal growth.

4.4. Storage Duration (Shelf Life) of *Injera*

The storage duration of *injera* without visible mold growth was determined by taking the time from day of baking to the moment in which the samples of a batch show any visible signs of molding. The samples were stored at average temperature of 25.6 ^{o}C (range 23.6 to 30.6 ^{O}C) and average relative humidity of 50.3% (range 26.8 to 62.7 %). The stored *injera* samples were daily observed to check mold growth on surface. The maximum shelf life is determined as one day before appearance of mold growth at least at one spot on the surface of *injera.*

The expected visible mold sign-free storage period of *injera* under unpreserved condition is 3-4 days (Ashagrie and Abate, 2012). The result in the current study also shows that the shelf life of *injera* packed with conventional package was 3 days. The results of current study shows that plastic packages coated with Ag and ZnONPs had a better visible mold sign-free storage period(>18 days) with above 70 % and above concentrations. The present result showed that treatment with Ag and ZnO nanoparticles have a better effect on elongation of *injera* shelf life. This result was even better than the result reported by Ashagrie and Abate (2012), in which addition of preservative sodium benzoate has increased shelf life of *injera* up to 12 days. It was also comparable with the result obtained by Abinet Terefe (2019) which proved that shelf life of vacuum packed *injera* can increase shelf life up to 15 days and above.

When comparing Ag and ZnONPs effects, ZnONPs has recorded the result similar to that of the control sample (4^{th} day). However, at same concentration level, the shelf life *injera* sample packed with plastic package coated with AgNPs at 10% has shelf life of 5 days. Also the result (23^{th} day) from AgNPs at 90% was greater than the result of ZnONPs at 90 % (19^{th} day).

However, in other treatments (30, 50, 70 %), ZnONPs has resulted in high shelf life than AgNPs. As shown in Fig 14 below, the shelf life of *injera* packaged with ZnONPs were 8, 15, and 21 days at 30, 50, and 70 % concentrations, respectively, whereas that of AgNPs was 5, 9 and 19th days, respectively. This shows that both nanoparticles can improve shelf life of *injera* to almost similar extents.

For both Ag and ZnONPs , the optimum concentration was 50% in which they increase *injera* shelf life for 10 days to 15 days with migration level of .34mg/Kg and 375mg/Kg, respectively.

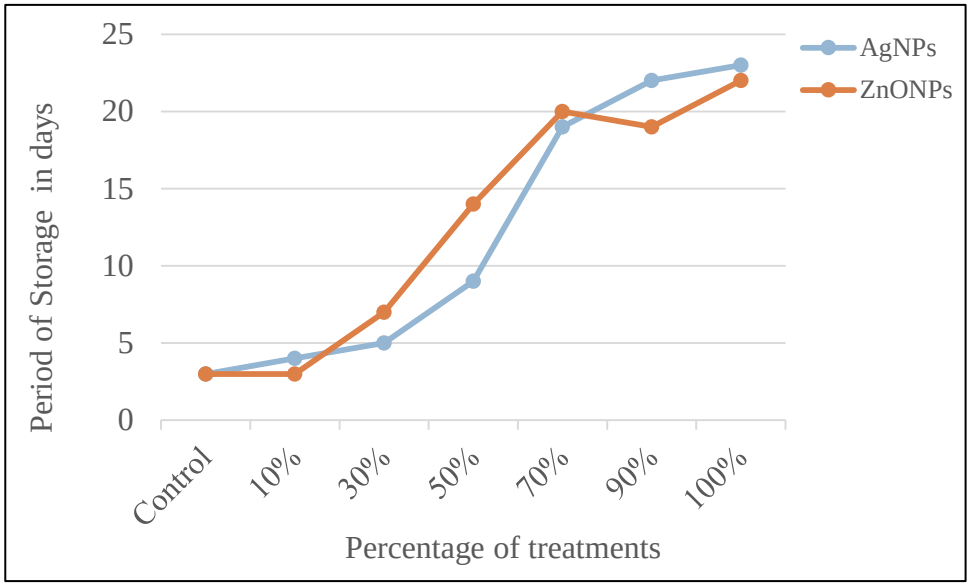


Figure 14: A graph showing the shelf life of *injera* samples

4.5. Internal Temperature

The result of internal temperature is shown in the Table 17 below. The data from infrared equipment was averaged for each treatment since it depends on day temperature.

Most of the internal temperature results were similar to that of control treatment. In addition, AgNPs 90 % was numerically different from ZnONPs 90 %, which were similar concentration level but different treatment types (Ag and ZnONPs). This difference may seem to come from *injera* thickness. There were numerically very similar results that indicating existence of similar internal temperature within the plastic packaging material currently used for *injera.*

Table 17: Internal Temperature of stored injera with plastic bag dip-coated with Ag and ZnONPs

Treatment	Internal Temperature
AgNPs	
AgNPs10%	25.22±0.02[ab]
AgNPs50%	25.36±0.06[ab]
AgNPs90%	25.18±0.04[a]
Ext+AgNPs	25.23±0.01[ab]
Control	0.03
SE	0.03
ZnONPs	
ZnONPs10%	25.31±0.06[ab]
ZnONPs50%	25.39±0.03[ab]
ZnONPs90%	25.43±0.15[b]
Ext+ZnONPs	25.25±0.02[ab]
Control	25.43±0.07[b]
SE	0.03

Data were interpreted as mean ± standard error. The means that share similar letter are not significantly (p>0.05) different. Ext =Extract, SE – standard Error

During storage of foods, internal temperature might be increased caused by degradation of food compositions that further increases microbial growth and inversely reduces the shelf life of that products. The results of reactions of food compositions within a given packaging material may increase temperature inside the products due to chemical bond breakage that may be a results of exothermal reaction of food composition in the that storage material. This reaction might be caused by entrance of O_2 into the packaging materials.

Abellana *et al*. (2001) compared the effect of temperature and water activity and their interactions on the rate of mycelia growth of *Penicillium SP*. and *Aspergillus* flavus on a sponge

cake. The growth rates showed dependence on temperature. According to Ajith and Sunita (2018), the dominant spoilage flora varies with the type of bread and the storage temperature. Based on the current data, it could be said that there is no temperature difference due to treatment of both types (Ag and ZnONPs). This means that there is no temperature difference that caused the activity of the treatments.The rate and extent of antimicrobial activity is impacted by temperature and the contact time (Metak, 2015). But, int current study, there was no significant difference caused by Ag and ZnONPs.

