Response of *Trigonella foenum-graecum* L. on CuO Nanoparticles Exposure

By

NOOR UL AIN
Reg. #: 02271411002

Department of Biotechnology
Faculty of Biological Sciences,
Quaid-I-Azam University Islamabad,
Pakistan
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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology

By

NOOR UL AIN

Reg. #: 02271411002

Supervised by: Dr. Muhammad Zia

Department of Biotechnology

Faculty of Biological Sciences,

Quaid-I-Azam University Islamabad,

Pakistan

2016

Certificate of Approval

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan accept the dissertation entitled "Response of *Trigonella foenum graecum* L. on CuO Nanoparticles Exposure." submitted by Noor ul Ain in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in Biotechnology.

Supervisor: _____

Dr. Muhammad Zia

External Examiner: _____

Dr. Muhammad Javaid Asad

Chairman: _____

Dr. Zabta Khan Shinwari

Date: April 01, 2016

Dedication

I dedicate this report to my beloved father Mr. Abdul Latif without whom none of my success would be possible

Declaration

I, Noor ul Ain, hereby solemnly declare that the work presented in this thesis entitled "Response of *Trigonella foenum-graecum* L. on CuO Nanoparticles Exposure" is original. I declare further that this work has not been submitted for any degree or diploma to any other university or institution. I am aware of the terms copyright, and plagiarism and I will be responsible for any copyright violation found in this work.

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Noor ul Ain

M.Phil (Biotechnology)

Department of Biotechnology

Quaid-I-Azam University, Islamabad

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Noor ul Ain

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List of Acronyms

2, 4-D	2,4-Dichlorophenoxyacetic acid
AAE	Ascorbic acid equivalent
BA	6-Benzylaminopurine
Cu-acetate	Copper acetate
CuONP	Copper oxide nanoparticles
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
F.G	Final germination
FC	Folin–Ciocalteu
FW	Fresh weight
GAE	Gallic acid equivalent
GI	Germination index
LSD	Least significant difference
MPFG	Mean period of final germination
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NPs	Nanoparticles
PEG	Polyethylene glycol
PI	Percent inhibition
PVP	Polyvinylpyrrolidone
QE	Quercetin equivalent
R.G	Rate of germination
ROS	Reactive oxygen species
SD	Standard deviation
T. foenum-graecum L.	*Trigonella foenum-graecum* L.
TAC	Total antioxidant capacity
TCA	Trichloroacetic acid
TFC	Total flavonoid content
TPC	Total phenolic content
TRP	Total reducing power
UV	Ultra violet

Abstract

Nanoparticles have gained much importance in last two decades, especially metal nanoparticles due to their unique physical, optical, electrical, magnetic and chemical properties. Nanoparticles interact with the plants and effect seed germination and growth of plant. *Trigonella foenum- graecum* L. (fenugreek) is one of the important medicinal plants containing important phytochemicals. The aim of this study was to evaluate the response of *T. foenum-graecum* L. to copper oxide nanoparticles using plant tissue culture technique at different concentrations along with polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) capped nanoparticles. The results showed that seed germination was not affected by CuONPs. However, growth of shoot and root was maximum in *T. foenum- graecum* L. seedlings at 50 mg/L of CuONPs, uncapped and capped and reduced with increase in concentration of NPs indicating toxicity. In a parallel experiment, Cu-acetate salt solution showed 100% seed germination but it restricted root and shoot elongation due to high salinity at 1%. Phytochemical screening of shoot and root extracts was also carried out showing maximum flavonoid and phenolic contents accumulation in Cu-acetate treated plantlets as compared to CuONP and control with maximum radical scavenging DPPH and antioxidant activity. PEG and PVP treated plantlets accumulated more flavonoid and phenolic contents as compared to CuONP-PEG and CuONP-PVP, indicating increased secondary metabolites production due to abiotic stress. But DPPH radical scavenging activity was higher in CuONP-PEG, 50mg/L and CuONP-PVP, 100 mg/L as compared to PEG and PVP.

Callus was induced from stem and leaf explants of *T. foenum-graecum* L. treated with CuONP, CuONP-PEG and CuONP-PVP. Maximum fresh and dry weight was found in control i.e. stem explant; 1.65g and 0.10g and leaf explant; 1.33g and 0.09g after 30 days as compared to explants treated with nanoparticles. Although growth was decreased in explants treated with CuONPs but results revealed maximum flavonoid and phenolic contents accumulation in explants treated with CuONP-PVP with maximum antioxidant activity.

Overall results indicate that CuONPs treatment affect the seedling growth of *T. foenum-graecum* L. depending on concentration without inhibiting seed germination. These experiments also showed that *in vitro* culture of *T. foenum-graecum* L. can be exploited for enhanced production of secondary metabolites.

Introduction

Nanotechnology represents an area with significant wide range of applications including food processing, wastewater treatment, biomedical products, healthcare, diagnostics and pharmaceuticals, as well as environmental, energy and material sciences. Therefore, nanotechnology has become a dynamic developing industry (Aslani *et al.,* 2014; Ma *et al.,* 2015). Nanoparticles are characterized by the materials with at least one dimension less than 100nm with large surface area (Burklew *et al.,* 2012). The small size of nanomaterials confers unique properties such as electrical conductivity, toughness and optical features. Due to these distinctive features, nanoparticles are increasingly been employed in industry and daily life by advancing the everyday materials and processes (Remédios *et al.,* 2012).

Nanoparticles (NPs) have also been used in agriculture as nanofertilizers to enhance plant growth and development in more effective way than applied fertilizers that are unable to reach plant due to leaching, hydrolysis and decomposition. These nanofertilizers also protect against biotic stresses for instance insect, fungi and bacteria. Hence nanofertilizers reduce the loss in fertilization and increase crop yielding through certain properties than are effective for crop plants (Ma *et al.,* 2010; Siddique *et al.,* 2015).

Nanoparticles interact with plants causing many morphological and physiological changes, depending on the properties of NPs. Efficacy of NPs is determined by their chemical composition, size, surface covering, reactivity, and most importantly the dose at which they are effective (Khodakovskaya *et al.,* 2012). There are number of studies showing both beneficial and negative effects of NPs on plant growth and development (Monica and Cremonini, 2009). Many studies have proved that zinc oxide NPs (ZnONPs) in peanut, soyabean, wheat and onion at low concentrations exhibiting beneficial effect on germination of seed (Prasad *et al.,* 2012; Sedghi *et al.,* 2013; Ramesh *et al.,* 2014; Raskar and Laware, 2014) . Carbon nanotubes have also gained importance due to unique properties in plant development by influencing the seed germination in various studies (Villagarcia *et al.,* 2012; Tiwari *et al.,* 2014). Silver nanoparticles have great impact on plant growth. A study was carried out to check the effect of biologically synthesized AgNPs on Bacopa monnieri, and results have revealed a significant effect on seed germination and induced the synthesis of protein and carbohydrate and decreased the total phenol contents and catalase and peroxidase activities (Krishnaraj *et al.,* 2012).

In another study, the effects of nanoscale titanium oxide (TiO_2) and silicon oxide (SiO_2) on soyabean plant were observed and these NPs improved the activity of nitrate reductase in soyabean, and germination and growth was apparently accelerated (Lu *et al.,* 2001).

Nanoparticles also elicit the production of secondary metabolites that are produced by plants under stress conditions. These bioactive compounds play important role in host immune system. *Aloe vera* L. is of the important medicinal plant due to its secondary metabolites, is treated with TiO_2 NPs and AgNPs to increase the production of secondary metabolites and it was concluded that NPs elicit the production of aloin in *Aloe vera* (Raei *et al.,* 2014).

Copper oxide nanoparticles (CuONPs) have gained special attention due to its simplicity and various physical properties including superconductivity, electron correlation effects, and spin dynamics. CuONPs are progressively used in various applications such as in catalysis, batteries, gas sensors, heat transfer fluids, and solar energy (Ahamed *et al.,* 2014). CuO crystal structures possess a narrowband gap, giving useful photocatalytic and photovoltaic properties (Chang *et al.,* 2012).

Although nanotechnology have huge number of beneficial applications, but technology always comes with a price i.e. there are environmental and healthcare concerns related to nanoparticles (Maynard *et al.,* 2006). Nanoparticles enter into the environment by intentional or unintentional means including waste streams from manufacture facilities, volcanic emissions, industrial processes and transportation (Klaine *et al.,* 2008; Dutschk *et al.,* 2014). Toxicity studies of NPs have been carried out aiming on biological and ecological effects but there are still gaps due to lack of knowledge release rates into the environment (Hansen *et al.,* 2008). NPs kill pathogenic bacteria effectively but on the other hand they have deleterious effects on helpful microbes in the environment such as plant growth promoting microbes, pollutants degrading and element cycling. Industrial wastes with NPs move into waters thus polluting oceans by accumulating on the surface of oceans and pose risk to marine life, birds and mammals (Kennedy *et al.,* 2004; Nowack and Bucheli, 2007).

T. foenum-graecum L. is one of the oldest plants in the world, commonly known as “fenugreek”. It is a self-pollinating, leguminous crop native to the Indian subcontinent and the Eastern Mediterranean region. Nearly 260 different varieties of *T. foenum-graecum* L. were suggested by Linnaeus but only 18 species have been traced. It is currently widely cultivated in central Asia, central Europe, Northern Africa, North America and parts of Australia, with India being the leading producer in the world (Pasricha and Gupta, 2014).

Most popular species of this genus is *Trigonella foenum-graecum* L. “Trigonella”, is a Latin word meaning “little triangle”, due to the triangular shape of its flowers and “foenum-graecum” stands for “Greek hay” which refers to its use as forage crop. It is also known as ‘ox horn’ or ‘goat horn’ because its two seed pods project in opposite directions from the nodes of the stem base and resemble an ox or goat horns. It is grown mainly as a spice crop (Morcos *et al.*, 1981; Patil *et al.*, 1997).

*T. foenum-graecum*L.exhibits antioxidant activity due to the presence of certain phytochemicals in the plant including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponins etc. Different studies proved that *T. foenum-graecum* L. extracts exhibit scavenging activity against free hydroxyl radicals that are associated with diseases like diabetes mellitus, atherosclerosis, cataract, rheumatism, and other auto immune diseases. Studies have also shown anti-bacterial activity possessed by *T. foenum-graecum* L. Extracts of *T. foenum-graecum* L. have shown to inhibit different bacterial species (Akbari *et al.*, 2012; Al-Asadi, 2014).

Objectives of the study

Objectives of this study were;

1. To evaluate the effects of CuONPs uncapped and caped with PVP and PEG on seed germination and plantlet length of *T. foenum-graecum* L.
2. To investigate the effects of CuONPs on stem and leaf explant culture
3. To determine antioxidant activity of shoot, root and callus extracts of *T. foenum-graecum* L. treated with CuONPs

Review of Literature

2.1. Nanoparticles

Nanotechnology is a developing field with a great potential to make new products incorporating distinctive properties and improving the products for various applications. There are many products of nanotechnology that are already Many nanotechnology-based products are already available in the market, including sporting goods, electronics, personal care, and automotive parts (Kang, 2010). According to another estimate the product sale incorporating nanotechnology will rise up to 15% in future, totaling $2.6 trillion (Thomas, 2006).

The term nanotechnology is a combination of two words; “nano” is a Greek word denoting a billionth and the word technology. Therefore nano scaled particles are considered to be the one with size less than 100nm at least one dimension (Bhushan, 2004; Sattler, 2010). This extremely small size of NPs, confers unique physical and chemical properties that results in higher reactivity with large surface area, making NPs feasible to utilize in a huge number of products for example paints, cosmetics, medicines, food and suntan lotions, as well as applications which directly release NPs into the environment, such as remediation of polluted environments (Aitken *et al.,* 2006).

2.1.1. Classification of Nanoparticles

Nanoparticles are classified into following three types:

i. *Natural NPs*; Natural nanoparticles have existed from the beginning of the earth’ history and still occur in the environment (volcanic dust, lunar dust, mineral composites)

ii. *Incidental NPs*; also defined as waste or anthropogenic particles, take place as the result of manmade industrial processes (diesel exhaust, coal combustion, welding fumes, etc.)

iii. *Engineered NPs*; are further grouped into four categories; a) carbon based NPs which include fullerene, single walled carbon nanotube (SWCNT) and multiwalled carbon nanotubes (MWCNT); b) metal based NPs including quantum dots, nanogold, nanozinc, nanoaluminum and nanoscales metal oxides like TiO_2, ZnO and Al_2O_3; c) dendrimers are nano-sized polymers built from branched units, which are capable to be designed to perform specific chemical function; d) composites which combine nanoparticles with other nanoparticles or with larger bulk-type materials (Lin and

Xing, 2007) and present different morphologies such as spheres, tubes, rods and prisms (Ju-Nam and Lead, 2008).

Engineered NPs have gained a lot of importance due to their wide range of applications in industry and improving the economy sectors such as consumer products, pharmaceutics, cosmetics, transportation, energy and agriculture etc. (Roco, 2003; Nowack and Bucheli, 2007).

Metal nanoparticles (MNPs) belong to the category of engineered NPs, have been in focus due to their unique features such as electronic, optical, mechanical, magnetic and chemical properties that can be significantly different from those of bulk materials (Boddu *et al.,* 2011). Metal NPs are synthesized employing various methods that confer different properties for desired purposes. Mostly methods that are used for synthesis of metal NPs are sol-gel, hydrosol/magnetic fluid, vacuum deposition and ball milling method (Lue, 2007).

2.1.2. Applications of Nanoparticles

The process of nanotechnology began with the generation, manipulation, and deployment of nanomaterials, representing an area holding significant promise for a wide range of applications. Nanotechnology has become a dynamically developing industry, with multiple applications in energy, materials, computer chips, manufacturing, health care, and medical diagnosis (Safari and Zarnegar, 2014; Neto, 2014).

Diagnostics and Therapeutics

Nanotechnology is an interdisciplinary field integrating engineering, chemistry, biology and medicine, thus making it useful for diagnostic and therapeutic purposes. It has been successfully used not only the diagnosis of cancer but also to treat cancer by using it as a drug delivery vehicle (Laroui *et al.,* 2013). Recent advances have shown that NPs have bioaffinity property making it a probe for molecular and cellular imaging, targeted NP drugs for cancer therapy, and integrated nano devices for early screening and detection of cancer. These developments offer exciting opportunities for the development of personalized therapy, in which the molecular profiles of an individual's genetic and protein biomarkers may be used to diagnose and treat the patient's cancer (Cai *et al.,* 2008).

Nanometrology

Nanometrology deals with the measurement of functionally important, mostly dimensional parameters and components with at least one critical dimension which is smaller <100 nm. Success in nanomanufacturing of devices will rely on new nanometrologies needed to measure basic materials properties including their sensitivities to environmental conditions

and their variations, to control the nanofabrication processes and materials functionalities, and to explore failure mechanisms (Nomura *et al.,* 2004).

Nano and Energy

Energy comes highest on the list of priorities in human needs (Ghoniem, 2011). World's primary energy demand will increase by 36% between 2008 and 2035 according to International Energy Agency (IEA). Due to carbon dioxide emission and climate changes that are influencing life and health, renewable energy resources needs to play vital role in developing more reliable and sustainable energy path. Solar energy is the most abundant, infinite and pure renewable energy source to date and it can be harnessed using photovoltaic cells. Although inorganic semiconductors including silicon, gallium arsenide and sulfide salts are primarily used but organic materials with nanostructures are more advantageous because of low cost and large scale manufacturing processes (Yu *et al.,*1995; Forrest, 2004).

Nanoparticles and Agriculture

Engineered NPs act as a smart tool for the efficient delivery of fertilizers, herbicides, pesticides and plant growth regulators etc. (Johnston, 2010). Applications of NPs has influenced the earlier plant germination as well as enhanced the yield of plants for food, fuel, and other uses (figure 2.1)(Ditta, 2012).

Nanoparticles have been employed in numerous researches to show crop improvement. Carbon nanotubes (CNT) have increased the seed germination in tomato (Khodakovskaya *et al.,* 2009). Other than CNT, metal nanoparticles including gold (Au), silver (Ag), copper (Cu), zinc (Zn), aluminum (Al), silica (Si), zinc oxide (ZnO), cesium oxide (Ce_2O_3), titanium dioxide (TiO_2) and magnetized iron (Fe) have also found to improve crop yield (Zhang and Webster, 2009).In a study effect of Au-NPs on *Brassica juncea* was checked at different concentrations and it was found that Au-NPs decreased overall growth of plant but increased the free radical stress supporting increase in biomarker antioxidative enzymes, proline and hydrogen peroxide (Gunjan *et al.,* 2014). In another study zinc and silver nanoparticles were employed on two important crops; *Zea mays* L. and *B. oleracea var. capitata* L. and both plants showed lower nanoparticle toxicity as compared to the free ions (Pokhrel and Dubey, 2013).

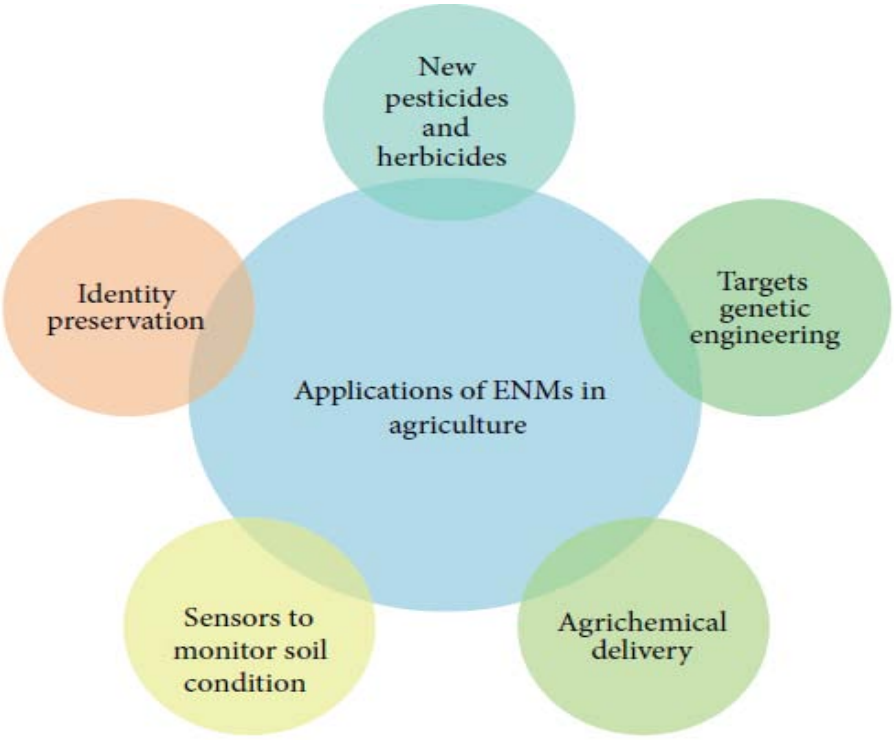

Figure 2.1: Applications of engineered nanoparticles in agriculture

2.1.3. Nanotoxicology

Increased production of NPs has raised the concern of its negative impact on the environment and interaction with the living organisms (Ye *et al.,* 2012). NPs enter living systems through intentional and unintentional releases such as solid/liquid waste streams from manufacture facilities and atmospheric emissions. Nanomaterials can come into contact with living organisms via multiple routes (figure 2.2), such as incidental release, direct release from industrial products or processes, as well as commercial products during intended uses that in turn enter the sewer-to-wastewater treatment plants (Grieger *et al.*,2009; Zhang and Fang, 2010).