4.6. Moisture Content and pH of the Stored *Injera*

4.6.1. Moisture content

Results of this investigation showed that there was a significant difference ($p<0.05$) on the moisture content of *injera* samples with different treatments. As showed in Table 18, the moisture content of *injera* samples were ranging from 52.6 % - 56.7%.

The moisture content of *injera* samples were decreased starting from beginning to the maximum shelf life. However, after maximum shelf life reached, it starts to be increased that may be due to deterioration caused by overgrowth of fungus and molds. There was no significance ($p>0.05$) difference between treatments and control in moisture content. However, the significant difference was seen between the control and other treatments starting from day 8 of storage.

Table 18: Moisture Contents of injera stored at different days

Teatment	Day2	Day 4	Day 6	Day 8	Day 10
AgNPs					
AgNPs10%	55.15±0.87[a]	54.08±1.02[a]	54.14±1.97[a]	-	-
AgNPs50%	54.95±0.33[a]	53.84±0.70[a]	53.72±0.70[a]	53.50±0.75[a]	53.68±0.58[a]
AgNPs90%	54.34±0.84[a]	54.11±0.67[a]	53.67±1.34[a]	52.83±0.43[a]	52.64±0.88[a]
Ext+AgNPs	55.05±1.04[a]	54.43±0.88[a]	53.94±0.78[a]	53.39±1.70[a]	52.72±1.80[a]
Control	54.25±1.03[a]	56.10±0.0.32[a]	55.64±0.62[a]	56.81±0.63[a]	56.43±0.27[a]
SE	0.34	0.36	0.49	0.64	0.65
ZnONPs					
ZnONPs10%	54.38±0.46[a]	54.46±0.44[a]	-	-	-
ZnONPs50%	55.20±0.83[a]	53.28±1.49[a]	54.37±0.68[a]	54.06±0.39[ab]	54.80±0.41[ab]
ZnONPs90%	56.20±0.78[a]	55.36±0.88[a]	55.36±1.06[a]	53.87±0.37[a]	54.66±0.53[ab]
Ext+ZnONPs	55.49±0.68[a]	54.71±0.80[a]	54.40±1.07[a]	55.05±1.05[ab]	54.65±0.90[ab]
Control	54.25±1.03[a]	54.81±0.98[a]	55.64±0.62[a]	56.81±0.63[b]	56.43±0.27[c]
SE	0.46	0.27	0.36	0.39	0.40

Data were interpreted as mean ±standard error. The means that share similar letter are not significantly ($P>0.05$*).different. Ext-Extract, SE – Standard Error, Control and ZnONPs were terminated on 4th day whereas AgNPs was on 6th day*

The reported result by Ashagrie and Abate (2012) has revealed that the moisture content of the control *injera* was 64.8 %. Other research reports on *injera* show that i*njera* from different cereal crops range between 45 to 55 %. Abinet Terefe (2019) has also reported that the moisture content of *injera* samples were 60.8 – 63.2 %. The difference between the previously reported and the present result may come from storage temperature and relative humidity difference. Thickness of the *injera* that is only manually controlled during baking may also be a factor for the difference. However, the report by Gedabo *et al* (2019) has

shown that moisture content of *injera* was from 45.2 to 56.2 %. This report is in agreement with this current result of *injera* moisture content.

Results of moisture content showed better maintenance of moisture content for breads stored by active coatings compared to the control one (bread without coating) (Noshirvania *et al*., 2017). In contradiction to this report, there is no difference between the control and the treatment samples of current study when compared in moisture contents.

Moisture is an important parameter in baked foods that significantly affects shelf life and growth of microbial contaminants (Teshome *et al.,* 2008). The report by Ayub *et al*., (2003), has shown that the overall mean moisture content of different kinds of bread made from wheat range from 37- 47 % during storage period. However, injera had higher moisture content that make it more perishable than most bread (Ashagrie and Abate, 2012). High Moisture content is a serious problem in many bakery products that can result in high microbial growth and leads to microbiological spoilage of foods.

However, using of active food packaging provides interaction between food and packaging material, inhibiting microbial growth more than other factors considered in storage of foods (De Azeredo, 2012). In current study, it was also observed that the control sample (uncoated plastic package) had recorded high number of colony forming unit per gram of *injera* whereas sample treated with either AgNPs or ZnONPs had a low colony-forming unit at each storage days based on their concentration. Even though *injera* has only 3-4 day shelf life when packed in conventional packages, it is possible to increase its shelf life to above 20 days using packaging plastic treated with Ag and ZnONPs(Fig 14).

4.6.2. pH of *injera*

The pH of the stored *injera* was measured at two days intervals. Table 19 shows the pH of *injera* at day 2, 4, 6, 8, and 10, respectively.

Table 19: The pH of injera stored for 10 days

Treatment	Day 2	Day 4	Day 6	Day 8	Day 10
AgNPs					
AgNPs10%	3.95±0.01[a]	3.96±0.00[a]	3.94±0.01[a]	-	-
AgNPs50%	3.96±0.01[ab]	3.97±0.01[a]	3.96±0.00[a]	3.99±0.03[a]	3.98±0.00[a]
AgNPs90%	3.97±0.01[c]	3.97±0.01[a]	3.96±0.01[a]	4.00±0.03[a]	4.00±0.01[a]
Ext+AgNPs	3.98±0.01[b]	3.97±0.00[a]	3.97±0.01[a]	3.97±0.02[a]	3.98±0.01[a]
Control	3.96±0.01[ab]	3.96±0.00[a]	3.96±0.00[a]	3.96±0.00[a]	3.95±0.01[a]
SE	0.00	0.00	0.00	0.01	0.01
AgNPs					
ZnONPs10%	3.96±0.01[a]	3.96±0.00[a]	-	-	-
ZnONPs50%	3.97±0.01[a]	3.98±0.01[a]	3.96±0.02[a]	3.99±0.03[a]	3.99±0.00[a]
ZnONPs90%	3.97±0.01[a]	3.98±0.00[a]	3.97±0.00[a]	4.00±0.04[a]	4.02±0.05[a]
Ext+ZnONPs	3.97±0.00[a]	3.97±0.01[a]	3.99±0.01[a]	4.01±0.04[a]	4.00±0.01[a]
Control	3.97±0.00[ab]	3.96±0.00[a]	3.96±0.00[a]	3.95±0.03[a]	3.95±0.01[a]
SE	0.00	0.00	0,01	0.02	0.00

*Data were interpreted as mean ±standard error. The means that share similar letter are not significantly (*P>0.05*).different.* Ext – Extract, SE – Standard Error, Control and ZnONPs were terminated on 4th day whereas AgNPs was on 6th day

As seen from this result, the pH of *injera* was not significantly (*p>0.05*) different from the control sample. However, as storage day increases the pH of *injera* treated with AgNPs and ZnONPs at different percentages increased whereas the result from the control (untreated plastic zipper bag) decreased numerically (acidity increased). This increment may be raised from reaction between metal nanoparticles and other compounds in *injera* samples.

When seen by storage day difference, at day 2, only the pH of *injera* samples stored in plastic bag treated with solution of Ext+AgNPs was significantly *(p≤0.05)* different from all other

treatments. This difference may come from any random error or from *injera* thickness difference which is not fully controlled during baking. At day 4, no treatments were significantly different even though the pH of most of treatments was appeared to be increased numerically. At day 6, *injera* stored in only plastic packages treated with 10%AgNPs was significantly different from *injera* samples stored in plastic packages coated with 90%ZnONPs. This result was random which may come from *injera* thickness difference or from small fraction of reaction with air due to small error during packaging. Although not significantly different, there is a numerical increase at day 8 and 10 as compared with other previous days, which may be due to reaction with these metals coated on the surfaces of the packages.

Abinet Terefe (2019) has reported that the pH values of the *injera* samples were found to be between 3.31 and 4.41. This report showed that *injera* samples under different treatments were not significantly ($p>0.05$) affected the pH value of the samples and this result is in agreement with the current result. The pH results of *injera* samples obtained in current study were comparable with the results reported in other related studies. Attuquayefio (2014) has reported that the result of different brands of *injera* was from 3.65 to 4.02. The result reported by Ashagrie and Abate (2012), also showed that the pH of the control (sample without preservatives) was 3.40. These reported results were closer to the results obtained in current study, which were from 3.94 to 4.02.

The pH values obtained was quite low (more acidic), when compared with the pH of bread, which is mostly between 4.7 and 7.4 (FDA, 2007*).* This makes *injera* favorable for yeast and mold growth in addition to its high moisture content that leads to decreased shelf life of *injera*. To overcome this problem in *injera* storage, the current study is promising to use biosynthesized Ag and ZnONPs on packaging of *injera* in a future.

4.7. Total Mold and Yeast Count

At day 2 the colony forming units (cfu/g) of each *injera* were not significantly ($p>0.05$) different. However, at day 4 the cfu/g of yeast and fungus for the control sample (3.0 log10) was highly significantly different from all the treatment samples. In contradiction to the control, the microbial results of treatments at 50 % and above samples were decreased. After Day 4, the results of all treatments were similar except the treatments at 10 % and the control. The

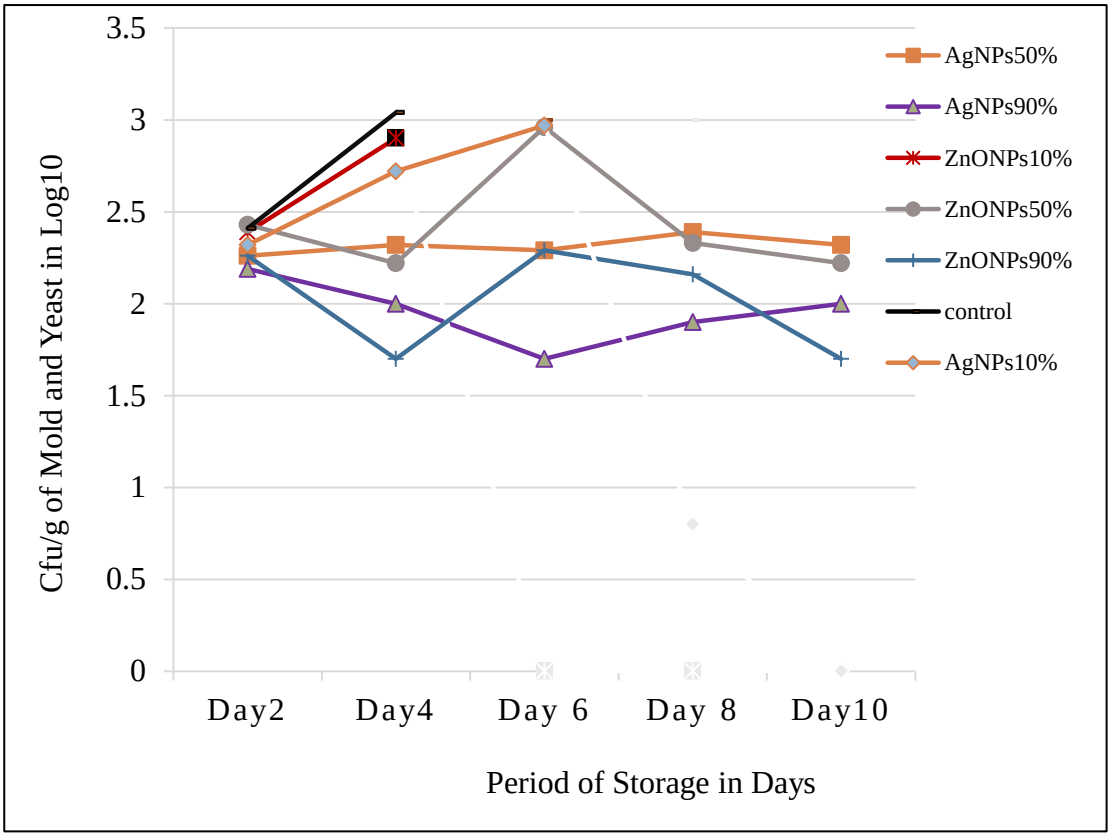
results from the control and treatments at 10 % were significantly increased as the storage day increased. The shelf life of *injera* stored in packages treated with AgNPs and ZnONPs increased when compared with the control sample. This shows that treating of *injera* with these biosynthesized nanoparticles can improves shelf life of *injera*.

According to Sanddozai and Samina,2009; Atlaw and Jha (2015) standard maximum permissible limits in ready-to-eat foods of baked products (cake, bread, and biscuit) for yeast and mold is <4 in log10).

Microbial contamination is caused either by ingredients or by external sources during or after processing (Degrirmencioglu, *et al.,* 2011). Active food packaging provides interaction between food and packaging material, inhibiting microbial growth more than other factors considered in storage of foods (De Azeredo, 2012). Some researchers (Moura *et al.,* 2012) have tried to benefit from bactericidal property of silver (Ag) to use it in food packaging by pairing with hydroxyl propyl methylcellulose (HPMC).