Nanoparticles are present in the atmosphere naturally but there is a little work done on ecological system and release of NPs in the air. They accumulate in to the atmosphere through diesel and it is concerning due to global warming aspect. The key species in the atmosphere are sulphuric acid, nitric acid and organic gases and producing secondary NPs that are volatile. Hence increasing particle number in the environment (Biswas and Wu, 2005; Klaine *et al.*, 2008).

Industrial products and wastes tend to end up in waterways, increasing the possibility of NPs contamination, although almost no current data on behavior and fate of NPs in aquatic

systems is available. Like metals, manufactured NPs tend to aggregate and subsequently settle. This process has positive effects on water bodies' purification, because this behavior results in a transfer of NPs from water column to sediments, resulting in a pollutant loss. Despite this possible pollutant loss, natural colloids interact with NPs and this will affect the NPs behavior, being more difficult to predict the NPs effect on the aquatic system (Nowack and Bucheli, 2007; Auffan *et al.*, 2010).

Soil and sediments are the ultimate sinks of NPs and, whether directly or indirectly released (e.g., via sewage treatment plants, aerial deposition, or waste handling), NPs will end up in soil; this system may present the most significant exposure avenue for assessing environmental risk. Being soil one of the main sinks, it raises concern about the entry of NPs into food webs and human access to contaminated agriculture (Lecoanet *et al.*, 2004; Unrine *et al.*, 2010).

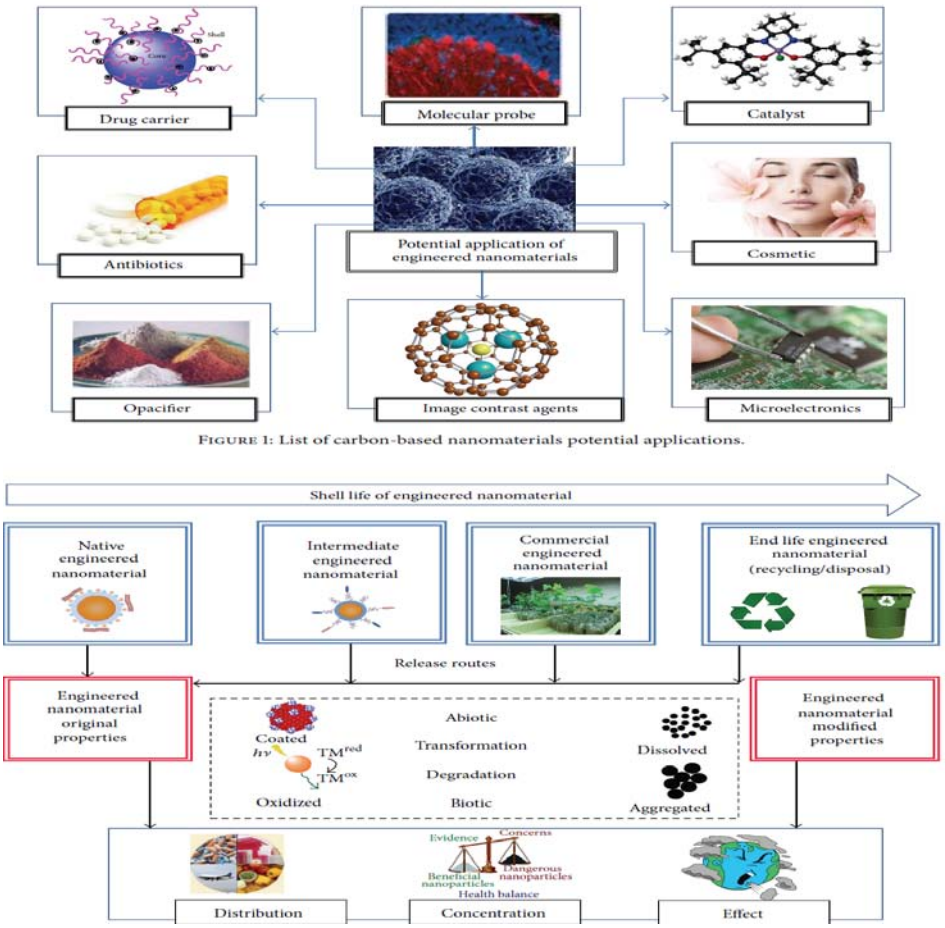

FIGURE I: List of carbon-based nanomaterials potential applications.

Figure 2.2: Nanomaterials in living systems

2.1.4. Copper oxide Nanoparticles

Copper (Cu) is an essential nutrient for the growth of plants and plays an important role in the photosynthetic reactions. It is essential component of protein and enzymes, primarily involved in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell wall and cytoplasm of plant cells (Nassar, 2004; Lee *et al.,* 2010). Since copper is a plant micronutrient, exposure to excessive concentration of Cu can cause a broad range of deleterious effects e.g. inhibition of photosynthesis and pigment synthesis, damage to plasma membrane permeability as well as other metabolic disturbances, either in field plants (Lanaras *et al.*, 1993; Ouzounidou *et al.,* 1993).

Copper Oxide NPs are largely used engineered NPs with important industrial applications including superconducting materials, catalytic agent, sensing materials, ceramics, thermoelectric materials, glass, etc. Due to excessive use of CuONPs in antifouling paints of boats signifies an important source of NPs contamination of aquatic ecosystems. These paints consist of a polymeric film made mostly of acrylic and styrenic monomers covering copper oxides NPs (Almeida *et al.,* 2007). These paints upon decomposition may release copper under soluble ionic forms or as NPs, both of which can be toxic to aquatic life (Sharma and Agrawal, 2005). Aquatic photosynthetic organisms represent the main source of biomass in the aquatic trophic chain and these organisms are very sensitive to the effects of copper species. It has been reported that copper may induce strong inhibition of photosynthetic electron transport processes and cause oxidative stress at cellular level (Dewez *et al.,* 2005;Knauert and Knauer, 2008).

Thus significant industrial production and applications of CuO NPs may lead to environmental exposure and causing toxicity to bacteria, nematodes, and other organisms. Studies on the toxicity of nano materials are still emerging and basically evidence several negative effects on growth and development of plants (Adhikari *et al.,*2012). CuNPs at various concentrations have been shown to improve the growth and yield of wheat as compared to control by improving chlorophyll content, leaf area, number of spikes and number of grains when applied to soil pots (Hafeez *et al.,* 2015).

2.2.*Trigonella foenum-graecum* L. (Fenugreek)

T. foenum-graecum L. is one of the oldest medicinal plants commonly known asFenugreek and local name “methi”, cultivated all over the world (Passano, 1995). *T. foenum-graecum* L. has been used as both food and medicine since ancient times in different regions of the world.

This crop is native to Indian subcontinent and Eastern Mediterranean region but also cultivated in central Asia, central Europe, northern Africa, North America and parts of Australia (Karaj, 2011). This wide distribution of its cultivation in the world is characteristic of its adaptation to variable climatic conditions and growing environments (Semalty *et al.,* 2009).

T. foenum-graecum L. is annual dicotyledonous herb belonging to family Fabaceae (Table 2.1) and its main producers are India, Pakistan, Bangladesh, Iran, Nepal, Egypt, Morocco, China, France, Spain, Turkey and Argentina (Basu *et al.,* 2004; Sheikhlar, 2013). Its height is up to 60cm. The characteristic features of this plant are trifoliate leaves, white flowers of triangular shape and roots bearing conspicuous root nodules (figure 2.3). Its seeds are of sickle pod shape and are of brown color with 3×4mm in dimensions (Morton, 1990; Acharya *et al.,* 2006).

Figure 2.3: *T. foenum-graecum* L. plant

2.2.1. Active Compounds

T. foenum-graecum L. has also been used as a medicinal plant since ancient times. The active constituents of *T. foenum-graecum* L. seed consist of carbohydrates (45-60%), proteins (20-30%) and lipids (5-10%). The carbohyrdates mainly contains galactomanans (mucilaginous fiber) that helps in decreasing the consumption of glucose and other calorie rich foods, thus plays an effective role to control blood glucose levels in diabetic patients. Glycosides produce steroidal sapogenins through hydrolysis (diosgenin, yamogenin,

tigogenin, neotigogenin) that aid in the production of synthetic sex steroids as well as used in the treatment of hypercholesterolemia. Other chemical compunds that are produced include spirostanol saponins, triterpenoids, trigonelline, flavonoids (e.g. atroside, quercetin, etc.) with presumed therapeutic effects. Apart from these pharmacological effects leaf extracts of *T. foenum-graecum* L. are used to treat head lice in human beings (Al Asadi, 2014).

Table 2.1: Classification of *T. foenum-graecum* L. plant

Kingdom	**Plantae**
Phylum	Anthophyta
Class	Dicotyledonae
Order	Fabales
Family	Fabaceae
Genus	Trigonella
Species	*Trigonella foenum-graecum*
Binomial name	*Trigonella foenum-graecum* L.

2.2.2. Medicinal Importance

T. foenum-graecum L. is a medicinal plant and it has therapeutic effects against various diseases like atherosclerosis, rheumatism, sugar lowering, blood lipids lowering and appetizer. It also contain antioxidant activity (Akbari *et al.,* 2012).

Hypoglycemic effects

Hypoglycemia refers to abnormal decrease in the blood sugar level in human body (Goswami, 2012). The seeds of *T. foenum-graecum* L. stimulate the glucose dependent insulin secretion from pancreatic beta cells thereby, exerting hypoglycemic effects in humans. It also inhibits the activity of two intestinal enzymes, alpha amylase and sucrase involved in metabolism of carbohydrate (Amin *et al.,* 1987; Ajabnoor and Tilmisany, 1988).

Hypocholesterolemic activity

Hypocholesterolemia is condition with abnormal deficiency of cholesterol level in blood. In a study it has been shown that administration of *T. foenum-graecum* L. seed extracts lower the serum cholesterol, triglyceride and low-density lipoprotein in hypercholesterolemia suffering patients and experimental models (Singhal *et al.,* 1982). It also reduces accumulation of

triglyceride in the liver without interfering with plasma insulin in rats suffering obesity (Smith, 2003).

Gastroprotective effect

T. foenum-graecum L. is effective against gastric ulcer and it is used for this purpose since old times. In a study ulcer protective effect of fenugreek seed was compared to omeprazole on ethanol-induced gastric ulcer in rats and seeds showed significant ulcer protective effects compared to those on omeprazole. Scientists have discovered that seeds of fenugreek has cytoprotective effect due to the anti-secretory action and to the effects on mucosal glycoproteins (Pandian *et al.,* 2002).

Antimicrobial activity

Antimicrobial activity of plant extracts is due to variety of compounds including aldehyde and phenolics. Antibacterial effects have also been reported in numerous studies. Researhers reported strong activity of *T. foenum-graecum* against 26 bacterial pathogens (Omoloso, 2001). In another study a high antimicrobial activity against peptic ulcer-linked *Helicobacter pylori* in the fenugreek sprout extract was observed (Randhir *et al.,* 2004). Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties (Hugo and Bloomfield, 1971). Methanol extract of fenugreek and coriander have shown increased antimicrobial activity against Pseudomonas spp., *Escherichia coli*, *Shigella dysentiriae* and *Salmonella typhi* (Dash *et al.*, 2011). Fatty oil of fenugreek seeds showed very significant antimycotic activity against *Aspergillus niger* and *A. fumigates* (Wagh *et al.,* 2007).

2.3. Tissue culture studies of *T. foenum-graecum* L.

Plant tissue culture provide propagation of plants which are rare or economically important and can be used to induce quantitative and qualitative modifications on the production of plant secondary metabolites by changing nutrient and hormonal media culture conditions (Collin, 2001). Plant tissue culture, is an important tool to understand physiological, biochemical, and morphological reactions taking place in cell under controlled conditions to specified factors in order to gain insight into the intact plant life to its natural environment (Neumann *et al.,*2009).*T. foenum-graecum* L. is an important medicinal plant producing several secondary metabolites that help in treating various disease conditions thus several studies have been conducted for the production of secondary metabolites from cell suspension cultures, callus and protoplast culture as well as organogenesis and genetic transformation.

Trisonthi *et al.* (1980) found effects of mevalonic acid on steroidal sapogenins synthesis in suspension cultures of *T. foenum-graecum* L. tissue and it should positive promoting effects. In another study cultures treated with nicotinic acid had increased trigonelline concentration (37%) (Ramesh *et al.,* 2010). Leguminous plants produce medicarpin, in response to biotic or abiotic elicitation from either their glycosidic conjugate pools or by de novo synthesis. Seedlings of *T. foenum-graecum* L. were treated with $CuCl_2$ to find out the origin of isoflavonoid pterocarpans, like medicarpin, medicarpin in response to copper elicitation. Accumulation of isoflavonoid aglycones and their glycosides were measured by using High Performance Liquid Chromatograpghy (HPLC) and their results showed the clear relationship between copper and de novo synthesis of medicarpin (Tsiri *et al.,* 2009).

2.3.1. Callus Culture

Plants can be regenerated through micropropagation, in vitro culture techniques including callus culture from shoot tips and axillary bud explants. Callus is an unorganized mass of cells or tissue derived from explants with the help of plant growth regulators. Plant regeneration from calli is possible by de novo organogenesis or somatic embryogenesis (Larkin and Scowcraft, 1981). Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the *in vitro* applied environmental stimuli, including growth factors added to the cultured cells (Flick *et al.,* 1983).

Callus cultures of *T. foenum-graecum* L.in the presence of Adenosine triphosphate (ATP) and $MgCl_2$ showed conversion of nicotinic acid and S. adenosylmethionine into trigonelline 3 to 4 times higher than seeds and 12-13 times higher than root and shoot cultures and successive stable subculturing was also obtained (Joshi and Handler, 1960). Radwan and Kokate (1980), obtained 15.6 mg/g of trigonelline from four-week-old callus cultures of *T. foenum-graecum*. The trigonelline concentration was found 3-4 folds more than seeds and 12-18 times more from roots and shoots of the parent plants.

2.4. Effect of Polyethylene glycol (PEG) and Polyvinylpyrrolidone (PVP) on Plants

Plants continue to grow and develop under variable environment and adapt to the conditions. Water deficiency can result into limited growth of plant often. It affects the shoot growth more than the root. Actually even under mild water deficit shoots may stop growing completely while roots continue to grow. Continued root growth allows the plant to plumb the soil for water and can be especially important for seedling establishment (Van der Weele

et al., 2000). Polyethylene glycol is often used to induce drought stress in seed germination studies and also influence growth of plant negatively as the concentration of PEG increases by reducing the oxygen availability (Hardegree and Emmerich, 1994).

Polyvinylpyrrolidone (PVP), is a versatile polymer, water soluble with excellent colloidal, stabilizing and complexing properties, being at the same time metabolic and physiologically inert. Previous studies have shown that PVP has positive effect on plant growth at various concentrations (Magnuson *et al.,* 1996).

2.5. Phytochemical Assays

Total flavonoid and phenolic content

Flavonoids and phenolics are important compounds of *T. foenum-graecum* L. and they accumulate at different stages of growth. Phenolic compounds are very important plant constituents exhibiting antioxiodant activity by inactivating lipid free radicals, or by preventing the decomposition of hydroperoxides into free radicals (Maisuthisakul *et al.*, 2007). Dietary flavonoids are usually glycosylated and can be classified as anthocyanidins, flavanols (catechins), flavones, flavanones, and flavonols which responsible for the orange, red and blue colors in fruits and vegetables (Merken and Beecher, 2000). Traditionally, deep colored fruits, vegetables or foods are recognized as more healthy to human body, especially in the oriental countries. There has been a growing interest in pigment components of fruits and vegetables, which may promote human health or lower the risk for disease (Lin and Tang, 2007).

2.6. Antioxidant Activity

Total antioxidant capacity

Antioxidants are present naturally in plants as well as supplements are also available (Chen *et al.,* 1992). Antioxidants of natural origin have benefits over synthetic because they do not side effects while synthetic were found to have genotoxic effects (Kahl and Kappus, 1993; Zheng and Wang, 2001). Hence determination of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous (Rohman *et al.,* 2010).

Generation of excessive reactive oxygen species (ROS) lead to the oxidative stress which plays an important role in development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular and neurodegenerative diseases. (Mandal *et al.,* 2011). Antioxidants inhibit and scavenge radicals and protecting against infections and degenerative diseases (Ebrahimzadeh *et al.,* 2010).

DPPH free radical scavenging activity

Due to increased interest in antioxidants, especially in those which prevent from deleterious effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases natural sources of antioxidants are preferred. Therefore various methods have also improved for the estimation of antioxidants including DPPH. The molecule of DPPH (2,2-diphenyl-1-picrylhydrazyl) is characterized as a stable free radical featuring delocalization of the extra electron over the molecule as a whole, preventing dimerization of molecules, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centered at about 520 nm (Molyneux, 2004).

Materials and Methods

3.1. Test Nanoparticles

Copper oxide nanoparticles (CuONPs) were used in this study to evaluate the effects on seed germination frequency of *T. foenum-graecum* L., shoot and root length as well as for phytochemical screening of plants.

Three types of CuONPs were tested; uncapped CuONPs and two others were capped with PEG and PVP (CuONP-PEG and CuONP-PVP) respectively. The NPs were provided by Miss Rabia Javed, PhD scholar at Department of Biotechnology Quaid-I-Azam University Islamabad. The NPs were characterized by X-ray diffraction, UV-visible spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) analysis. The size of all NPs that are employed was <50nm (Table 3.1).

Table 3.1: Size of test nanoparticles

Sr. No.	Nanoparticles	Size
1	CuONP	46nm
2	CuONP-PEG	47nm
3	CuONP-PVP	40nm

3.2. Preparation of Nanoparticles, Salt, PEG and PVP Dilutions

The test NPs were suspended directly in distilled water and dispersed by ultrasonic vibration (100W, 40 kHz) for 30 min. Different doses of NP suspensions 50, 100, 200 and 400 mg/L, were prepared for the seed germination experiment.

PEG and PVP solutions were also prepared with concentrations; 10, 20, 40 and 80 mg/L. Along with these, salt solutions of copper acetate, 0.5 and 1% were also prepared by dissolving in distilled water.

3.3.Seeds

Seeds of *T. foenum-graecum* L. were purchased from National Agricultural Research Centre (NARC), Islamabad. Selection of the seeds was based on ethnobotanical, traditional medicinal importance and least exploration. Seeds were cleaned, freed from dust and foreign material and then kept in dark and dry place before use.

3.4.Seed Germination and Seedling Parameters

Chemicals and Apparatus

Ethanol (Sigma-Aldrich Germany), mercuric chloride, vacuum oven (Vacucell, Einrichtungen GmbH), Whattmann filter paper, petri plates, appendorf tubes, pipettes, beakers and sonicator (SweepZone technology, USA).

All glassware (flasks, beakers, petri plates) and other instruments including scalpels, cutter, forceps used in this experiment were rinsed with distilled water, wrapped in paper and then autoclaved. Before inoculation all these instruments were treated with UV light in laminar flow hood for 10 min.

Media preparation

Following four different media were prepared for seed germination experiment;

i. Half strength i.e. 2.2 g/L of Murashige and Skoog medium (MS), containing 0, 50, 100, 200 and 400 mg/L CuONP, CuONP-PEG and CuONP-PVP
ii. Half MS media containing 10, 20, 40 and 80 mg/L of PEG and PVP
iii. Half MS media containing 0.5 and 1% copper acetate
iv. Simple half strength MS media (control)

Media was also supplemented with 3% sucrose and 0.44% gelrite as solidifying agent. The pH of media was adjusted to 5.7. The media was sonicated for 30 min, heated to dissolve gelrite, shaked well and 30 ml was poured in 100 ml conical flasks. The flasks were plugged and autoclaved at 121°C, 15 psi and for 20 min.

Under aseptic conditions, seeds were immersed in freshly prepared 0.1% mercuric chloride solution for 3-4 min for surface sterilization. Subsequently washed thrice with distilled water. Five seeds per flask were inoculated and kept in dark in growth room at 25°C. Seed germination parameters were recorded after 5 days and flasks were transferred at 16 hr photoperiod condition for growth of plants.

Seed Germination Parameters

After 5 days of seed inoculation seed germination data was collected.

Percentage germination frequency

Final percentage (%) germination was calculated after five days using formula;

$$Final\ percentage\ germination\ (\%) = \frac{number\ of\ germinated\ seeds}{total\ number\ of\ seeds\ planted} \times 100$$

Rate of germination (RG)

$$Rate\ of\ germination = \sum Ni/Di$$

Where;

N= daily increase in seedling number

D= number of days from seed placement

Mean period of final germination (MPFG)

$$Mean\ period\ of\ final\ germination = \sum (NiDi)/S$$

Where;

N= daily increase in seedling number

D= number of days from seed placement

S= total number of seeds germinated

Germination index (GI)

$$Germination\ index\ (GI) = \frac{\%germination\ against\ sediment\ sample}{\%germination\ in\ control}$$

Percentage inhibition (%)

$$Percentage\ inhibition\ (\%) = 1 - \{\frac{final\ \%germination\ in\ sample}{final\ \%germination\ in\ control}\} \times 100$$

Seedling growth parameters

Length of root and shoot

After 15 days of inoculation plantlets were separated from the medium. Root and shoot lengths of plantlets were measured using a ruler in centimeter (cm) and average was taken.

Fresh and dry weight

Five seedlings were taken from each flask after 15 days and their fresh weight was recorded using analytical weighing balance and average fresh weight was calculated. For dry weight plantlets were dried in oven at 70°C for 24 hr and then weighed.

3.5. Callus Induction

For callus induction, seeds of *T. foenum-graecum* L. were first inoculated on MS media. Plantlets were collected after 15 days.

Media preparation for callus initiation

Media was prepared for callus initiation containing 20ml MS media in 100ml flask supplemented with plant growth regulators 2 mg/L 1-Naphthaleneacetic acid (NAA), 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-Benzylaminopurine (BA). In addition to plant growth regulators (PGRs) CuONP, CuONP-PEG and CuONP-PVP were also added into the medium at concentrations of 0 (control), 2.5, 5 and 10 mg/L. The media was autoclaved prior to induction at 121°C for 20 min.

Callus initiation

Callus was initiated by cutting stem of a plantlet into pieces of 8 mm ±1 mm as well as leaf was also cut into size of 5 mm ±1 mm using a sterilized scalpel. After inoculation the flasks were transferred to growth room where temperature is maintained at 25°C with 16 hr of photoperiod.

The calli were generated over a time period of four weeks from both stem and leaf explant. After 30 days calli were separated from the media and fresh weight was calculated using analytical balance. The callus was then subjected to drying in a vacuum oven at 45°C for three days. After drying, callus was once again weighed and readings were noted.

3.6. Phytochemical Screening

Extract preparation

Fresh weight extracts of shoot and root were prepared in ethanol. The dried extracts were dissolved in DMSO at 20 mg/ml for antioxidative analysis.

Dry weight callus extracts were prepared in ethanol and then dissolved in DMSO at concentration of 100 mg/ml.

Chemicals and apparatus

Quercetin, Potassium acetate, Aluminium chloride, Folin–Ciocalteu reagent (FC), (Riedel-da Haen, Germany), Gallic acid, Methanol, 2, 2-diphenyl-1-picryhydrazyl (DPPH), Ascorbic acid, Sulfuric acid, Ammonium molybdate, Sodium phosphate, Phosphate buffer (Riedel-de-Haen, Germany), Potassium ferricyanide, Trichloroacetic acid (TCA), Ferric chloride and Dimethyl sulfoxide (DMSO) were purchased from Sigma (Sigma Aldrich, USA), Incubator IC83 (Yomato, Japan), 96 well plate (SPL life science, Korea), Microplate reader (Biotek USA, Elx 800) and Micropipette (Sartorius, France).

Determination of total flavonoids contents (TFC)

Total flavonoid contents of the test samples were identified according to the method previously described by Almajano *et al.* (2008).

20 µL test sample, standard and blank were taken in to 96 well microplate, followed by adding 10 µL of aluminum chloride solution and then 10 µL of potassium acetate (1 M). 160 µL of distilled was added to attain final volume of 200 µL. The plate was then incubated for 30 min at room temperature. The absorbance was measured by using microplate reader (Bioteck, USA) at 415 nm.

Determination of total phenolic contents (TPC)

Total phenolic contents of the test samples were determined by using method described by Astill *et al.* (2001).

Test sample 20 µL, positive control (gallic acid) and DMSO (negative control) were added to 96 well microplate, followed by addition of 90 µL of FC reagent and incubated for 5 min at room temperature. After incubation 90 µL of sodium carbonate was added into the plate. Readings were taken at 630 nm wavelength of microplate reader.

3.7. Antioxidant Activity

DPPH free radical scavenging assay

The free radical scavenging activity of the test samples against 2, 2-diphenyl-1-picrylhydrazyl reagent (DPPH) was determined according to the protocol described by Clarke *et al.* (2013).

In a 96 well microplate, 10 µL of test sample, standard (ascorbic acid) and blank were added. Then 190 µL of DPPH reagent was added and incubated for 1 hr at 37°C. Further readings were noted at 517 nm wavelength on microplate reader.

Total antioxidant capacity determination (TAC)

The total antioxidant capacity of the fresh weight extracts was determined by Clarke *et al.*(2013).

In appendorf tubes, 100 µL of test sample, standard and blank was taken and mixed by adding 900 µL of antioxidant reagent. The tubes were then incubated at 95°C for 90 min. After incubation the reaction mixture was cooled to room temperature and 200 µL of sample was transferred to microplate. Optical density was measured at 630 nm on microplate reader.

Total reducing power determination (TRP)

The reducing power of the test samples was investigated according to the protocol described by Jafri *et al.* (2014).

100 µL of test sample, positive control (ascorbic acid) and negative control (DMSO) were taken and then 200 µL of phosphate buffer was added, followed by addition of 250 µL of 1% potassium ferricyanide into the eppendorf tubes. The mixture was then incubated at 50°C for 20 min. After incubation, 200 µL of 10% TCA was added. The reaction mixture was centrifuged at 3000 rpm for 10 min. Supernatant layer, 150 µL was picked and poured into microplate well and then 50 µL of 0.1% ferric chloride was added. Readings were taken at 630 nm using microplate reader.

3.8. Statistical Analysis

All the experiments were performed in triplicate. To analyze the effect of CuONPs on seed germination, 15 seeds were treated with each concentration while 10 explants each of leaf and stem were inoculated on MS media for callus induction on each treatment. The results are presented as mean with standard deviation. The means were further analyzed by Analysis of Variance (ANOVA) and Least Significant Difference (LSD) at 0.05 probability.

Results

Objectives of this study, were to determine the effects of CuONPs on seed germination frequency of medicinally important plant *T. foenum-graecum* L. as well as on growth parameters including shoot and root elongation. CuONPs were used as elicitors to predict either they increase or decrease seed germination. Three different types of CuONPs were used to test the effect; CuONP, CuONP-PEG and CuONP-PVP at four different concentrations. Seed germination frequency data was collected after 5 days of inoculation and root and shoot length data was observed after 15 days. Later, phytochemical and antioxidant activities were performed.

4.1. Effect of CuONPs on seed germination

Seeds were germinated on four different concentrations of CuONP, CuONP-PEG and CuONP-PVP i.e.50, 100, 200 and 400 mg/L, PEG and PVP; 10, 20, 40 and 80 mg/L, two different salt concentrations of copper acetate (Cu-acetate); 0.5 and 1% and control 0 (simple MS medium). Seed germination frequency was 100% in control, CuONP-PEG, PEG, PVP and Cu-acetate at all concentrations. Whereas it was 90 and 80% in CuONP at concentrations 50, 100 and 400 mg/L respectively and 100% at 200 mg/L. Seed germination frequency of CuONP-PVP was 90% at 50 and 400mg/L while 100% at 100 and 200 mg/L (Table 4.1). Seed germination frequency results of seeds inoculated on PEG and PVP were also 100% at all concentrations.

Mean period of final germination (MPFG), of seeds was also calculated and it was found to be 0.5 in all treatments at all concentrations. Whereas percent inhibition was almost 0 in almost all cases except seeds inoculated on CuONP (50, 100 and 400 mg/L), CuONP-PVP (50 and 400 mg/L) and PVP (10 mg/L).

Table 4.1: Seed germination frequency of *T. foenum-graecum* L.

Treatment	Concentration	FG (%)	RG	MPFG	GI	PI
CuONP (mg/L)	50	90[b]	1.8	0.5	0.9	10
	100	80[c]	1.6	0.5	0.8	20
	200	100[a]	2.0	0.5	1.0	0
	400	80[c]	1.6	0.5	0.8	20
CuONP-PEG (mg/L)	50	100[a]	2.0	0.5	1.0	0
	100	100[a]	2.0	0.5	1.0	0
	200	100[a]	2.0	0.5	1.0	0
	400	100[a]	2.0	0.5	1.0	0
CuONP-PVP (mg/L)	50	90[b]	1.8	0.5	0.9	10
	100	100[a]	2.0	0.5	1.0	0
	200	100[a]	2.0	0.5	1.0	0
	400	90[b]	1.8	0.5	0.9	10
PEG (mg/L)	10	100[a]	2.0	0.5	1.0	0
	20	100[a]	2.0	0.5	1.0	0
	40	100[a]	2.0	0.5	1.0	0
	80	100[a]	2.0	0.5	1.0	0
PVP (mg/L)	10	90[b]	1.8	0.5	0.9	10
	20	100[a]	2.0	0.5	1.0	0
	40	100[a]	2.0	0.5	1.0	0
	80	100[a]	2.0	0.5	1.0	0
Cu-acetate (%)	0.5	100[a]	2.0	0.5	1.0	0
	1	100[a]	2.0	0.5	1.0	0
Control	0	100[a]	2.0	0.5	1.0	0

*Same letters are showing similar values otherwise differ significantly at $P<0.05$

4.2. Average Shoot and Root Length

Average root and shoot length of plantlets were recorded after 15 days of inoculation on all the concentrations. In control average shoot length of plantlet was 7.2 cm and root length 4.1 cm (Table 4.2). Maximum shoot length was found at CuONP, 50 mg/L i.e. 7.7 cm and gradual decrease as the concentration of nanoparticles increased. Data has shown the significant decrease in shoot length treated with Cu-acetate at 0.5%, 4.9 cm and 1%, 4.3 cm as well as significant decrease in root length i.e. 2.3 cm at 0.5% and 1.6 cm at 1% (figure 4.1). Whereas maximum root length was 6.8 cm at 50 mg/L of CuONP-PEG and trend gradually decreased as concentration of nanoparticles increased (figure 4.2) and same results were found for CuONP-PVP (figure 4.3). Maximum shoot length of PEG and PVP treated plantlets was found at 10 mg/L i.e. 6.2 and 6.6 cm respectively and shoot length reduced as the concentration of PEG and PVP in the media increased. However, reduction in root length was also observed in PEG and PVP treated plantlets with increasing concentration but maximum root length was found at 10 mg/L i.e. 3.4 and 3.7 cm (Table 4.2).

Figure 4.1: Effect of CuONP and Cu-acetate on root and shoot length of *T. foenum-graecum* L. at different concentrations

Figure 4.2: Effect of CuONP-PEG and PEG on root and shoot length of *T. foenum-graecum* L. at different concentrations

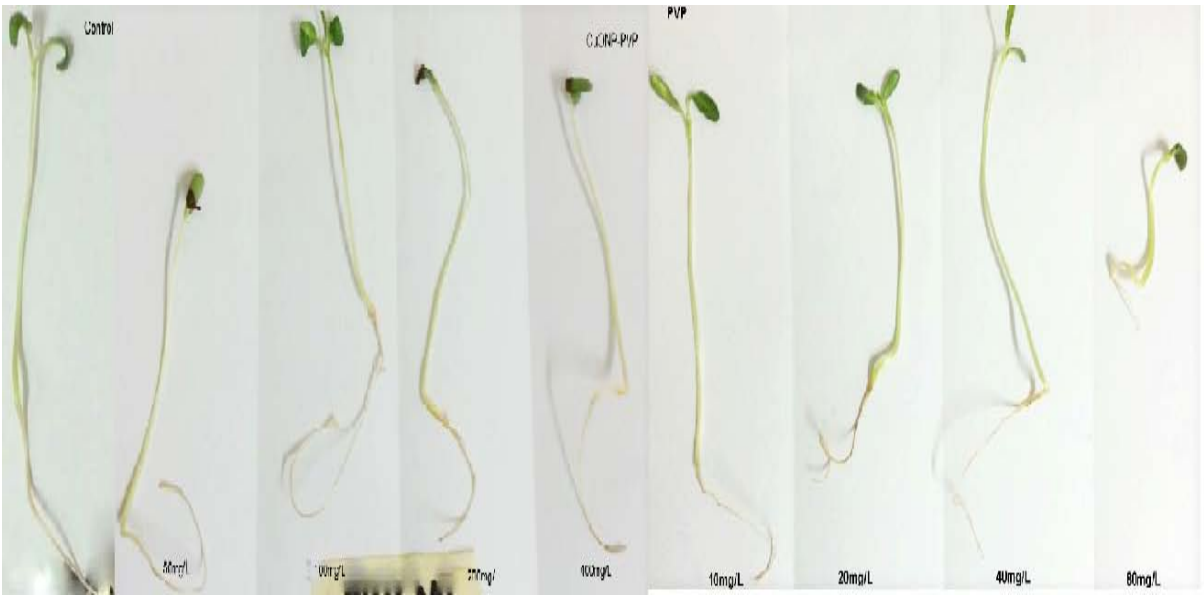

Figure 4.3: Effect of CuONP-PVP and PVP on root and shoot length of *T. foenum-graecum* L. at different concentrations

Table 4.2: Average shoot and root length of *T. foenum-graecum* L. plantlets

Treatment	Concentration	Average shoot length (cm)	Average root length (cm)
CuONP (mg/L)	50	7.7^{a}	6.6^{a}
	100	6.5^{c}	5.1^{c}
	200	6.4^{c}	4.4^{d}
	400	6.3^{c}	4.3^{d}
CuONP-PEG (mg/L)	50	7.0^{b}	6.8^{a}
	100	6.9^{b}	6.1^{b}
	200	6.8^{bc}	6.0^{b}
	400	5.4^{bc}	3.0^{f}
CuONP-PVP (mg/L)	50	7.0^{b}	5.9^{b}
	100	6.7^{bc}	5.8^{b}
	200	6.6^{bc}	4.6^{d}
	400	6.4^{c}	3.4^{ef}
PEG (mg/L)	10	6.2^{cd}	3.4^{ef}
	20	6.1^{cd}	3.3^{ef}
	40	6.0^{cd}	2.2^{g}
	80	5.9^{d}	2.1^{g}
PVP (mg/L)	10	6.6^{bc}	3.7^{e}
	20	6.5^{c}	3.6^{e}
	40	6.2^{cd}	3.6^{e}
	80	5.3^{e}	3.5^{e}
Cu-acetate (%)	0.5	4.9^{f}	2.3^{g}
	1	4.3^{g}	1.6^{h}
Control	0	7.2^{b}	6.6^{a}

*Same letters are showing similar values otherwise differ significantly at $P<0.05$

4.3. Average Fresh and Dry Weight

Average fresh weight of whole plantlet was calculated after 15 days of inoculation and dry weight was calculated after overnight period of drying in vacuum oven. Average fresh weight of plantlets was recorded maximum at CuONP 50 mg/L, 0.47 g as well as maximum dry weight, 0.05 g (Table 4.3). Whereas plantlets treated with capped CuONPs (CuONP-PEG and CuONP-PVP) showed reduced average fresh and dry weight as compared to uncapped CuOONPs. Average fresh weight of plantlets grown on PEG and PVP was maximum at 10 mg/L i.e. 0.28 and 0.32 g and it reduced with increase in concentration.

Table 4.3: Average fresh and dry weight of *T. foenum-graecum* L. plantlets

Treatment	Concentration	Average fresh weight (g)	Average dry weight (g)
CuONP (mg/L)	50	0.47[a]	0.05[a]
	100	0.42[ab]	0.03[b]
	200	0.13[c]	0.01[c]
	400	0.11[c]	0.01[c]
CuONP-PEG (mg/L)	50	0.34[b]	0.04[ab]
	100	0.32[b]	0.03[b]
	200	0.20[bc]	0.02[bc]
	400	0.31[b]	0.03[b]
CuONP-PVP (mg/L)	50	0.43[ab]	0.04[ab]
	100	0.31[b]	0.03[b]
	200	0.32[b]	0.03[b]
	400	0.31[b]	0.02[bc]
PEG (mg/L)	10	0.28[bc]	0.04[ab]
	20	0.26[bc]	0.03[b]
	40	0.24[bc]	0.03[b]
	80	0.22[bc]	0.03[b]
PVP (mg/L)	10	0.32[b]	0.04[ab]
	20	0.29[bc]	0.04[ab]
	40	0.27[bc]	0.03[b]
	80	0.13[c]	0.01[c]
Cu-acetate (%)	0.5	0.22[bc]	0.02[bc]
	1	0.19[c]	0.01[c]
Control	0	0.28[bc]	0.04[ab]

*Same letters are showing similar values otherwise differ significantly at $P<0.05$

4.4. Phytochemical Screening of Plantlets

4.4.1. *Total flavonoid contents*

According to the results obtained after performing total flavonoid activity, the trend has shown maximum flavonoids in Cu-acetate treated plantlets at 1% (shoot extracts; 0.1 µg QE/mg FW and root extracts; 0.3 µg QE/mg FW) as compared to control and CuONPs treatment (figure 4.4). Whereas in plantlets treated with PEG have shown significantly higher flavonoid content in comparison with CuONP-PEG and control (figure 4.5). In addition to these, CuONP-PVP and control have shown much lower contents of flavonoid and phenolic as compared to PVP treatment (shoots; 0.3 µg QE/mg FW and roots; 1.7 µg QE/mg FW) (figure 4.6).