Since there are no available previous findings that deal with storage of *injera* with nano packaging, the results of this study are compared with findings on related products. The report by Khanom *et al.,* (2016) has shown that the total yeast and mold count of packed bread from the supermarket and unpacked bread samples were compared. Unpacked bread samples had a bit higher yeast and mold count than packed bread samples, which were $1.01x10^5$(5 in log10) and $1.05x10^5$(5.02 in log 10) cfu/g, respectively. Also as reported by Rodriguez (2000), the maximum total yeast and mold count of bread packed with normal atmospheric air was 5.98 log cfu/g, which is higher than that of bread samples packed under absence of oxygen, which was 2.00 log cfu/g. The report by (Noshirvania *et al.,* 2017) shows that the results of microbial tests revealed an increase in shelf life of sliced wheat bread from 3 to 35 days for package incorporated with 2 % ZnO NPs compared to the control. According to Godebo *et al.,* (2019), yeast and mold count of 1.83, 2.26, and 2.39 log 10 cfu/g were recorded at 1^{st} , 2^{nd} and 4^{th} days of storage, respectively, for control sample whereas the *injera* added with 5 % fenugreek has recorded 1.22 to 1.29 log10 of mold and yeast at 4^{th} day of storage. Tewodros *et al*., (2015), has also found 2.67, 2.50, and 2.27 log cfu/g yeast and mold in *injera* substituted with flax seed at 3, 6 and 9 %, respectively.

Rayman, (1981) for the adequacy of baked products, made an interesting proposal. According to their criteria, in terms of molds and yeasts, the limit between acceptable quality and marginal quality is 2 x 10^3(3.30 log10) Cfu/g and the limit separating marginal quality from unacceptable quality is 5 x 10^4 (4.5 log10) cfu/g.



The shelf life of injera stored with control and 10% ZnONPs' were terminated at day 4 whereas that 10% AgNPs was at day 6

Figure 15: Colony count of mold and yeast in log 10 during *injera* storage

High moisture level promotes the growth of yeasts and molds in bakery products (Jay *et al*.**,** 2005**).** Generally, molds are tolerant of acid conditions and favor an acidic pH (3.5-5.5). Therefore, foods with pH value<4.5 are not usually spoiled by bacteria but are more susceptible to mold spoilage (Khan *et al*., 2013). Even though shelf life *injera* is affected more by moulds, using of Ag and ZnONPs were proved to reduce its growth significantly in present study.

4. 8 Migration Result

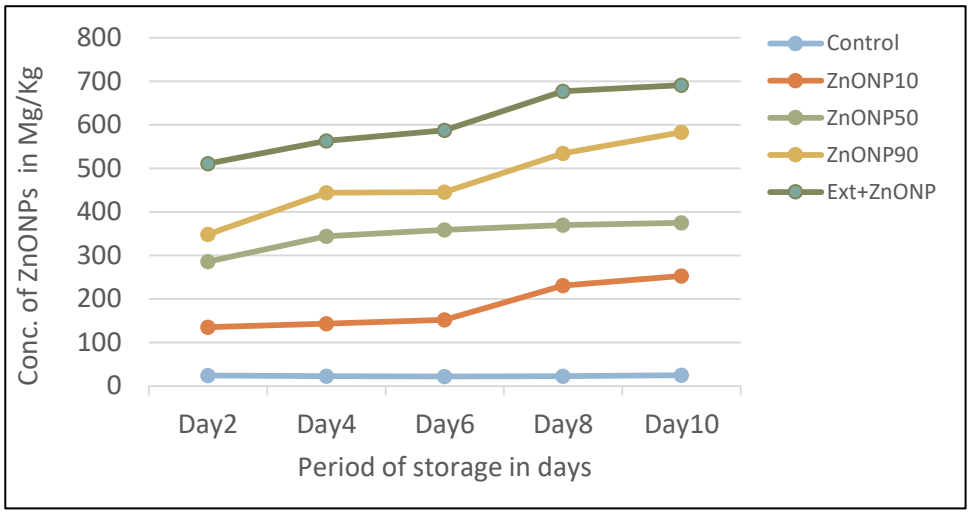
Migration test was conducted to determine the amount of Ag and Zn in the stored *injera* samples. Figure 16 shows the amount of silver and zinc migration from plastic package to *injera*.

The migration level of the control and AgNPs (10 %) were 0 and 0.02 mg/Kg, respectively (Fig 16b). Whereas other treatments' results were higher i.e. 0.45, and 0.59 mg/Kg for 50 %AgNPs and 90 % AgNPs, respectively. The result of the control samples at different days shows similar results showing absence of Ag in Teff *injera.* At day 2 of storage, 10 % AgNPs (0.02 mg/Kg) records lower amount of migration followed by Ext +AgNPs (0.22 mg/kg). There was significant ($P \leq 0.05$) difference between each treatments and the control sample except 10 % AgNPs. The control sample was significantly different from all other treatments at day 6 and 8 storage days. The results of 10 % AgNPs and Ext + AgNPs were similar at both days. But, the results of 50 % AgNPs and 90 % AgNPs were highly significantly different from other treatments. At day 10, all treatments were statistically different from the control sample.

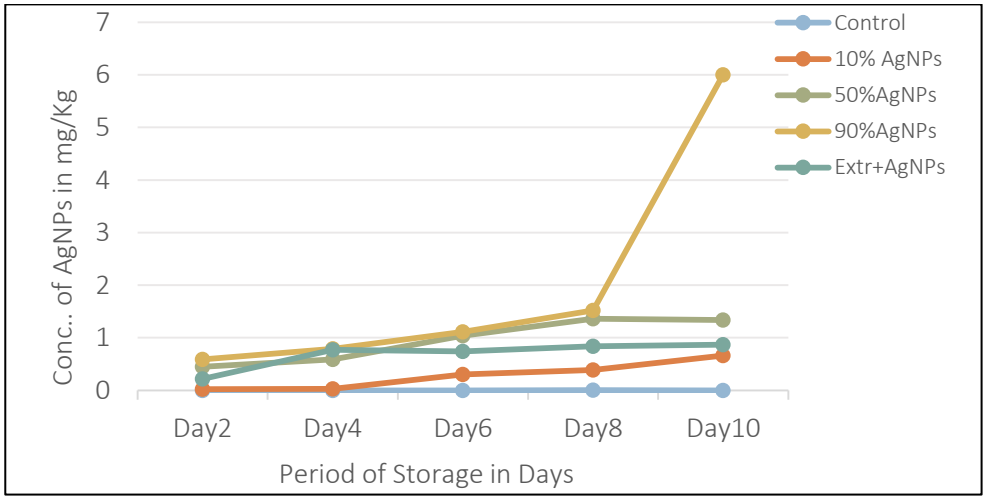
For ZnONPs (Fig 16a), there was less migration at day 2 than other days after. Each treatments was significantly ($P \leq 0.05$) differ from each other at same storage day, which is due to its high concentration gap. The control sample had about 23.6 Zn (mg/Kg) content whereas treatments at 10, 50 and 90% had 135.41, 285.61, 348.27mg/Kg, respectively. Similarly, Ext + ZnONPs has recorded higher migration(511.29mg/kg) as compared to other treatments. At day 4 the result from the control sample was similar with results at day 2. However, all other treatments had recorded a higher migration results with their respective order of increased concentration. This shows that migration has increased with increasing of storage duration. For instant, the result of 10% ZnONPs and 90 % ZnONPs had increased from 135.41 and 285.61mg/Kg (day 2) where it reaches 253 and 583.36mg/Kg, respectively at day 10. Similarly, the results of Ext + ZnONPs has also increased with increasing of storage duration

This increment of migration of both AgNPs and ZnONPs may be caused by moisture content of *injera* which rinses the nano particles on the surface of the plastic bags and loosen the attachement of nanoparticles to the surface. The nanoparticles are very small in sizes and can be transferred to products from packaging materials facilitated by this moisture. Also there is

a degradation of the products at slow condition which may also be the cause for this increased migration through reaction with this AgNPs.



a) Migration of ZnONPs to stored *injera* sample



b) Migration of AgNPs to stored *injera* samples

Figure 16: Migration results of both Ag and ZnONPs

According to Huang *et al.*, (2011), the amount of migrated silver increased with time and temperature in which 0.75 – 4 µg (0.0045 – 0.0244 mg/Kg) silver migration range was recorded after 15 days of storage. This study is also in agreement with present finding since migration AgNPs increased with time.

Dame (2018) has reported that white teff which was used in this study has 9.58mg/Kg Zn. According to Abebe *et al*. (2007), Zn concentration in white teff was 4.02 mg/100g (40.2mg/Kg). These results are somewhat similar with the results of control sample in this study. However, the amounts found in current study were above the Zn content of the control sample that confirms presence of migration that increased with increasing of nano particticle concentration.

Using of nanoparticles in the food industry might pose risk that do not occur in macro size materials (Sozer, and Kokini, 2009). The report by Weil *et al* (2011) has mentioned some specific problems, such as certain nano-composites migrating from packaging to the food, and as a result consumed with foods. The other problem is inhalation since nano-composites are very small and can permeate the lung and reach the blood. After consumption with food or inhalation, toxicity occurs when nanomaterials react with oxygen within cells, increasing oxidative stress (Weil *et al.,* 2011).

EU and USA food safety authorities regulate the application of AgNPs in food packaging. European Food Safety Authority (ESFA, 2011) recommended upper limits of Ag migration from packaging to be less than 0.05 mg/kg in food. However, in current study, the amount of migration results of AgNPs is above this limit that needs further study to use it for injera packaging.

Eventhough ZnO is considered "generally recognized as safe (GRAS) (Rasmussen *et al.,* 2010), characteristics of its nano form may be different due to its increased surface area. Nevertheless, there is no any tangible information concerning its safety and maximum limitation for recommended application of food.

This limitation of using nanoparticles because of migration may be solved by incorporation of them as ingredients of plastic polymers. According to Radusin *et al.,* (2016), nanoparticles incorporated into polymeric matrices have shown a great potential of antimicrobial for prolonging the shelf life of foodstuff. The study by Eslami *et al*., (2016) has shown that, silver nanoparticles were effective as antimicrobials when incorporated into polymer matrix. Also, Azlin-Hasim, *et al*.(2015) has concluded that the incorporation of AgNPs into LDPE can be used in food contact materials for low migration levels.

Nano sized metal oxides are also effective as antimicrobial agents when incorporated into polymer matrix (Chaudhry and Castle, 2011). In particular, ZnONPs is commonly used as effective antimicrobial agent in polymers (Espitia *et al, (*2012). They can be coated, absorbed, or directly incorporated in the synthesis processes (Martinez-Abad *et al*, 2012).

4.9. Effect of Treatment Type

The average of data from different days and different concentrations was taken to evaluate the effect of AgNPs, Ext+AgNPs, ZnONPs and Ext+ZnONPs. The following Table 20 shows the effects treatment types by comparison of grand mean.

Table 20: **The effect of type on different injera parameters**.

Treatment Type	Moisture Content	pH	Mold and Yeast count(log10)
AgNPs	53.97±0.27^{a}	3.96±0.01^{a}	2.32±0.21^{b}
Ext+AgNPs	53.74±0.17^{a}	3.98±0.00^{a}	2.25±0.0.04^{b}
ZnONPs	54.62±0.24^{ab}	3.98±0.01^{a}	2.29±0.21^{b}
Ext+ZnONPs	55.51±0.65^{bc}	4.00±0.01^{b}	1.70±0.13^{a}
Control	56.08±0.37^{c}	3.96±0.00^{a}	2.92±0.04^{c}
SE	0.28	0.00	0.12

Data were interpreted as mean ±standard error. The means that share similar letter are not significantly ($P>0.05$*).different. Ext +AgNPs –eucalyptus Extract + AgNPs before centrifugation, Extr + ZnONPs – Digita extract + ZnONPs before centrifugation, SE – Standard*

As seen from the Table 20, moisture content is not affected by type of treatment used. The control sample is significantly different from others which is because of accelerated reaction in the package caused by over microbial growth. Ext+ZnONPs is also statistically diffferent from both AgNPs and Ext+AgNPs. But, when seen numerically it was very similar showing that the difference may come from other factors like thickness of injera. According to Gedabo et al (2019), the moisture content of injera was from 45.2 to 56.2 %. This report is in agreement with this current result of *injera* moisture content revealing that all treatment types have no effect on moisture content of *injera.*

The pH of *injera* stored in plastic package treated with Ext +ZnONPs is significantly different from all other types. This diffference may rise from basiciy of ZnONPs which was more deposited on the surface of the package caused by oily nature of digita extract. Abinet Terefe (2019) has reported that the pH values of the *injera* samples were found to be between 3.31 and 4.41. Attuquayefio (2014) has also reported that the result of different brands of *injera* was from 3.65 to 4.02. All the the current results were in the reported range showing that pH of *injera* treated with Ag And ZnONPs) were not significantly ($p>0.05$) affected.