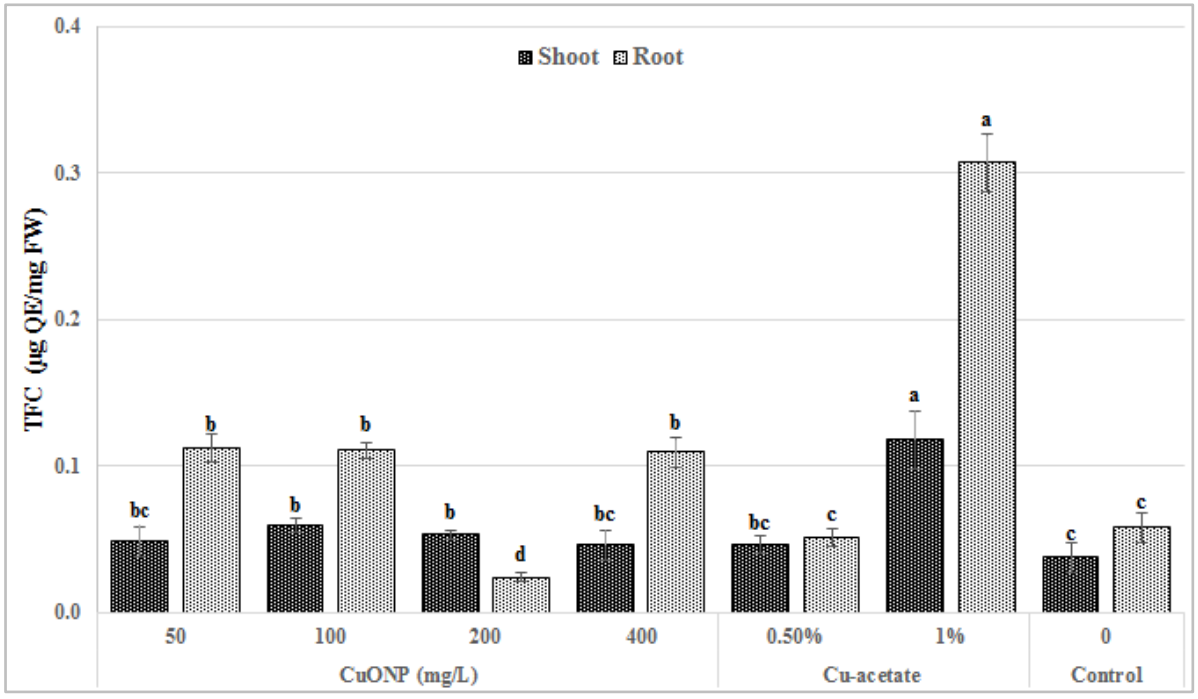

Figure 4.4: Effect of CuONP and Cu-acetate on total flavonoid content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

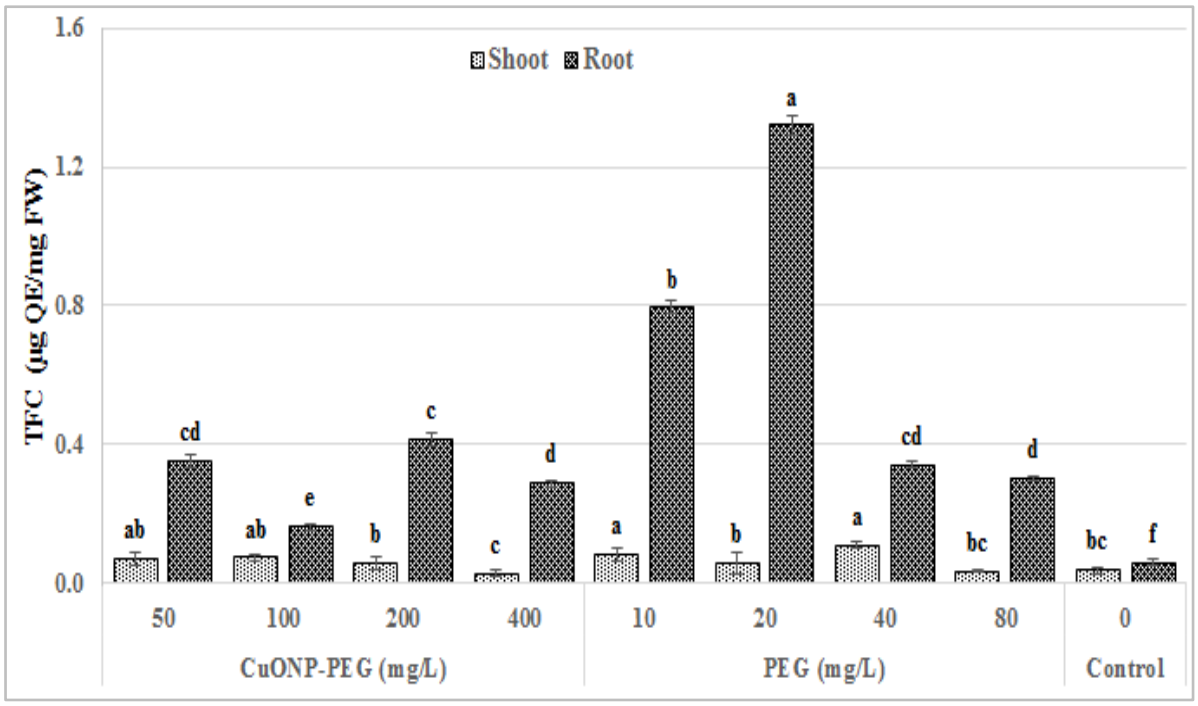

Figure 4.5: Effect of CuONP-PEG and PEG on total flavonoid content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

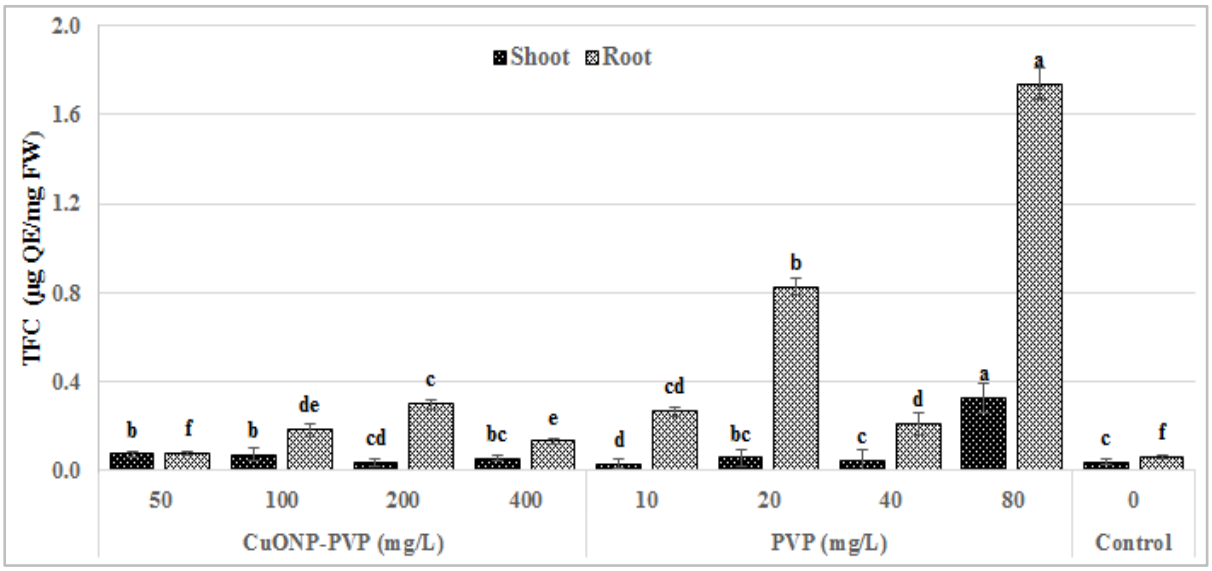

Figure 4.6: Effect of CuONP-PVP and PVP on total flavonoid content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.4.2. *Total phenolic contents*

Total phenolic contents were determined in the plant extracts treated with CuONPs and results showed that maximum total phenolic contents were accumulated in plantlets in Cu-acetate treated plantlets at 1%, 0.3 µg GAE/mg FW both in root and shoot extracts. Root extracts showed maximum phenolic content accumulation treated with CuONP at 50 mg/L, 0.3 µg GAE/mg FW and it was decreased as concentration increased (figure 4.7).

Whereas root extracts treated with PEG showed maximum phenolic content at 40 mg/L (2.2 µg GAE/mg FW). CuONP-PEG treated plantlets also had more phenolic content as compared to control at 100 mg/L (0.2 µg GAE/ mg FW in shoot) and at 200 mg/L (1.6 µg GAE/mg FW in root) (figure 4.8). PVP treated plantlets accumulated maximum phenolic contents at 80 mg/L (3.4 µg GAE/mg FW) as compared to plantlets treated with CuONP-PVP (figure 4.9).

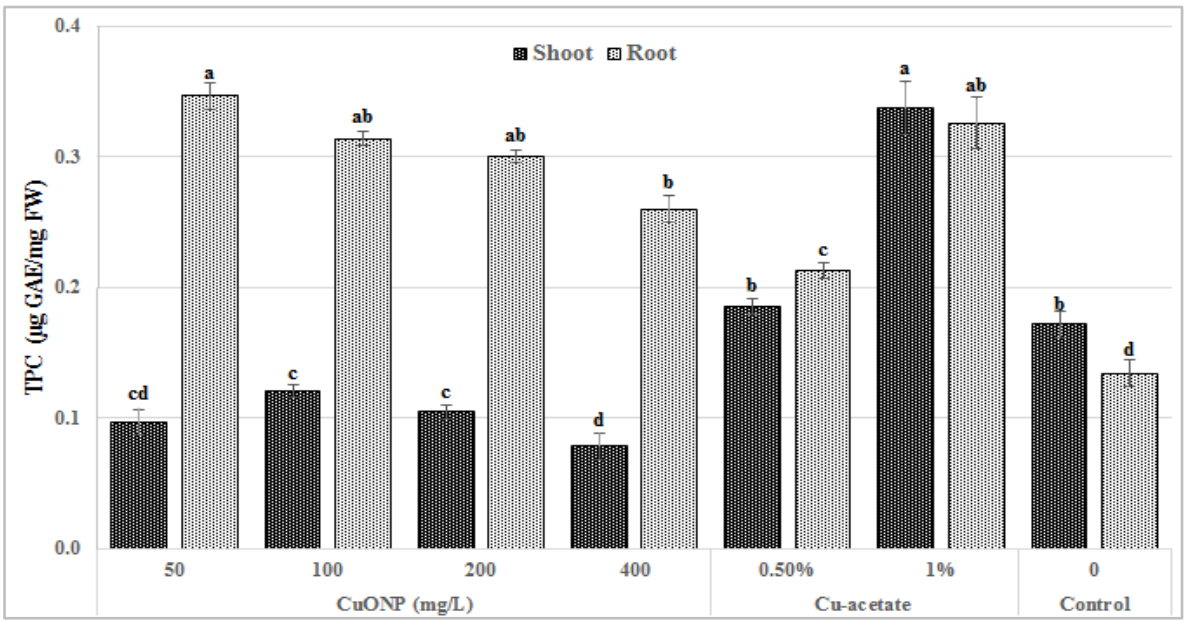

Figure 4.7: Effect of CuONP and Cu-acetate on total phenolic content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

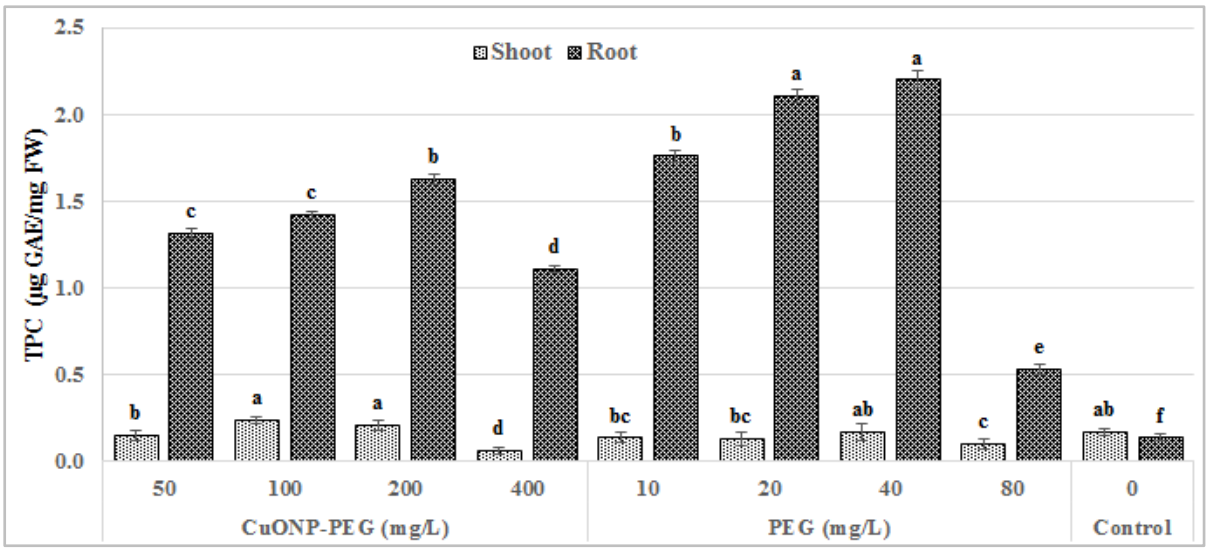

Figure 4.8: Effect of CuONP-PEG and PEG on total phenolic content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

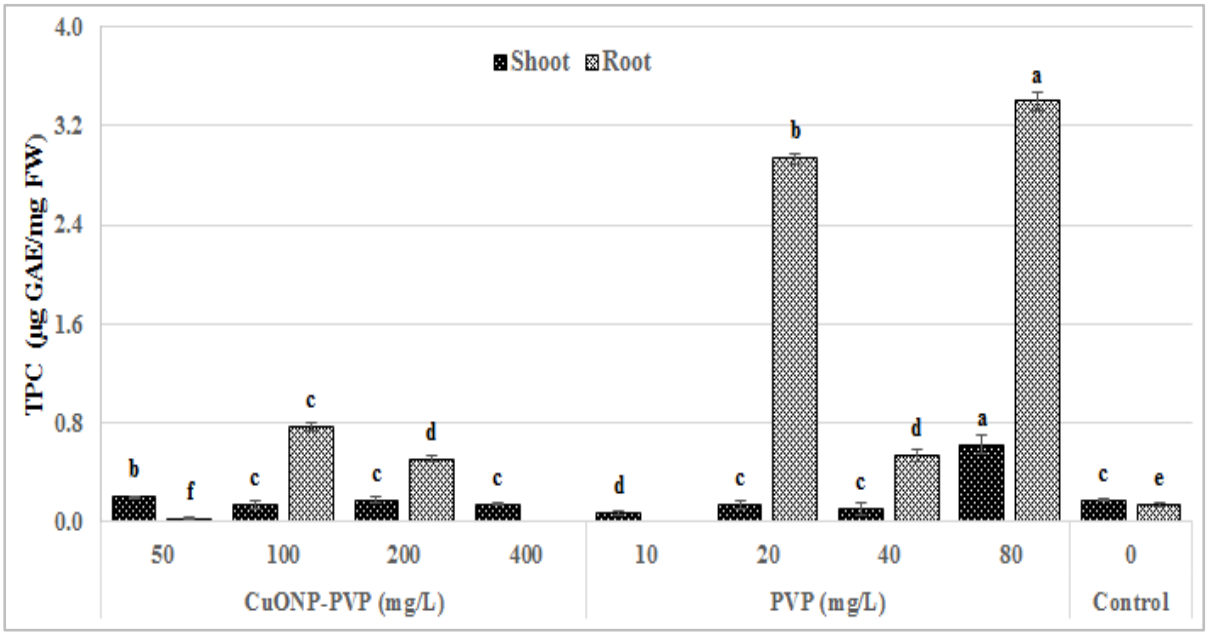

Figure 4.9: Effect of CuONP-PVP and PVP on total phenolic content derived from shoot and root extracts of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.5. Antioxidant Activity of Plantlets

4.5.1. *DPPH radical scavenging activity*

DPPH activity was determined in plantlets treated with nanoparticles, PEG, PVP and compared with control. The results have shown that Cu-acetate at concentration of 1% has maximum radical scavenging activity in both root (71%) and shoot (59%) extracts as compared to CuONP and control (figure 4.10). But in case of CuONP-PEG, PEG and control, CuONP-PEG has shown maximum scavenging activity at 50mg/L, (64%) and trend gradually decreased as concentration of nanoparticles is increased (figure 4.11). The free radical scavenging activity of CuONP-PVP was found to be maximum in root extracts at concentration 100mg/L, (70%) whereas seedlings grown on PVP have also shown increased DPPH radical scavenging activity as compared to control (figure 4.12).

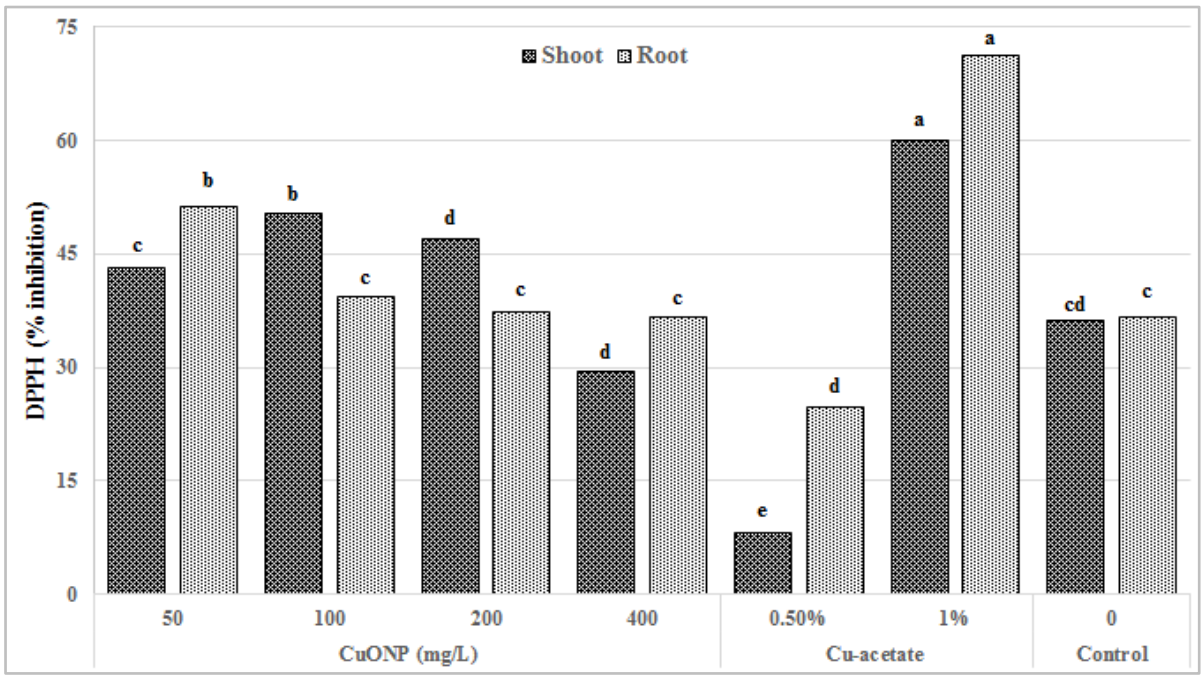

Figure 4.10: DPPH radical scavenging activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP and Cu-acetate. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

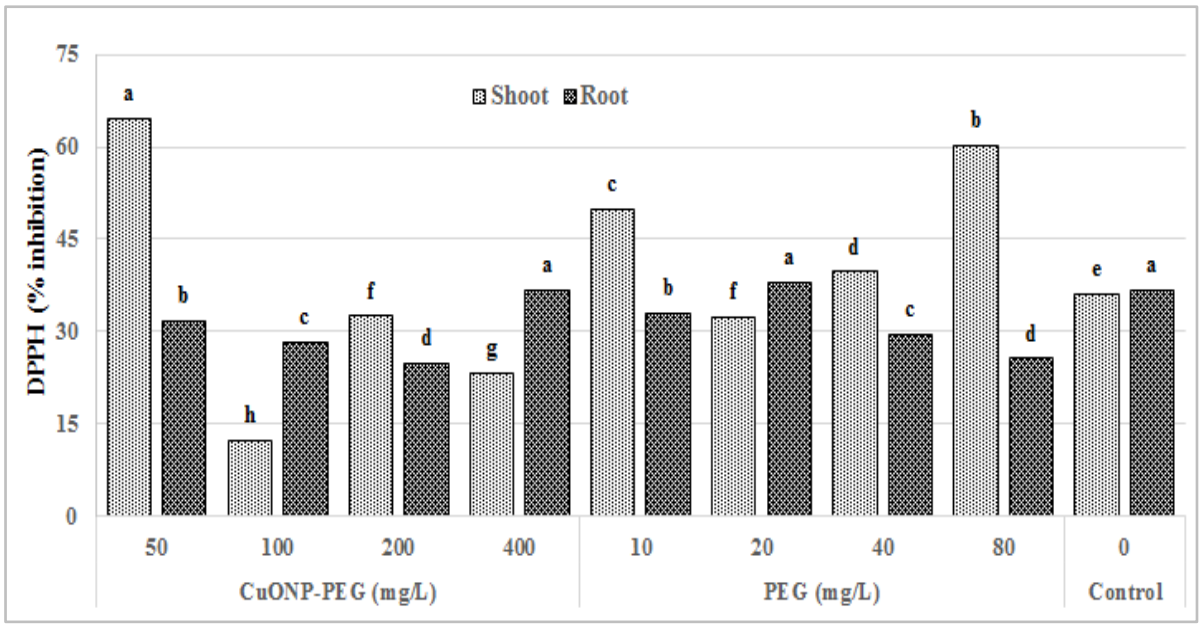

Figure 4.11: DPPH radical scavenging activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PEG and PEG. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

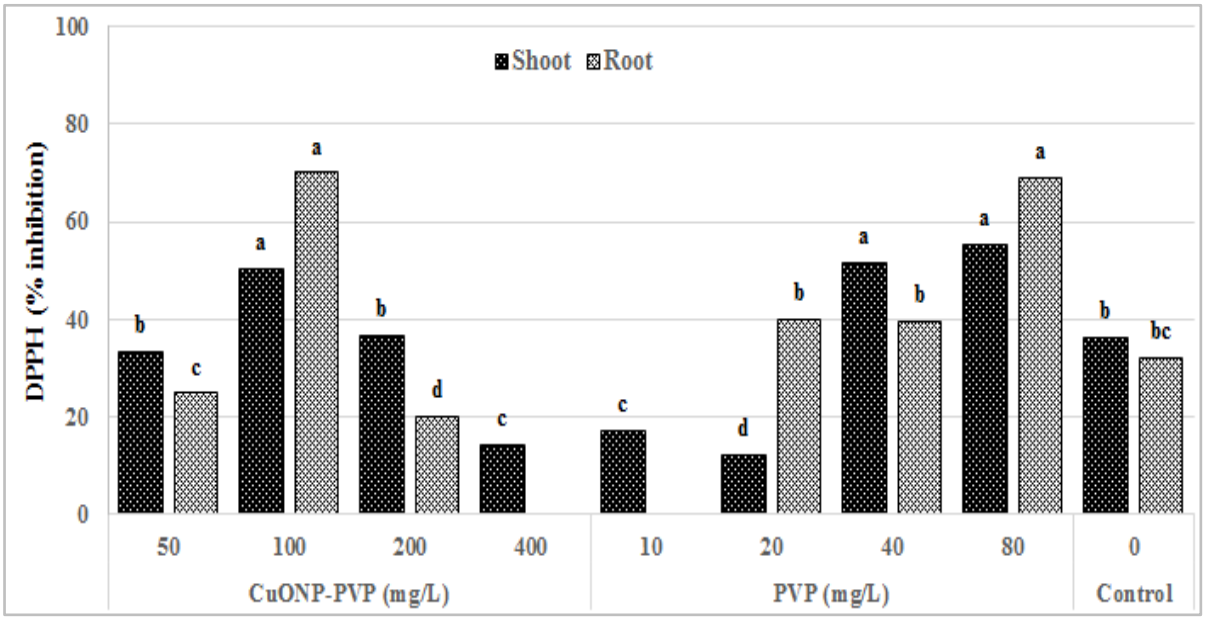

Figure 4.12: DPPH radical scavenging activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PVP and PVP. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.5.2. *Total antioxidant capacity*

Total antioxidant assay results have shown maximum activity in plantlets tested on 1% Cu-acetate (2.2 µg AAE/mg FW) as compared to CuONP derived shoot extracts but roots extract data revealed maximum antioxidant activity in CuONP at 50 mg/L, (1.5 µg AAE/ mg FW) but with increasing concentration of nanoparticles trend tend to decrease (figure 4.13). CuONP-PEG tested root (5.7 µg AAE/mg FW) and shoot (1.0 µg AAE/mg FW) extracts have shown maximum antioxidant activity as compared to PEG and control (figure 4.14). Maximum TAC activity was observed in PVP treated plantlets as compared to CuONP-PVP and control (figure 4.15).

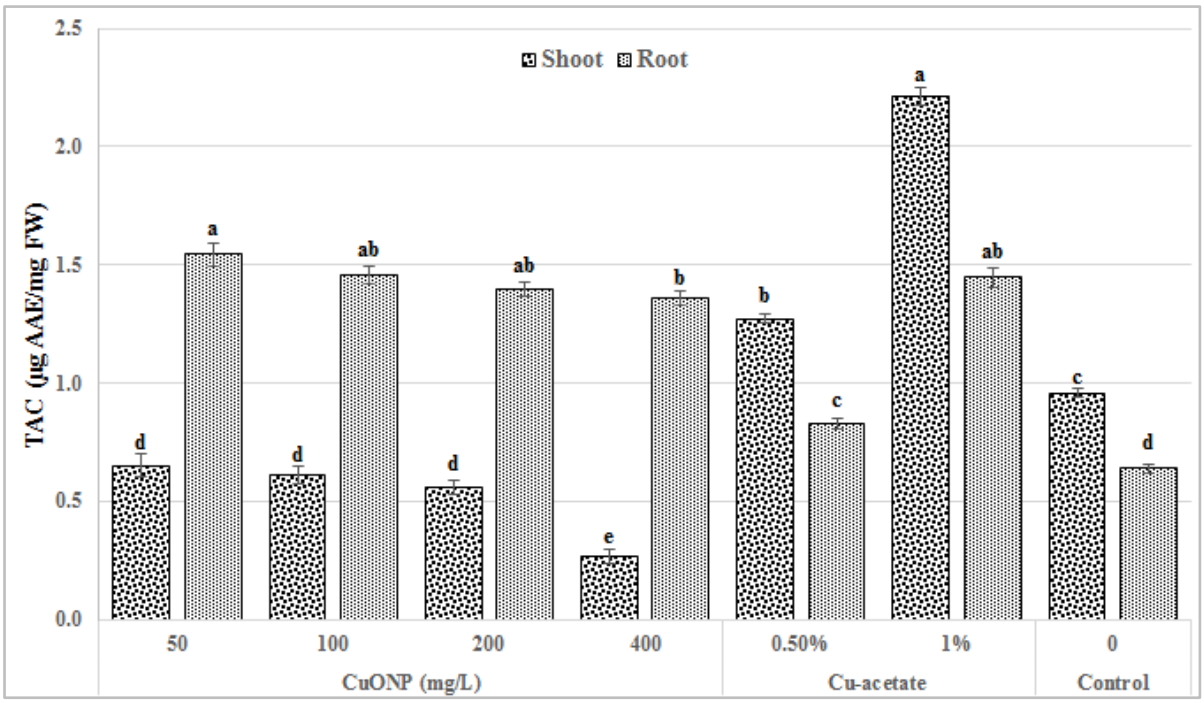

Figure 4.13: Total antioxidant activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP and Cu-acetate. Bars sharing the same alphabetical letters are similar otherwise differ significantly at $P<0.05$

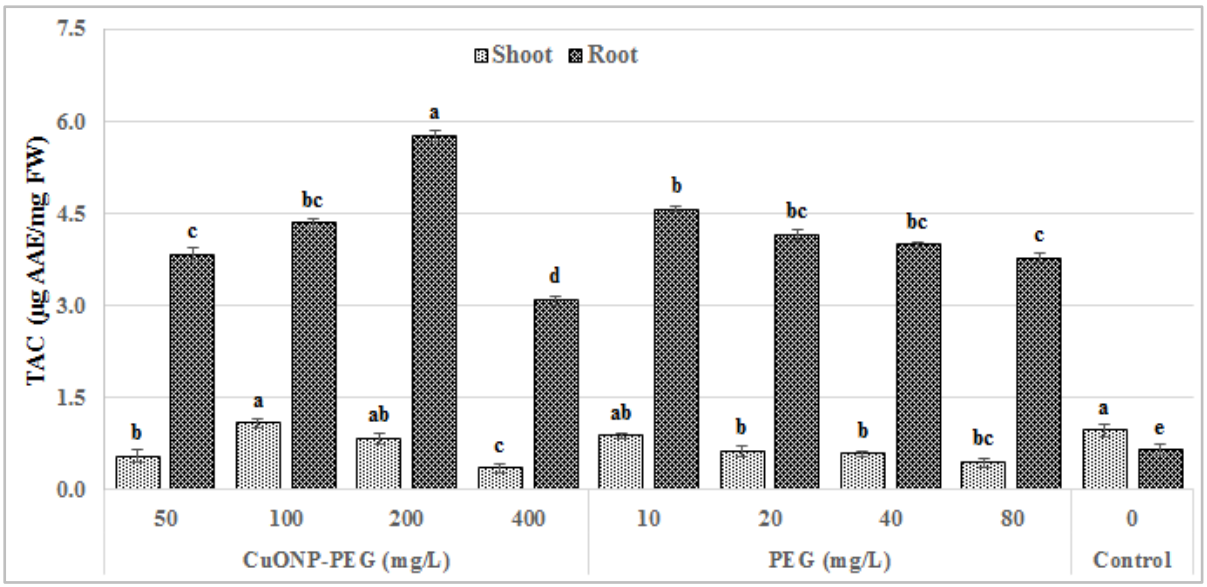

Figure 4.14: Total antioxidant activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PEG and PEG. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

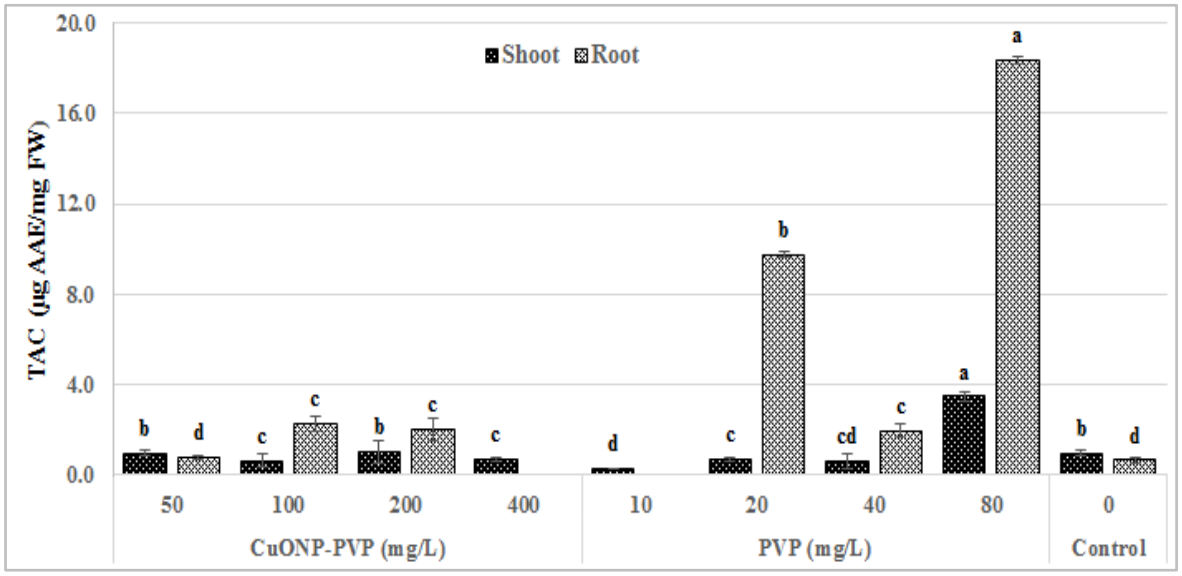

Figure 4.15: Total antioxidant activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PVP and PVP. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.5.3. *Total reducing power*

According to the results of total reducing power assay, maximum activity was found in shoot extracts of Cu-acetate (1.9 µg AAE/mg FW) and maximum reducing power of root shown by CuONP (2.1 µg AAE/mg FW) as compared to control (figure 4.16). CuONP-PEG have shown maximum total reducing power as compared to PEG and control (figure 4.17). Whereas, in case of CuONP-PVP, PVP and control, PVP have increased reducing power activity as compared to others (figure 4.18).

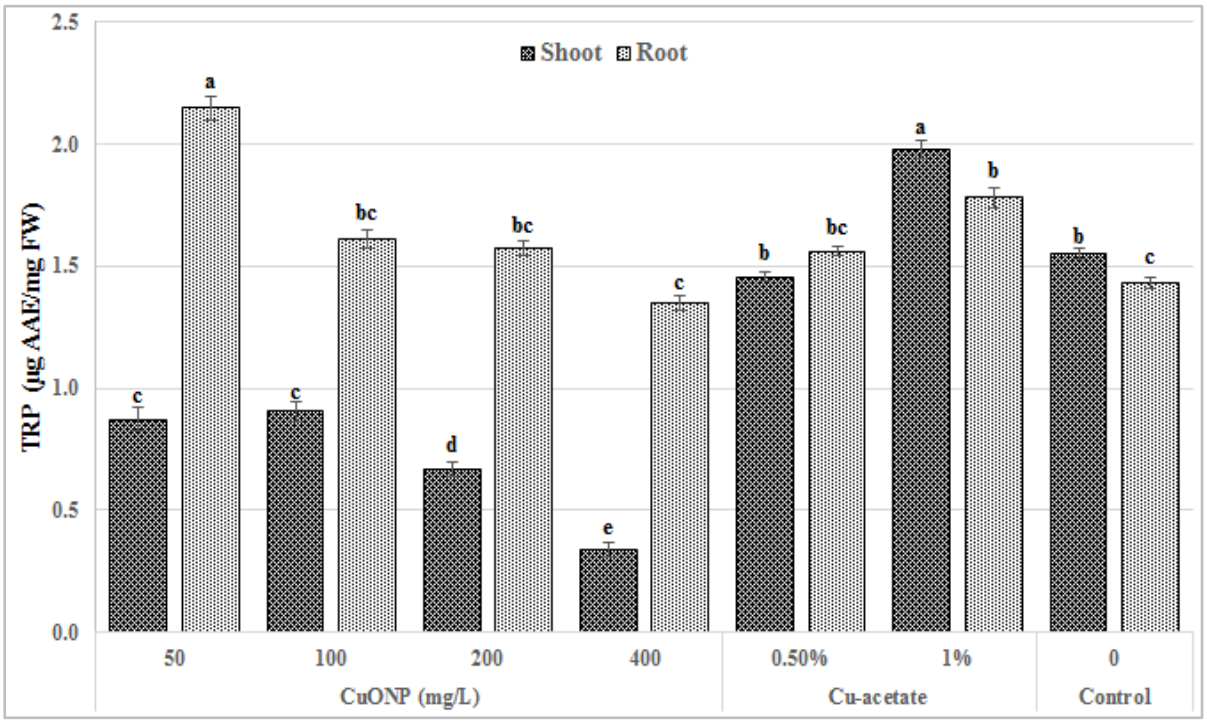

Figure 4.16: Total reducing power activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP and Cu-acetate. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

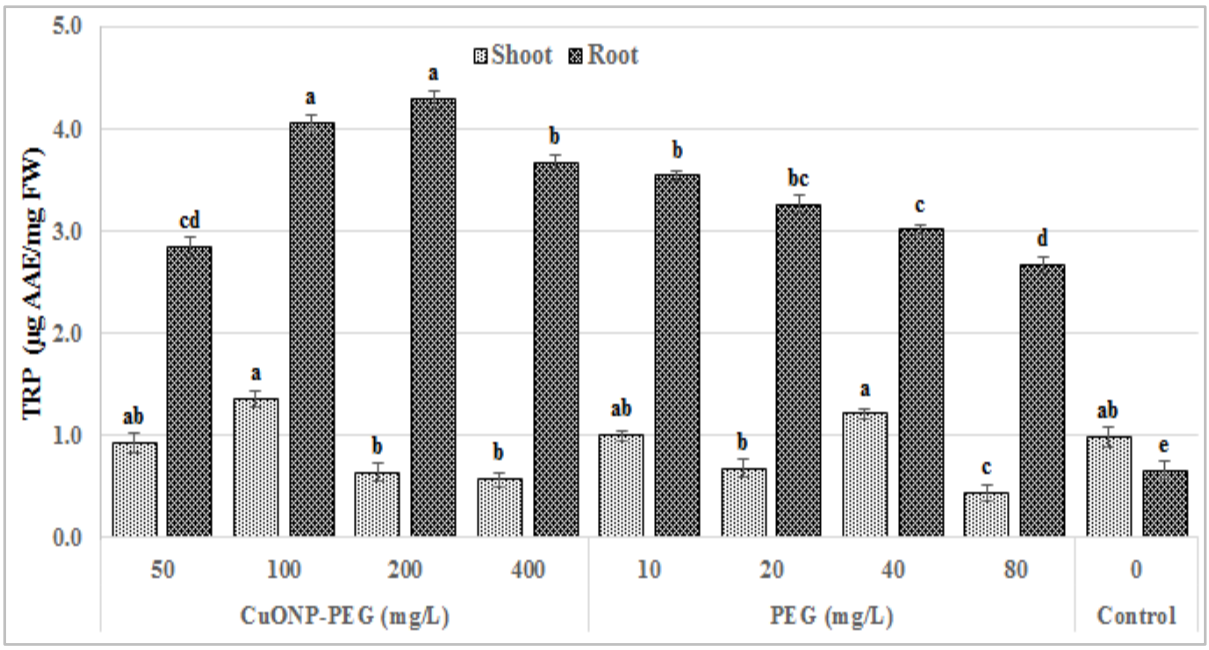

Figure 4.17: Total reducing power activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PEG and PEG. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

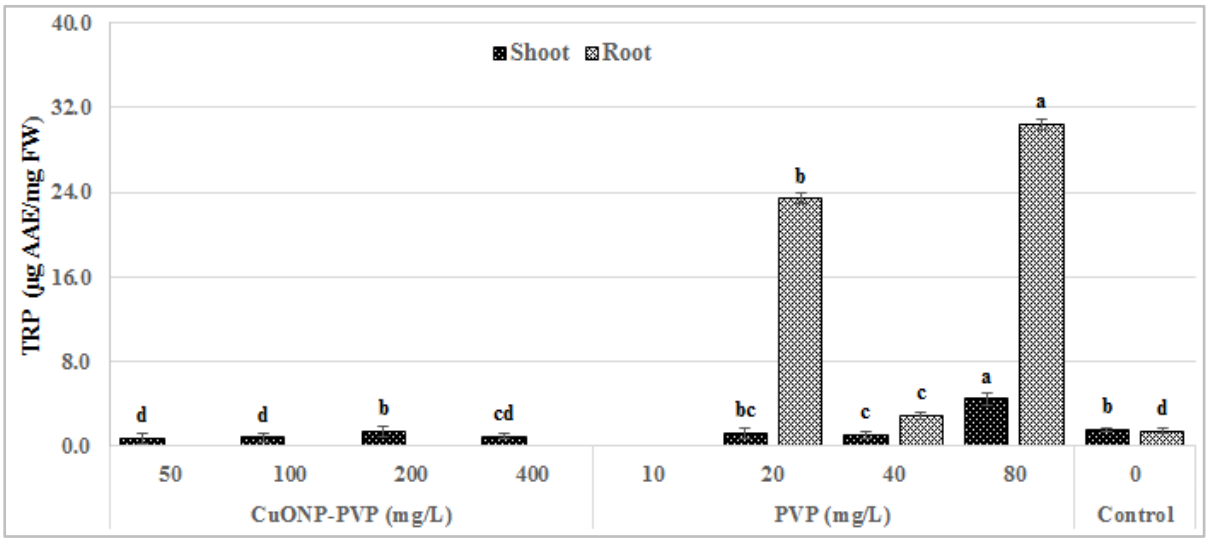

Figure 4.18: Total reducing power activity of root and shoot extracts of *T. foenum-graecum* L. against different concentrations of CuONP-PVP and PVP. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.6. Callus Induction

Effect of CuONP, CuONP-PEG and CuONP-PVP was determined at three concentrations; 2.5, 5 and 10 mg/L on callus induction from shoot and leaf explant. The experiment was conducted over a time period of 30 days. After 30 days, callus was collected (figure 4.19 and 4.20) and then average fresh weight and dry weight were calculated followed by determination of biochemical profiling.