Ext+ZnONPs are statically different all from treatments in mold and yeast counts. The oily nature of digita extract may cause the nano particles to be deposited more on the surface of plastic package and released during storage aided by moisture from *injera.* This more deposition was confirmed by migration test being very high in Zn content than other treatments. The other reason may also be the smallness (64.25nm) in size of ZnONPs which shows better performance in antimicrobial test of present study. All type of treatments were significantly different from the control sample showing that all of them have antimicrobial effects.

According to Ahmed *et al.,(*2017), the inhibition zone of 4 and 6% ZnONPs applied againist *Aspergillus niger* was 9-12mm, respectively. Banerjee and Nath (2015) have also reported that 10-15µL of 1mg/ml solution of AgNPs applied against Bacillus subtilis has recorded 11 and 15mm of inhibition zone. These two reports show that both AgNPs and ZnONPs have better antimicrobial effects which was also confirmed in present study.

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Injera is Ethiopian daily stable foods, which is very perishable due to its high moisture content and its acidic nature that favors fast mould and yeast growth. Traditional preservation methods are not convenient for *injera* storage. However, nano technology is becoming a promising work in elongation of shelf life of food, which can also be, perform for *injera*. In this study, biosynthesized Ag and ZnONPs were used for *injera* preservation with objective to increase its shelf life. The study was conducted first to identify antimicrobial activity of these nanoparticles against *Aspergillus Niger, Penicillium sp. and Rhizopus sp*. on PDA after confirmation of nano particles formation and secondly to evaluate the performance and migration level of plastic bag coated with these nanoparticles through shelf life.test.

First, the production of nano particle was confirmed by color change and laboratory equipment (UV – Vis, XRD, and SEM).The AgNPs and ZnONPs have the size of 84.07 and 64.25nm respectively.

It was observed that both Ag and ZnONPs had better antimicrobial activity against all fungus studied. ZnONPs have somewhat more antimicrobial activity compared with AgNPs, which may be caused by smallness in size. From the three fungus Aspergillus sp. is more inhibited than penicillium sp. and rhizopus sp., respectively.

The Performance evaluation of these nanoparticles showed that shelf life of *injera* was well increased without affecting other *injera* qualities. Both NPs increased shelf life of *injera* to more than 10 days at concentrations above 50% when packed aseptically. However, the amount of migration of Ag and ZnONPs was relatively high that may be due to direct contact between *injera* and dip coated plastic package.

Some studies show that AgNPs has health effects if consumed with food. For this, European Union recommends that the concentration of AgNPs in food products must be below 0.05 mg/kg. However, the current study has recorded relatively higher results than this ranges. Because, before using this nano particles, further research will be needed to decrease its migration without affecting its anti-microbial properties.

It has been 15 years since the first wave of international research programs were launched to understand the potential health impacts of engineered nanomaterials. Today, concrete conclusions regarding the health and risks of most nanomaterials and products in which they are contained are lacking, and regulations struggle to keep pace with the rapidly evolving science that support nanomaterial risk assessment. In this context, research designed to support regulatory decision making should be encouraged in order to make concert conclusions on how to use nano-enabled packaging materials.

5.2. Recommendations

Even though nano coated plastic package found to be one way of *injera* preservation methods, the method of nano particle production is time consuming. In addition to this, way of storage for *injera* is difficult since it is piled on the same storage container which accelerates the growth of molds. An other difficulty in using of this dip coated plastics for *injera* storage is migration wich is accerated with *injera* moisture and transferred to *injera* easily. Based on these, the following recomenadtion were suggested to solve the problem as possible.

- ✓ It would be advisable to find improved way of using biosynthesized nanoparticles in packing of *injera* which may be inclusion of these nanoparticles as ingredients of packaging materials during processing. It may be possible to decrease migration by resisting release of the nano materials from the package to food.
- ✓ It would be better if improved method for for bulk production will be devised for synthesis of these nano particles to safe time and manual power.
- ✓ Storage of *injera* is in bulk form which makes more affected by fungal gowth. For this, more confortible method needs to be searched to decrease the effects of molds caused by this storage method problem.

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7. APPENDEX

1. ANOVA Tables of Studied Parameters

a) Moisture content of *injera*

AgNPs -Day 4

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
AgNPs10%	3	54.08	1.76741	1.02041	49.6928	58.4738	52.23	55.75
AgNPs 50%	3	53.84	1.20972	.69843	50.8315	56.8418	52.57	54.98
AgNP90%	3	54.11	1.15993	.66968	51.2319	56.9948	53.02	55.33
Control	3	56.10	.55940	.32297	54.7070	57.4863	55.67	56.73
Ext+AgNPs	3	54.43	1.52923	.88290	50.6345	58.2321	52.72	55.66
Total	15	54.51	1.39172	.35934	53.7420	55.2834	52.23	56.73

Moisture content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.948	4	2.487	1.449	.288
Within Groups	17.168	10	1.717		
Total	27.116	14			

ZnONPS- Day 4

Moisture Content

Moisture Content

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	3	56.0967	.55940	.32297	54.7070	57.4863	55.67	56.73
Ext+ZnONP	3	54.7133	1.38356	.79880	51.2764	58.1503	53.15	55.78
ZnONP10%	3	54.4600	.75901	.43822	52.5745	56.3455	53.75	55.26
ZnONP50%	3	53.2833	2.58365	1.49167	46.8652	59.7015	50.30	54.78
ZnoNP90	3	55.1800	1.50330	.86793	51.4456	58.9144	53.83	56.80
Total	15	54.7467	1.60761	.41508	53.8564	55.6369	50.30	56.80

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.705	4	3.176	1.353	.317
Within Groups	23.477	10	2.348		
Total	36.182	14			

2. Mold and Yeast Cfu/g in log10

AgNPs Day 4

	N	Mean	Std. Deviation	Std. Error	95% CI		Minimum	Maximum
					Lower Bound	Upper Bound		
AgNPs10%	3	2.7200	.10392	.06000	2.4618	2.9782	2.60	2.78
AgNPs50%	3	2.3200	.15100	.08718	1.9449	2.6951	2.18	2.48
AgNPs 90%	3	2.0000	.00000	.00000	2.0000	2.0000	2.00	2.00
Ext+AgNPs	3	2.1600	.15100	.08718	1.7849	2.5351	2.00	2.30
Control	3	2.9967	.04509	.02603	2.8847	3.1087	2.95	3.04
Total	15	2.4393	.39105	.10097	2.2228	2.6559	2.00	3.04