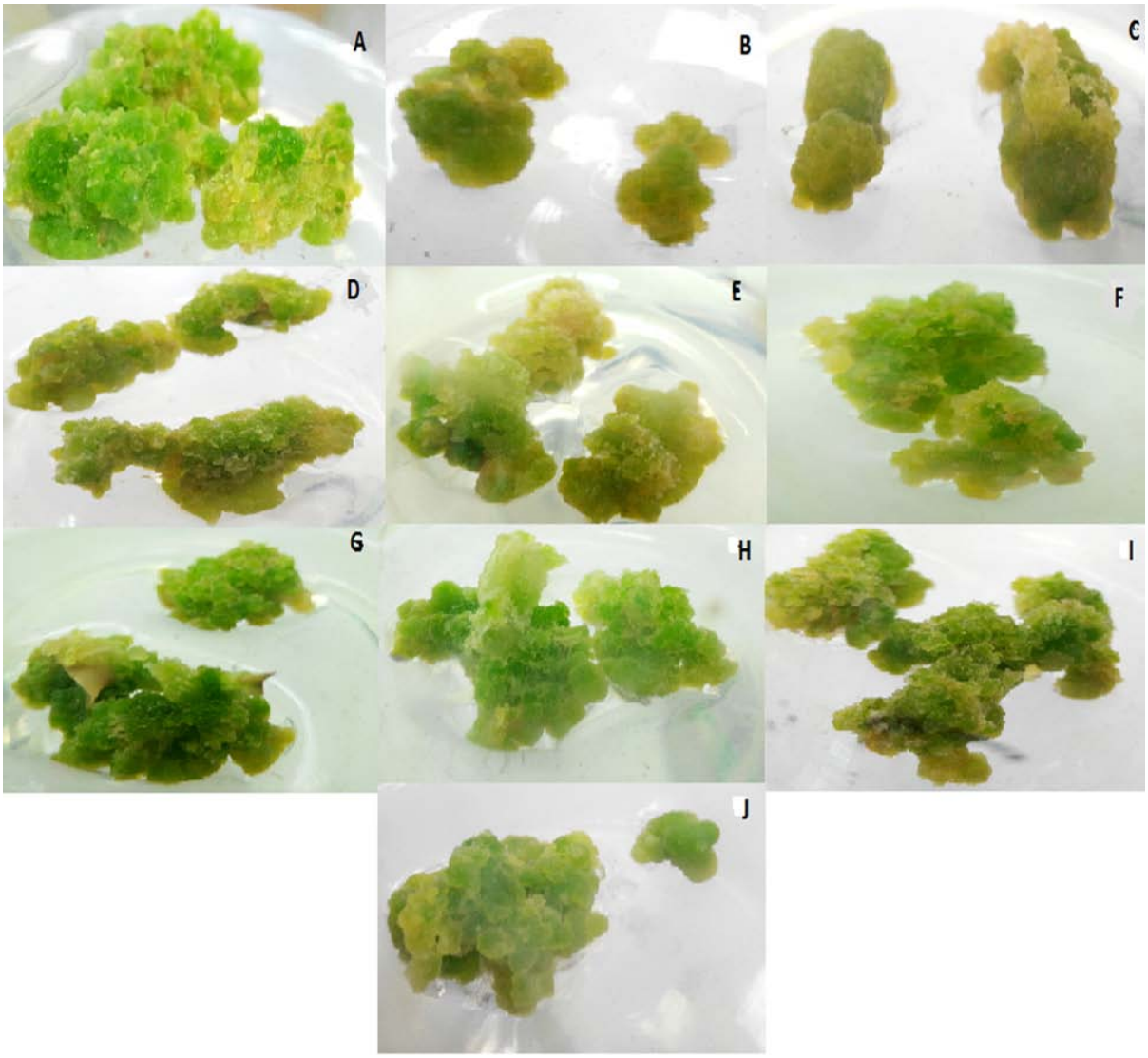

Figure 4.19: Callus induction from stem explant of *T. foenum-graecum* L at different concentrations of CuONP, CuONP-PEG and CuONP-PVP; (A) control, (B-D) CuONP: 2.5, 5, 10 mg/L, (E-G) CuONP-PEG: 2.5, 5, 10 mg/L, (H-J) CuONP-PVP: 2.5, 5, 10 mg/L

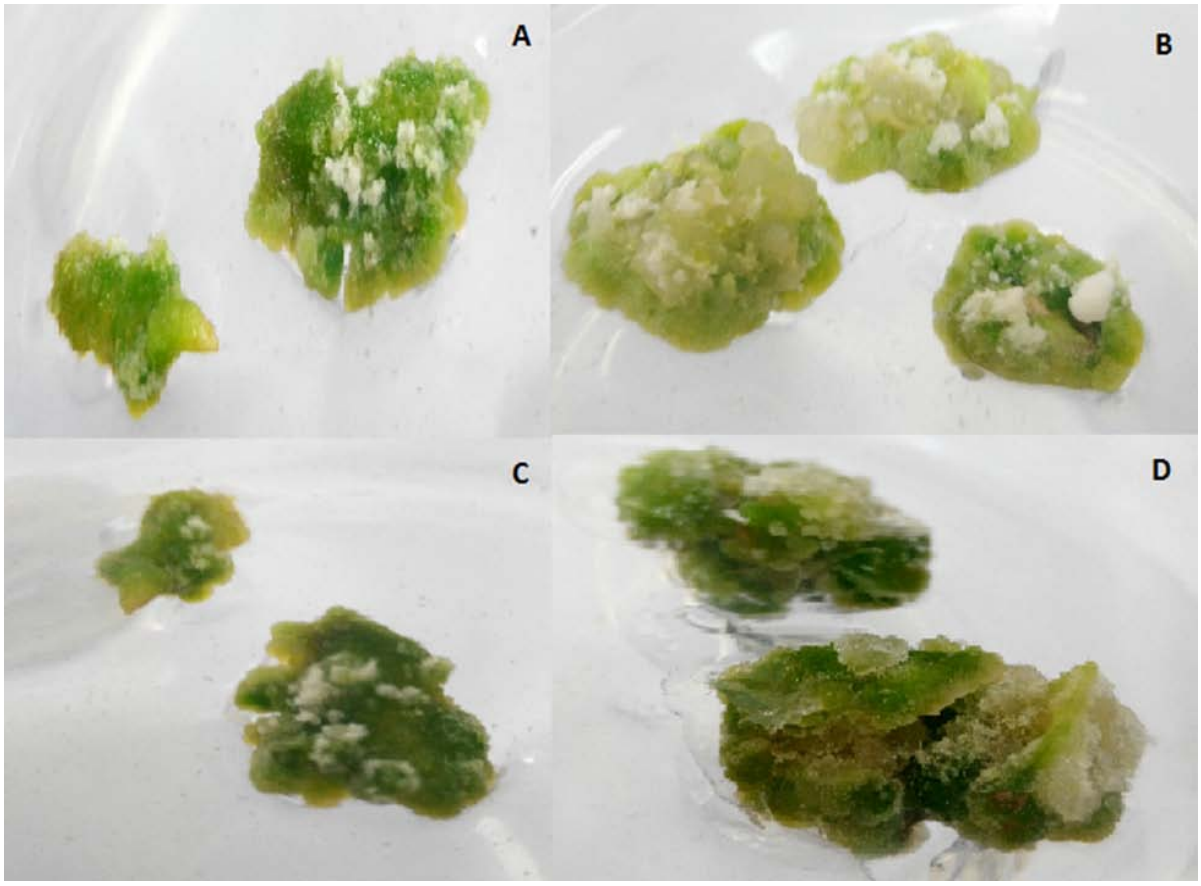

Figure 4.20: Callus induction from leaf explant of *T. foenum-graecum* L on CuONPs; (A) control, (B-D) CuONP: 2.5, 5, 10 mg/L

4.6.1. Average Fresh and Dry Weight of Explant

Average fresh weight and dry weight were calculated for both stem and leaf explants. According to the results obtained average fresh weight of control both in stem, 1.65 g and leaf, 1.33 g explants was higher as compared to the explants treated with different concentrations of nanoparticles. Whereas dry weight was also found to be higher in control i.e. 0.10 g for stem explant and 0.09 g for leaf explant (Table 4.4).

Table 4.4: Average fresh and dry weight of *T. foenum-graecum* L. callus treated with CuONPs

Treatment	Concentration (mg/L)	Average fresh weight/explant (g)		Average dry weight/explant (g)	
		Stem	Leaf	Stem	Leaf
CuONP	2.5	0.67^{c}	0.85^{ab}	0.05^{b}	0.07^{ab}
	5	0.25^{c}	0.12^{c}	0.02^{c}	0.01^{c}
	10	1.33^{ab}	0.13^{bc}	0.07^{ab}	0.01^{c}
CuONP-PEG	2.5	1.25^{b}	0.11^{c}	0.08^{ab}	0.01^{c}
	5	0.99^{bc}	0.33^{b}	0.05^{b}	0.02^{b}
	10	0.82^{bc}	0.21^{b}	0.06^{b}	0.02^{b}
CuONP-PVP	2.5	1.13^{b}	1.24^{a}	0.07^{ab}	0.08^{a}
	5	1.31^{ab}	$0.14b^{c}$	0.08^{ab}	0.02^{b}
	10	1.42^{a}	0.12^{c}	0.09^{a}	0.01^{c}
Control	0	1.65^{a}	1.33^{a}	0.10^{a}	0.09^{a}

*Same letters are showing similar values otherwise differ significantly at $P<0.05$

4.7. Phytochemical Screening of Explants

4.7.1. *Total flavonoid contents*

According to the data, total flavonoid contents were found to be maximum in shoot explants treated with CuONP-PVP at all three concentrations 2.5, 5 and 10 mg/L, 3.51, 3.83 and 3.88 µg QE/mg DW respectively (figure 4.21). CuONP-PVP treated leaf explants also showed maximum flavonoid content accumulation at 10 mg/L (3.46 µg QE/mg DW) as compared to CuONP, CuONP-PEG and control (figure 4.22).

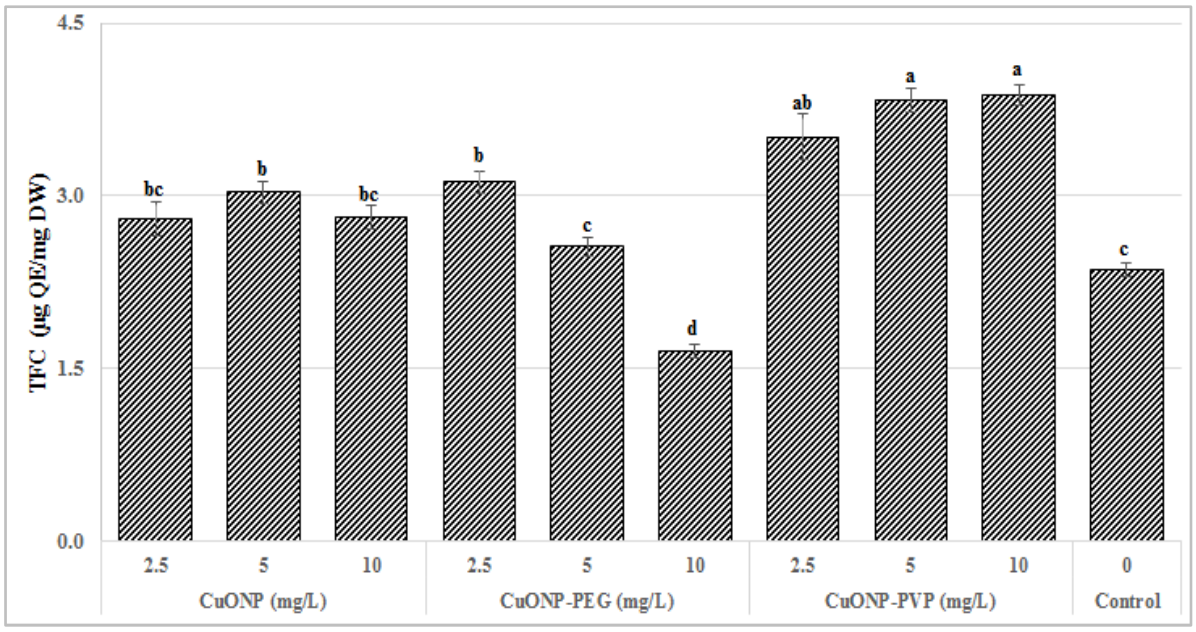

Figure 4.21: Effect of CuONP, CuONP-PEG and CuONP-PVP on total flavonoid content derived from shoot explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

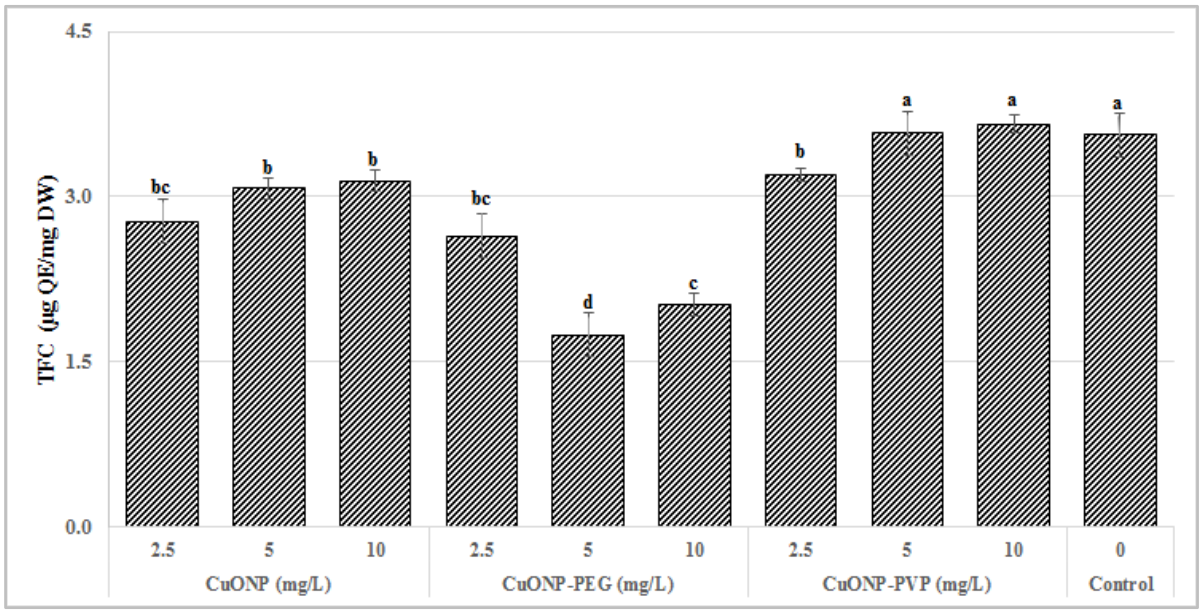

Figure 4.22: Effect of CuONP, CuONP-PEG and CuONP-PVP on total flavonoid content derived from leaf explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.7.2. *Total phenolic contents*

According to the results TPC, maximum phenolic contents were accumulated in explants of shoot treated with CuONP at 5 mg/L (4.0 µg GAE/mg DW) and CuONP-PEG at 2.5 mg/L (3.9 µg GAE/mg DW). Whereas shoot explants grown on CuONP-PVP showed maximum phenolic contents at all three concentrations 2.5, 5 and 10 mg/L (3.9, 4.0 and 3.9 µg GAE/mg DW) (figure 4.23). Results of leaf explants treated with 2.5 mg/L of CuONP-PEG showed more phenolic contents accumulation 3.5 µg GAE/mg DW i.e. equivalent to control (figure 4.24).

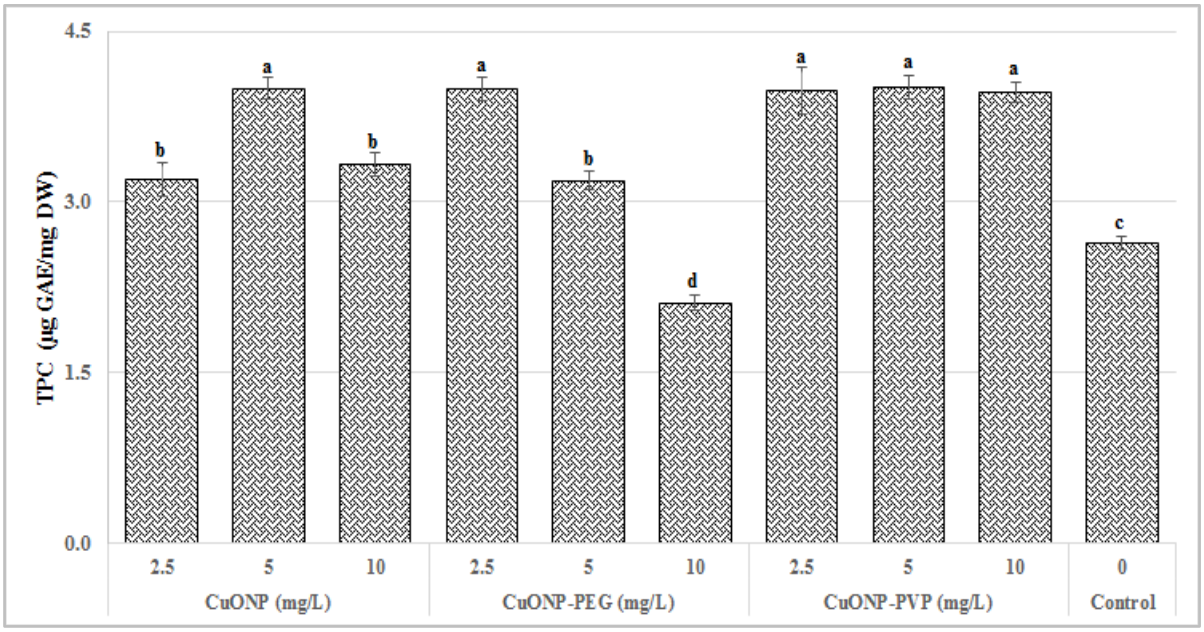

Figure 4.23: Effect of CuONP, CuONP-PEG and CuONP-PVP on total phenolic content derived from shoot explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

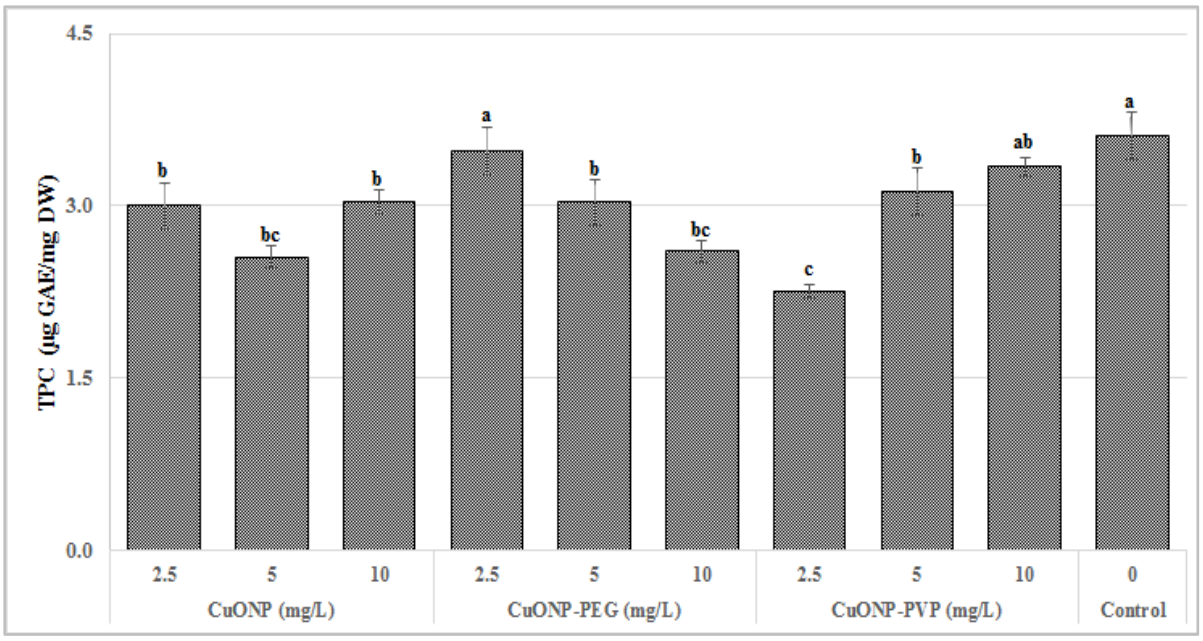

Figure 4.24: Effect of CuONP, CuONP-PEG and CuONP-PVP on total phenolic content derived from leaf explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.8. Antioxidant Activity of Explants

4.8.1. *DPPH radical scavenging activity*

DPPH radical scavenging activity was found maximum in shoot explants treated with CuONP-PEG 5 mg/L (56%), CuONP-PVP 5 and 10 mg/L (55 and 56%) but decreased activity was observed in explants grown on CuONP (figure 4.25). Whereas maximum scavenging activity was found in leaf explants treated with CuONP-PEG at 5 mg/L (72%) compared to others (figure 4.26).

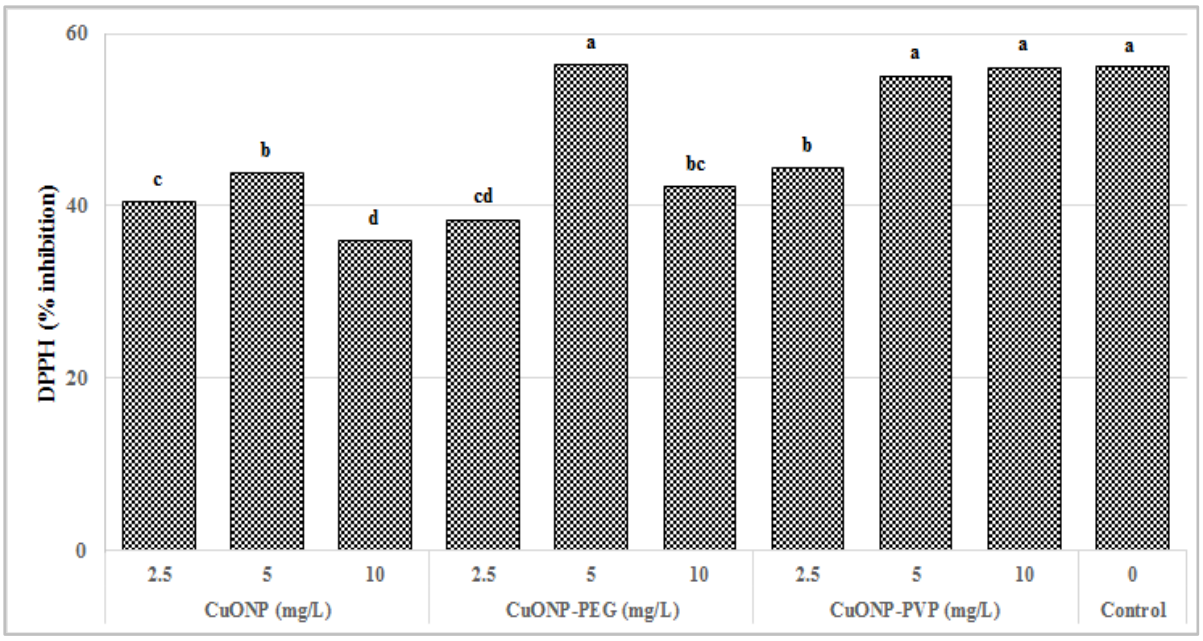

Figure 4.25: Effect of CuONP, CuONP-PEG and CuONP-PVP on DPPH radical scavenging activity derived from shoot explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

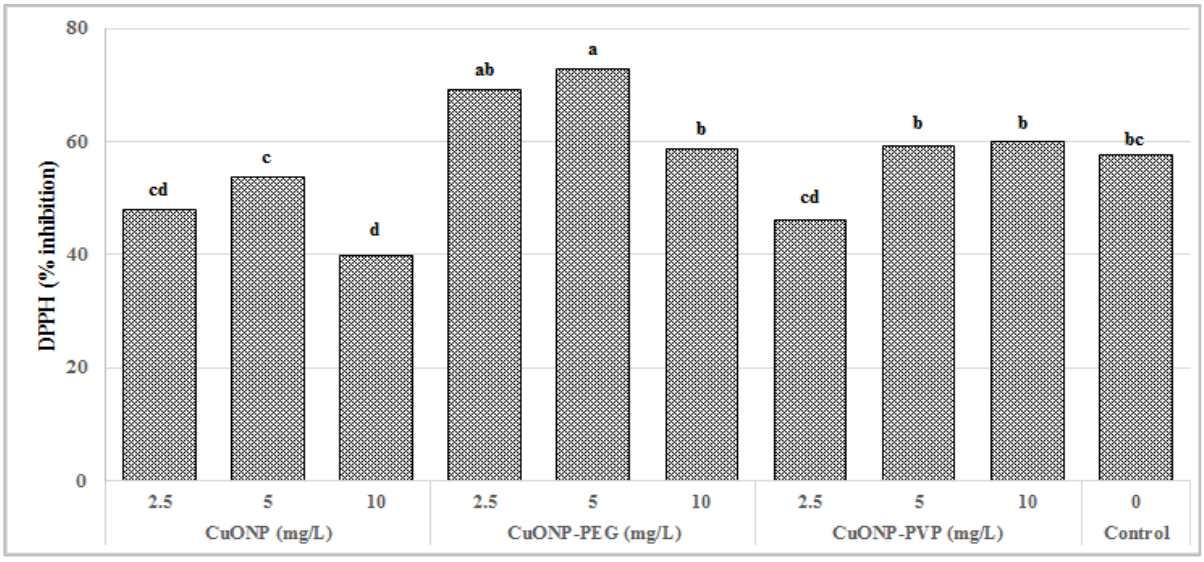

Figure4.26: Effect of CuONP, CuONP-PEG and CuONP-PVP on DPPH radical scavenging activity derived from leaf explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.8.2. *Total antioxidant capacity*

Total antioxidant capacity was found maximum in control (7.1 µg AAE/mg DW) and CuONP-PVP (6.5 µg AAE/mg DW at 10 mg/L) treated shoot explants as compared to other nanoparticles treatment (figure 4.27). The trend of total antioxidant capacity derived from leaf explants was found maximum in CuONP (9.5 µg AAE/mg DW at 5 mg/L) and CuONP-PEG (9.9 µg AAE/mg DW at 2.5 mg/L) treated explants as compared to CuONP-PVP and control (figure 4.28).

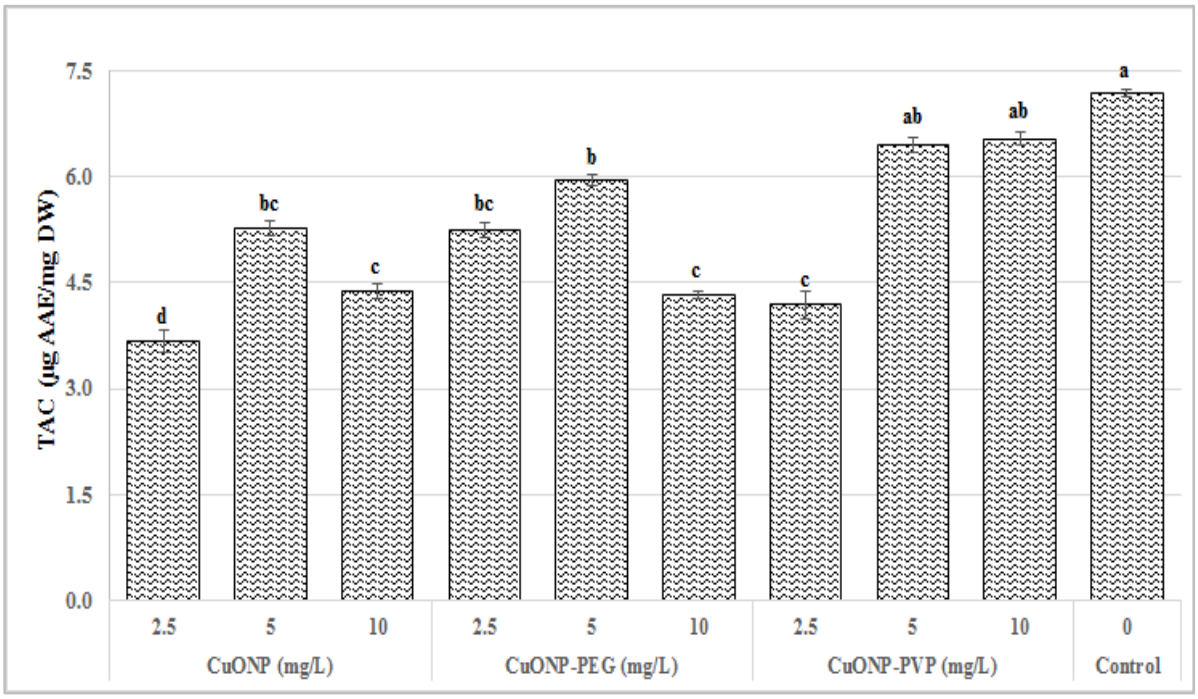

Figure 4.27: Effect of CuONP, CuONP-PEG and CuONP-PVP on total antioxidant capacity derived from shoot explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

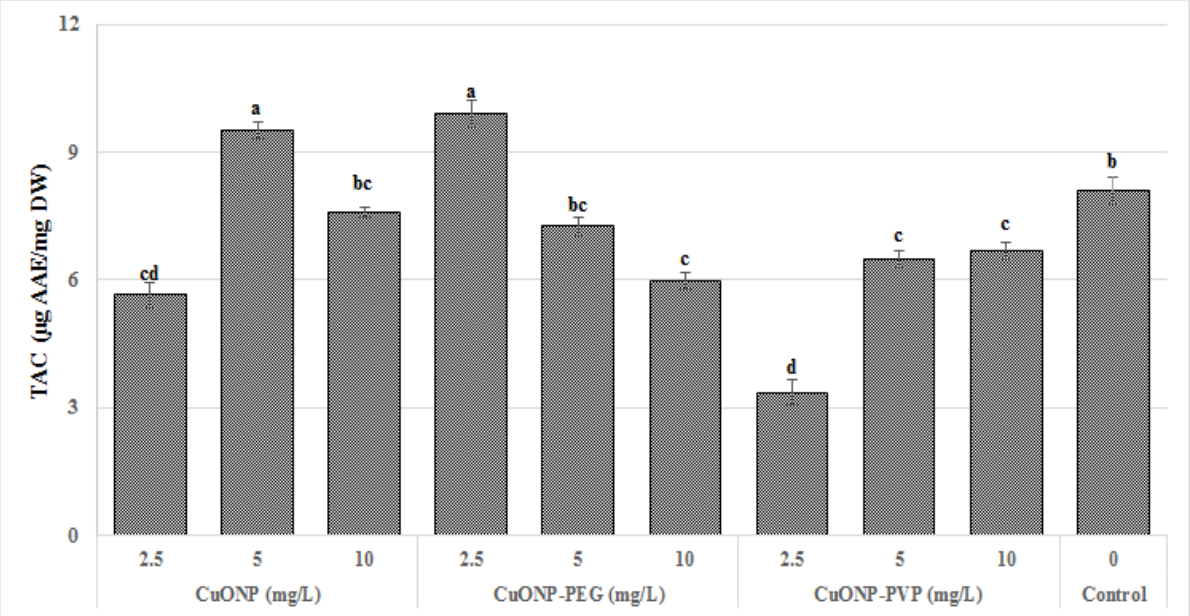

Figure 4.28: Effect of CuONP, CuONP-PEG and CuONP-PVP on total antioxidant capacity derived from leaf explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

4.8.3. *Total reducing power*

Total reducing power assay results showed maximum activity in shoot explants treated with CuONP-PVP (10.5 µg AAE/mg DW) as compared to control, CuONP and CuONP-PEG (figure 4.29). In case of leaf explants, maximum reducing power was found in CuONP treated explants (10 µg AAE/mg DW at 2.5 mg/L) (figure 4.30).

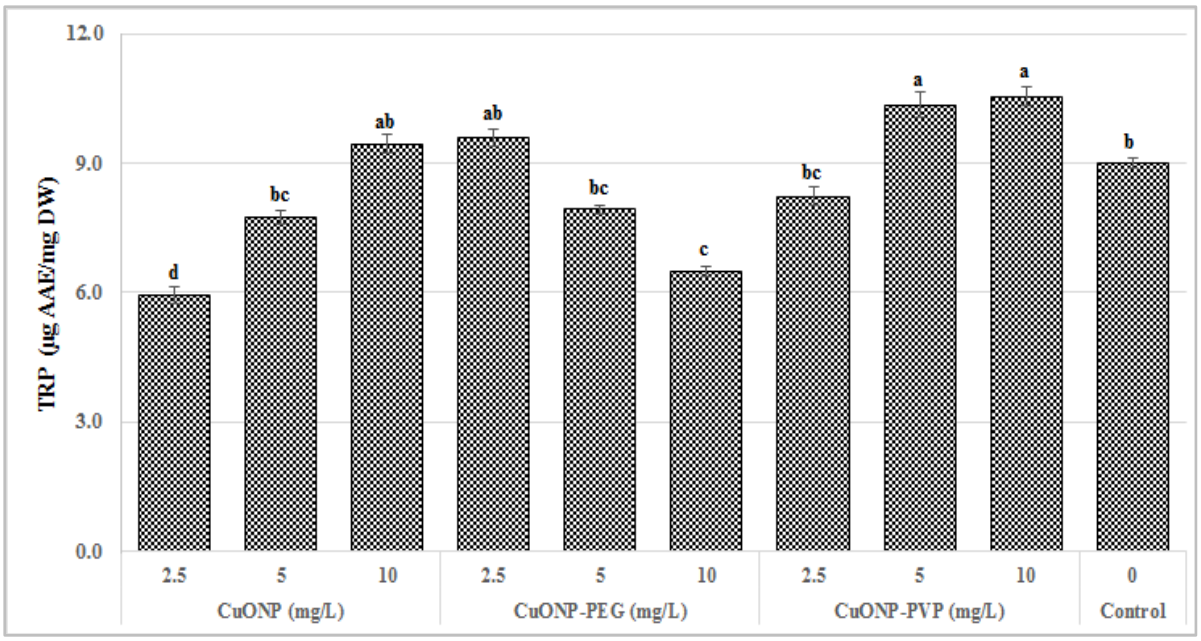

Figure 4.29: Effect of CuONP, CuONP-PEG and CuONP-PVP on total reducing power derived from shoot explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.05

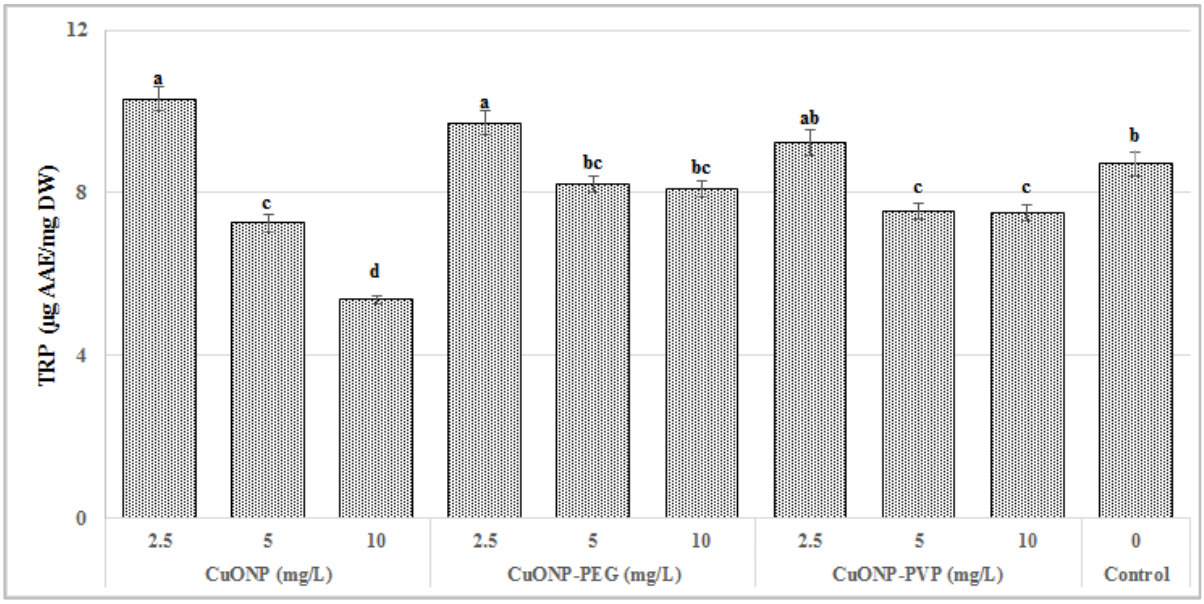

Figure 4.30: Effect of CuONP, CuONP-PEG and CuONP-PVP on total reducing power derived from leaf explant of *T. foenum-graecum* L. Bars sharing the same alphabetical letters are similar otherwise differ significantly at P<0.0

Discussion

Nanoscience has expanded greatly in the past decade due to unique properties exhibited by nanoparticles. Thus, nanoparticles are becoming main source of revolutionizing the industry and playing significant role in economy (Rosei, 2004). Nanotechnology has also permitted advanced research in areas including biotechnology and agriculture to improve crop yield, therefore using nanoparticles as fertilizers. Nanoparticles possess features that are beneficial to crops, release the nutrients on-demand, controlled release of chemical fertilizers that regulate plant growth and enhanced target activity (DeRosa *et al.,* 2010; Nair *et al.,* 2010). Nonetheless, technology always comes with price, due to widespread use of nanoparticles there are concerns related to environment and health i.e. taken up by living organisms especially plants and entering into food chain (Lecoanet *et al.,* 2004; Auffan *et al.,* 2011).