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.024	4	.506	43.298	.000
Within Groups	.117	10	.012		
Total	2.141	14			

ZnONPs Day 4

	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum
ZnONP10%	3	2.9333	.05774	.03333	2.7899	3.0768	2.90
ZnONP50%	3	2.2200	.06928	.04000	2.0479	2.3921	2.18
ZnONP90%	3	1.7000	.00000	.00000	1.7000	1.7000	1.70
Ext+ZnONP	3	1.9000	.17321	.10000	1.4697	2.3303	1.70
Control	3	2.9967	.04509	.02603	2.8847	3.1087	2.95
Total	15	2.3500	.55300	.14279	2.0438	2.6562	1.70

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.201	4	1.050	130.739	.000
Within Groups	.080	10	.008		
Total	4.281	14			

2. Images of Stored *Injera* dip coated with Ag and ZnONPs in Plastic Bags

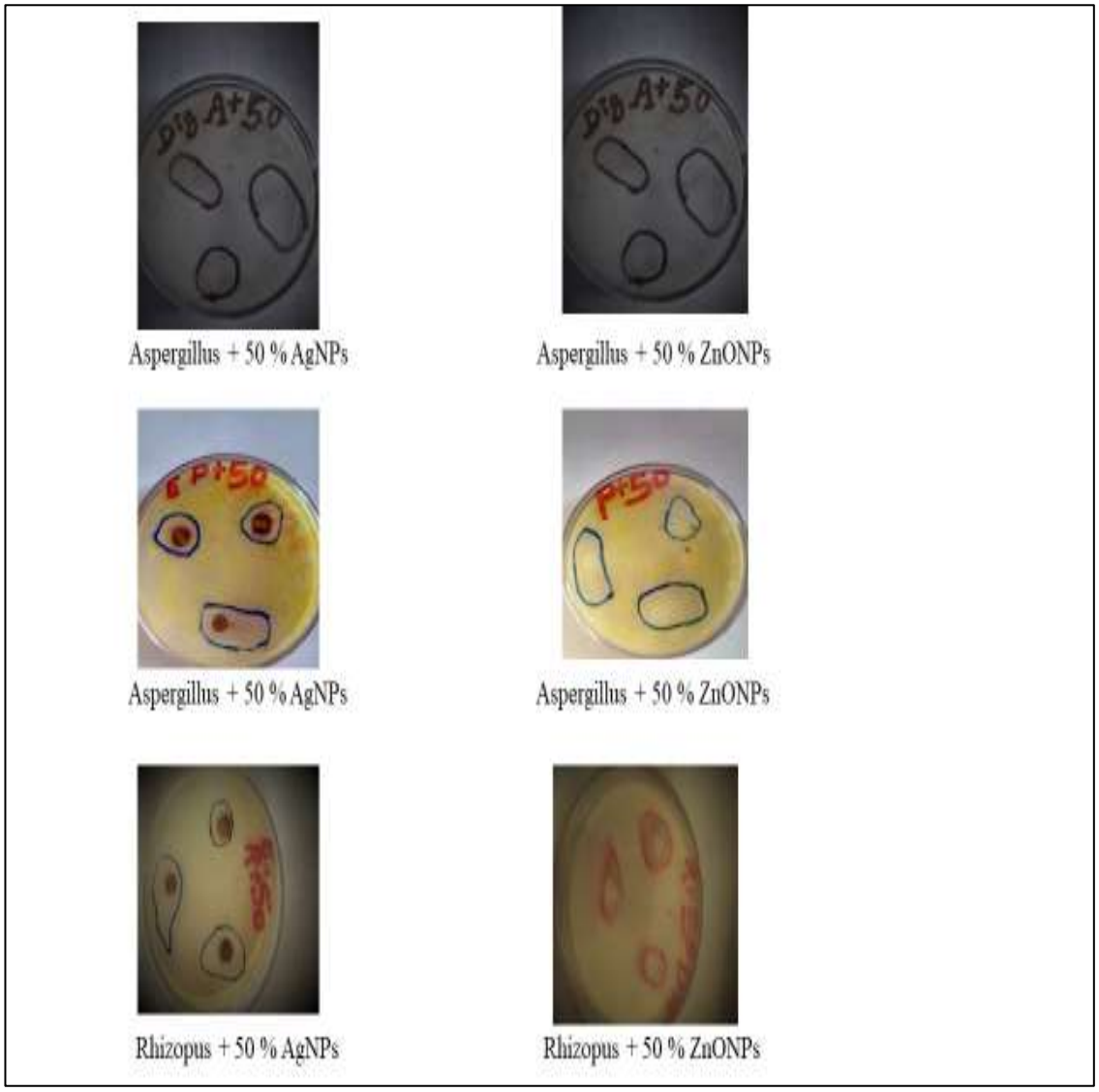
70%ZnO at day 21

70%AgNPs at day 19

100% ZnONPs at day 23

90% AgNPs at day 24

Images of Plates Showing Inhibition Zone of Antimicronial activities of Ag and ZnONPs



4. Arranged Data Antimicrobial Test and Ag and ZnONPs Migration from ANOVE Tables

a) Antimicrobial activities of both Ag and ZnONPs

Treatment	Silver Ag Nanoparticles(AgNPs)			Zinc Oxide ZnO Nanoparticles(ZnONPs)		
%	Aspergillus Niger	Penicillinium Sp.	Rhizopus	Aspergillus Niger	Penicillinium Sp.	Rhizopus Sp.
10%	1.47 ± 2.02^{g}	1.08 ± 0.54^{f}	0.57 ± 0.43^{f}	3.61 ± 0.39^{g}	3.49 ± 1.25^{f}	2.65 ± 0.47^{f}
20%	2.06 ± 0.89^{fg}	2.82 ± 1.36^{f}	0.87 ± 0.34^{f}	6.38 ± 0.63^{fg}	8.20 ± 0.82^{ef}	3.43 ± 0.54^{gf}
30%	5.93 ± 0.24^{efg}	4.86 ± 1.87^{ef}	2.49 ± 0.52^{ef}	10.1 ± 0.83^{ef}	11.6 ± 0.49^{de}	6.74 ± 1.53^{fg}
40%	6.15 ± 7.82^{efg}	5.50 ± 1.00^{def}	3.73 ± 0.99^{def}	12.12 ± 7.5^{def}	11.89 ± 1.34^{de}	8.25 ± 0.82^{ef}
50%	7.31 ± 7.57^{defg}	5.53 ± 1.21^{def}	5.29 ± 2.54^{def}	13.49 ± 6.18^{cde}	14.17 ± 4.02^{cd}	9.01 ± 1.80^{def}
60%	8.88 ± 0.80^{def}	8.78 ± 2.98^{cde}	7.51 ± 2.31^{cde}	13.11 ± 1.00^{de}	15.08 ± 1.80^{cd}	11.85 ± 0.51^{cde}
70%	9.38 ± 1.16^{cde}	9.34 ± 3.44^{cde}	8.30 ± 1.93^{bcd}	14.04 ± 2.25^{cde}	15.39 ± 3.47^{cd}	11.96 ± 1.52^{cd}
80%	10.07 ± 3.87^{cde}	9.87 ± 3.22^{cd}	11.05 ± 4.03^{bc}	16.27 ± 3.95^{bcd}	18.63 ± 1.69^{bc}	16.47 ± 3.77^{ab}
90%	13.31 ± 4.02^{bcd}	12.06 ± 4.91^{bc}	12.77 ± 2.94^{abc}	19.54 ± 3.55^{bc}	18.48 ± 4.80^{bc}	17.32 ± 2.45^{ab}
100%	18.69 ± 5.34^{ab}	16.13 ± 4.0^{ab}	13.44 ± 5.50^{ab}	21.75 ± 099^{ab}	21.97 ± 3.97^{ab}	18.87 ± 3.01^{a}
Ext+A/E+Z	15.89 ± 2.31^{abc}	$13.70\pm2.23^{b}c$	8.24 ± 7.03^{bcd}	21.61 ± 2.39^{ab}	18.72 ± 0.58^{bc}	15.21 ± 3.75^{bc}
Control	21.84 ± 3.81^{a}	19.75 ± 3.19^{a}	17.01 ± 3.28^{a}	27.36 ± 4.31^{a}	24.95 ± 4.12^{a}	18.05 ± 1.95^{ab}
SE	1.17	0.98	0.97	1.21	1.04	0.96

Data were interpreted as mean ±standard error. The means that share similar letter are not significantly ($P>0.05$*).different. Ext-Extract, SE – Standard Error, Control and ZnONPs were terminated on 4th day whereas AgNPs was on 6th day*

A Table showing mold and yeast colony count (Cfu/g) in log 10

Treatment	Day2	Day4	Day 6	Day 8	Day10
AgNPs					
AgNPs10%	2.32±0.1^{a}	2.72±0.1^{c}	2.97±0.0^{d}	-	-
AgNPs50%	2.26±0.1^{a}	2.32±0.1^{b}	2.29 ±0.1^{c}	2.39±0.1^{b}	2.32±0.1^{c}
AgNPs90%	2.19±0.1^{a}	2.00±0.0^{a}	1.70±0.0^{a}	1.9±0.1^{a}	2.00±0.0^{a}
Extr+AgNPs	2.33±0.1^{a}	3.00±0.0^{ab}	2.00±0.0^{b}	1.96±0.1^{a}	2.16±0.1^{ab}
control	2.41±0.1^{a}	3.00±0.0^{d}	3.00±0.0^{d}	3.00±0.0^{c}	3.00±0.0^{c}
SE	0.04	0.1	0.13	0.14	0.11
ZnONPs					
ZnONPs10%	2.39±0.1^{ab}	2.93±0.0^{d}	-	-	-
ZnONPs50%	2.43±0.1^{c}	2.22±0.0^{c}	2.22±17^{c}	2.33±0.1^{b}	2.22±0.0^{c}
ZnONPs90%	2.26±0.1^{a}	1.70±0.0^{a}	1.90±17^{b}	2.16±0.1^{ab}	1.70±0.0^{a}
Ext+ZnONPs	2.30±0.0^{ab}	1.90±0.0^{b}	1.70±0^{a}	1.96±0.1^{a}	1.90±0.1^{b}
control	2.41±0.07^{b}	3.00±0.03^{d}	3.00±0.03^{d}	3.03±0.03^{d}	3.00±0.03^{d}
SE	0.02	0.14	0.15	0.12	0.15

*Data were interpreted as mean ±standard error. The means that share similar letter are not significantly (*P>0.05*).different. Ext -Extract SE – Standard Error, Control and ZnONPs were terminated on 4th day whereas AgNPs was on 6th day.*

b) Migration of both Ag and ZnONPs during storage

2) Migration of AgNPs

Treatment	**Silver(Ag) in mg/kg**				
	Day2	Day4	Day6	Day8	Day10
Control	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.002±0.00[a]	0.00±0.00[a]
10%AgNPs	0.02±0.00a	0.03±0.00a	0.30±0.04[b]	0.39±0.03[b]	0.66±0.06[b]
50%AgNPs	0.45±0.04[b]	0.59±0.08[c]	1.04±0.03[d]	1.36±0.0.07[c]	1.34±0.05[c]
90%AgNPs	0.59±0.05[c]	0.79±0.06[d]	1.11±0.04[d]	1.52±0.06[d]	6.00±0.26[d]
Ext+AgNPs	0.22±0.00[b]	0.77±0.04[d]	0.74±0.04[b]	0.84±0.0.04[b]	0.87±0.04[c]
SE	0.08	0.08	0.11	0.17	0.59

*Data were interpreted as mean ±standard error. The means that share similar letter are not significantly (*P>0.05*).different.* Ext - Extract, SE – Standard Error

2) Migration of ZnONPs

Treatment	**Zinc(Zn) in mg/kg**				
	Day2	Day4	Day6	Day8	Day10
Control	23.65±0.48[a]	22.79±0.81[a]	21.68±1.05[a]	22.52±0.65[a]	24.33±1.15[a]
ZnONP10	135.41±1.83[b]	143.10±6.56[b]	151.69±0.96b	230.43±0.41[b]	253±23.32[b]
ZnONP50	285.61±3.0[c]	344.32±2.67[c]	358.80±0.69[c]	370±0.69[c]	375±1.65[c]
ZnONP90	348.27±2.84[c]	444.18±4.52[d]	445.25±3.64[d]	534.48±10.57[d]	583.36±2.46[d]
Ext+ZnONP	511.29±1.19[e]	563.183±1.17[e]	587.96±2.18[d]	677.62±8.69[d]	691.36±10.64[e]
SE	62.92	59.22	61.26	50.73	49.30

*Data were interpreted as mean ±standard error. The means that share similar letter are not significantly (*P>0.05*).different, Ext = Extract*