According to the previous reports engineered nanoparticles in general and metal nanoparticles in particular affects plant's morphology and physiology depending on the properties of nanoparticles. The objectives of current study were to investigate the effects copper oxide nanoparticles on seed germination, root and shoot elongation of *T. foenum-graecum* L. as it is one of the important medicinal plants and also a food crop. Response of seedlings to CuONPs was analyzed by antioxidant assays.

Seed germination is a physiological process and widely used as phytotoxicity test because it is sensitive, simple and low cost and it depends on nanoparticle-plant physical interactions (Wang *et al.,* 2001; Munzuroglu and Geckil, 2002). Seed germination is a process that starts with water imbibition by seeds and ending with the emergence of rootlet (Wierzbicka and Obidzińska, 1998). In this study seeds showing emergence of radical or cotyledon out of seed coat were recorded as being germinated. The findings of this study showed 100% seed germination without any significant adverse effect at all concentrations. This data agrees with a study conducted by Adhikari *et al.* (2012), showing 100% germination of seeds of soyabean and chickpea grown on CuONP. In addition to this, in another experiment, fenugreek seeds were treated with AgNPs and results showed increased seed germination enhancing the seed potential by increasing the characteristics of seed germination (Hojjat, 2015). In this case seed germination was 100% without showing any toxic effects grown on CuONPs. It is probably due to the seed coat which act as protector for the embryo and plays important role in selective permeability.

Major factors that contribute to reduced crop yields are drought, salinity and high temperatures. It is threatening for sustainable agriculture and global food security. Osmotic

stress can be achieved by growing plants in a media containing varying concentrations of polyethylene glycol (PEG). PEG modifies the osmotic potential of nutrient solution culture inducing plant water deficit in a relatively controlled manner (Araus *et al.,* 2008). In previously reported studies, PEG is used to control the water potential and induce drought stress in seed germination. In this study different concentrations of PEG were used to determine the response of *T. foenum-graecum* L. to osmotic stress. The data showed no inhibitory effects on seed germination frequency. These findings are in accordance with the research of Hardegree and Emmerich (1990), PEG contact with seed had no detrimental effects on seed germination in four plant species. However in 1994 another study by Hardegree and Emmerich has shown germination is affected by increase in immersion and PEG solution depth. The imbibition path of the bare membrane treatment is limited to the solution volume associated with the capillary interface between seed and membrane surface.

Shoot and root elongation results were varying at different concentrations of NPs and phytotoxicity was evident. CuONP showed maximum shoot elongation i.e. 7.7cm at 50mg/L as compared to control but trend gradually decreased as concentration of nanoparticles increased and trend was followed in plantlets treated with CuONP-PEG and CuONP-PVP. But significant shoot inhibition has been observed in seedlings treated with Cu-acetate salt i.e. 4.3cm at 1% concentration. Thus indicating minimal toxicity of copper oxide nanoparticles on shoot growth with increase in concentration in contrast to copper acetate salt. Media containing PEG, average shoot length also decreased at higher concentrations and in accordance to previous study in which PEG has significantly reduced the shoot growth in two populations of *Anthxanthum odoratum* performed by Anwer *et al.* (2004). Average shoot elongation was decreased from lower to higher concentrations of PVP.

Plantlets grown on capped CuONPs and Cu-acetate evidently inhibited root length as compared to plantlets obtained from media containing uncapped CuONPs. At lower concentrations CuONPs showed maximum root length at lower concentrations but it was inhibited with increase in concentrations of NPs in the media showing phytoxicity and markedly reduced root development. As roots are the first target tissue to confront the excess concentrations of pollutants hence toxic symptoms seem to appear more in roots as compared to shoots. Therefore comprehensive phytotoxicity profile should be investigated in higher plants for nanoparticles (Lin and Xing, 2007). Although mechanism of nanoparticles toxicity is yet not clear but it can be postulated that NPs possess ability to cross permeable membranes including seed coat and cellular membranes of roots, NPs may coagulate on the root surface and altering root chemistry, Cu ions can be released from NPs that can change

ionic balance, pH and cellular components and it also depends on mass to size ratio, shape and surface properties of nanoparticles. It is also observed in some reports that it also depends on species of plant and type of nanoparticles employed (Sresty and Rao, 1999; Brunner *et al.,* 2006; Xu *et al.,* 2006).

Copper is an essential micronutrient but exposure to excess copper salt had detrimental effects on plant growth. Copper ions tend to accumulate in the root tissues with little translocation to the shoot, thus principle effect of Cu toxicity on root growth (Sheldon and Menzies, 2004). Similar results were found in a study conducted by Thounaojam *et al.* (2012), in which reduction in root and shoot length was observed due to accumulation of Cu in seedlings of rice, directly co-related with the toxicity in the plant. Results of current study reconfirmed the earlier report showing co-relation of Cu tolerance and its greater accumulation in roots as compared to shoots due to poor translocation.

Root length is an important feature against drought stress in plant varieties; in general, variety with longer root growth has resistant ability for drought (Leishman and Westoby, 1994). In present study seedlings grown on PEG showed reduction in root length that attributes to the water stress induced by PEG at higher concentrations which affected root growth negatively. Similar results were reported by Kaydan and Yagmur (2008), PEG and NaCl affected seedlings growth of Presto negatively and showed reduction in shoot and root length. Whereas plantlets treated with PVP also showed reduced shoot and root length as concentration was increased (Table 4.1).

Average fresh and dry weight was found to be maximum in CuONP at 50 mg/L as compared to control but gradual decreasing trend was observed (table 4.2). The increase in biomass at certain concentration suggests the optimum dose limit for the growth of *T. foenum-graecum* L. seedlings. However, decrease in biomass beyond this concentration suggested the toxic effect of CuONPs. Plantlets treated with PEG and PVP also showed decrease in biomass with increase in stress. The reduction in biomass can be due to reduced availability of water and other nutrients and disturbance in normal cellular function required for proper growth.

Flavonoids and phenolics play an important role in detoxification of ROS. Total flavonoid and phenolic contents were determined and results showed that extracts of root accumulated more flavonoids and phenols as compared to control root extracts while plantlets treated with Cu-acetate salt has accumulated maximum phenolic contents indicating the elicitation of secondary metabolites production in the presence of abiotic stress. The same trend was seen in the shoot extracts and CuONPs have shown variations in the flavonoid and phenolic

content accumulation. These results depict that ROS generated inside the plant on exposure to CuONPs.

ROS are proposed to be responsible for negative effects of NPs but toxicity mechanism of NPs has not yet been clearly understood (Applerot *et al.,* 2012). Antioxidant activity of *T. foenum-graecum* L. treated with CuONPs was determined in the current study. According to results obtained showed less significant difference on exposure to CuONP, CuONP-PEG and CuONP-PVP as well as reducing power. NPs induce oxidative stress that damages cell (Hossain *et al.,* 2015). DPPH results depict that CuONPs are responsible for oxidative stress and interfere in normal growth through length, fresh weight and dry weight of the plants. Therefore plants activate their defense mechanism to protect themselves from damage. And failure of defense mechanism leads to lipid peroxidation, mitochondrial perturbation, DNA damage and eventually apoptosis of cell (Li *et al.,* 2004). So, antioxidants play an important role to combat stress.

Callus Induction

Micropropagation is an interesting method that can be economically exploited for medicinal as well as ornamental purposes. Callus culture, a type of plant tissue culture consisting of undifferentiated tissues, constitutes an important tool in plant biotechnology. It can be used in numerous ways, for example, for organogenesis, indirect somatic embryogenesis, and generation of somaclonal variations (Jattana *et al.,* 2008; Homhuan *et al.,* 2008). *T. foenum-graecum* L. is a plant with medicinal properties and callus culture is an alternative method to enhance the production of secondary metabolites like flavonoids, phenolics and antioxidants.

The results of this research showed that best growth of callus was obtained in control i.e. simple MS medium without supplementation of nanoparticles as compared to explants grown on CuONPs and it was determined by fresh weight, 1.65g (stem explant) and 0.10g (leaf explant) after 30 days of growth. These results are contradictory to a study performed by Yahya and Al-Salih (2014) in which ZnO nanoparticles were used to observe the uptake of nanoparticles by *Prosopisfarcta* L. and results showed maximum growth at 100 mg/L as compared to lower concentrations of nanoparticles. But results of present study are in accordance with results obtained from callus cultures of banana, showed reduction in callus growth due to presence of ZnO nanoparticles in the medium (Helaly *et al.,* 2014).

Although callus growth was decreased due to presence of NPs in the medium but flavonoid and phenolic contents were increased. Phytochemical screening of callus cultures showed more flavonoid and phenolic contents accumulation in explants of stem and leaf treated with capped CuONP i.e. CuONP-PVP as compared to other two forms of NPs and control. While

DPPH radical scavenging activity and antioxidant activity was evidently higher in explants treated with CuONP-PEG. These results indicate that capped nanoparticles have more phytotoxic effects on explants as compared to simple CuONP.

Conclusion

Overall experimental results illustrated that presence of CuONPs affects the growth of *T. foenum-graecum* L. seedlings at different concentrations. The maximum growth was found at 50 mg/L for *T. foenum-graecum* L. seedlings. The effective growth at certain optimum concentration and inhibited growth beyond this concentration may be attributed to the accumulation and uptake of CuONPs by the roots. It was found that the accumulation and uptake of NPs were dependent on the exposure concentrations. In particular, the exposure of plants to nanomaterials and the impacts of such an exposure on plant systems could open a new direction research on nanotechnology. Future studies should be directed towards understanding the mechanism uptake and translocation of nanoparticles, physical and chemical properties of nanoparticles in rhizosphere. It is also concluded from above mentioned results of antioxidant activity elicitation is an effective method for the synthesis of secondary metabolites including flavonoids and phenolics. Therefore, in order to enhance the production of secondary metabolites in *T. foenum-graecum* L. abiotic elicitors can be suggested.

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Appendix

Table 1: Phytochemical screening and antioxidant activity of shoot and root extracts of *T. foenum-graecum* L. treated with CuONPs

	Sample ID (mg/L)	TFC (µg QE/mg FW)		TPC (µg GAE/mg FW)		TAC (µg AAE/mg FW)		TRP (µg AAE/mg FW)		DPPH (% inhibition)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	50	0.048	0.112	0.097	0.347	0.652	1.543	0.873	2.147	43.26	51.28
	100	0.060	0.111	0.121	0.314	0.611	1.456	0.903	1.612	50.37	39.39
	200	0.053	0.024	0.105	0.300	0.559	1.400	0.670	1.570	46.90	37.30
CuONP (mg/L)	400	0.046	0.109	0.078	0.260	0.264	1.360	0.340	1.350	29.37	36.58
	0.50%	0.046	0.052	0.185	0.213	1.273	0.828	1.456	1.560	8.20	24.64
Cu-acetate	1%	0.118	0.307	0.337	0.326	2.214	1.447	1.977	1.780	59.99	71.14
Control	0	0.038	0.058	0.172	0.134	0.958	0.638	1.550	1.430	36.20	36.65
	Sample ID (mg/L)	**TFC (µg QE/mg FW)**		**TPC (µg GAE/mg FW)**		**TAC (µg AAE/mg FW)**		**TRP (µg AAE/mg FW)**		**DPPH (% inhibition)**	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	50	0.068	0.353	0.150	1.315	0.543	3.834	0.924	2.845	64.54	31.82
	100	0.073	0.162	0.233	1.423	1.079	4.334	1.349	4.051	12.29	28.20
	200	0.056	0.415	0.210	1.626	0.831	5.752	0.632	4.284	32.50	24.93
CuONP-PEG (mg/L)	400	0.028	0.288	0.058	1.106	0.343	3.080	0.567	3.670	23.22	36.77
	10	0.084	0.795	0.134	1.758	0.873	4.563	0.998	3.540	49.91	33.00
	20	0.058	1.320	0.130	2.105	0.619	4.145	0.673	3.260	32.44	38.00
	40	0.109	0.340	0.170	2.200	0.579	3.987	1.207	3.010	39.67	29.50
PEG (mg/L)	80	0.035	0.302	0.097	0.530	0.436	3.776	0.438	2.670	60.10	25.60
Control	0	0.038	0.058	0.172	0.134	0.958	0.638	0.980	0.654	36.20	36.65
	Sample ID (mg/L)	**TFC (µg QE/mg FW)**		**TPC (µg GAE/mg FW)**		**TAC (µg AAE/mg FW)**		**TRP (µg AAE/mg FW)**		**DPPH (% inhibition)**	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	50	0.073	0.075	0.198	0.026	0.959	0.760	0.692		33.41	24.82
	100	0.068	0.182	0.134	0.767	0.618	2.253	0.779		50.31	70.00
	200	0.033	0.298	0.174	0.507	0.987	2.013	1.346		36.71	19.98
CuONP-PVP (mg/L)	400	0.055	0.131	0.142		0.672		0.908		14.11	35.2
	10	0.028	0.265	0.070		0.262				17.07	30
	20	0.056	0.827	0.135	2.938	0.666	9.748	1.223	23.350	12.24	40.00
	40	0.044	0.211	0.108	0.534	0.598	1.943	1.048	2.791	51.45	39.61
PVP (mg/L)	80	0.325	1.738	0.621	3.400	3.461	18.355	4.420	30.435	55.38	68.92
Control	0	0.038	0.058	0.172	0.134	0.958	0.638	1.550	1.430	36.20	32.00

Table 2: Phytochemical screening and antioxidant activity of stem explant of *T. foenum-graecum* L. treated with CuONPs

	CuONP (mg/L)			CuONP-PEG (mg/L)			CuONP-PVP (mg/L)			Control
	2.5	5	10	2.5	5	10	2.5	5	10	0
TFC (µg QE/mg DW)	2.80	3.03	2.82	3.12	2.56	1.65	3.51	3.83	3.88	2.35
TPC (µg GAE/mg DW)	3.20	4.00	3.33	3.99	3.19	2.11	3.98	4.01	3.96	2.64
TAC (µg AAE/mg DW	3.67	5.26	4.37	5.24	5.94	4.33	4.19	6.45	6.53	7.18
TRP (µg AAE/mg DW)	5.94	7.73	9.45	9.60	7.93	6.49	8.23	10.35	10.55	9.00
DPPH (% inhibition)	40.54	43.86	35.94	38.27	56.39	42.33	44.41	55.00	56.00	56.24

Table 3: Phytochemical screening and antioxidant activity of leaf explant of *T. foenum-graecum* L. treated with CuONPs

	CuONP (mg/L)			CuONP-PEG (mg/L)			CuONP-PVP (mg/L)			Control
	2.5	5	10	2.5	5	10	2.5	5	10	0
TFC (µg QE/mg DW)	2.78	3.07	3.14	2.64	1.75	2.02	3.20	3.58	3.46	3.45
TPC (µg GAE/mg DW)	3.00	2.55	3.03	3.50	3.03	2.60	2.26	3.13	3.47	3.50
TAC (µg AAE/mg DW)	5.64	9.51	7.59	9.91	7.25	5.99	3.36	6.48	6.5	8.15
TRP (µg AAE/mg DW)	10.31	7.25	5.38	9.72	8.22	8.10	9.24	7.55	7.65	8.22
DPPH (% inhibition)	48.07	53.81	39.90	69.11	72.87	58.66	46.19	59.26	60	58.00

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paper text:

Abstract Nanoparticles have gained much importance in last two decades, especially metal nanoparticles belonging to class of engineered nanoparticles due to their unique physical, optical, electrical, magnetic and chemical properties. Nanoparticles interact with the plants and effect seed germination and growth of plant. Fenugreek is one the important medicinal plants of Indian and Mediterranean origin containing important phytochemicals that can be elicited by biotic and abiotic factors including nanoparticles. The aim of this study was to evaluate the response of fenugreek to copper oxide nanoparticles using plant tissue culture technique at different concentrations 50, 100, 200 and 400mg/L along with PEG and PVP, 10, 20, 40 and 80mg/L for five days. The results of the study have shown that seed germination wasn't affected by

CuONPs and it was 100%. But shoot and root growth of fenugreek varied with concentration of nanoparticles. Maximum shoot growth was found 7.7cm in plantlets treated with CuONP at 50mg/L as compared to control 7.2cm but elongation was evidently inhibited as concentration of CuONPs increased. Whereas as root elongation was found to be maximum in CuONP-PEG 50mg/L, 6.8 cm as compared to control 4.1cm but root elongation was inhibited as the concentration increased indicating toxicity in roots. In a parallel experiment, Cu-acetate salt solution have shown 100% seed germination but it restricted root and shoot elongation. Phytochemical screening of shoot and root extracts was also carried out showing maximum flavonoid and phenolic contents in Cu-acetate treated plantlets as compared to CuONP and control with maximum radical scavenging DPPH and antioxidant activity. PEG and PVP treated plantlets accumulated more flavonoid and phenolic contents as compared to CuONP-PEG, CuONP-PVP and control, indicating more secondary metabolite production due to osmotic stress. But radical scavenging activity was higher in CuONP-PEG, 50mg/L and CuONP-PVP, 100 mg/L as compared to PEG, PVP and control. Callus was induced from stem and leaf explants of T. foenum graecum L. treated with CuONP,CuONP-PEG and CuONP-PVP. Maximum fresh and dry weight was found to be in control i.e. stem explant; 1.65g and 0.10g and leaf explant; 1.33g and 0.09g after 30 days as compared to explants treated with nanoparticles. Phytochemical screening of callus extracts derived from leaf and stem explants have shown more flavonoid and phenolic contents accumulation in CuONP-PVP treated explants as well as antioxidant activity. Therefore, these results indicate that copper oxide nanoparticles affect the growth of seedling of fenugreek depending on size, concentration as well as species of the plant without inhibiting seed germination. Chapter 1 INTRODUCTION Nanotechnology represents an area with significant wide range of applications including food processing, wastewater treatment, biomedical products, healthcare, diagnostics and pharmaceuticals, as well as environmental, energy and material sciences. Therefore, nanotechnology has become a dynamic developing industry and it is also estimated that 15% of all products worldwide would have incorporated nanotechnology within their production processes via 2014 (Remedios

39**et al., 2012;** Aslani **et al., 2014;** Nhan **et al.,**

2015) Nanoparticles are characterized by the materials with at least one dimension less than 100nm and large surface area (Burklew et al., 2012). The small size of nanomaterials confers unique properties such as electrical conductivity, toughness and optical features. Due to these distinctive features, nanoparticles are increasingly been employed in industry and daily life by advancing the everyday materials and processes. (Remedios et al., 2012) Nanoparticles (NPs) have also been used in agriculture as nanofertilizers to enhance plant growth and development in more effective way than applied fertilizers that are unable to reach plant due to leaching, hydrolysis and decomposition. These nanofertilizers also protect against biotic stresses for instance insect, fungi and bacteria. Hence nanofertilizers reduce the loss in fertilization and increase crop yielding through certain properties than are effective for crop plants. (Ma et al., 2009; Nhan et al., 2015; Siddique et al., 2015) Nanoparticles interact with plants causing many morphological and physiological changes, depending on the properties of NPs. Efficacy of NPs is determined by their chemical composition, size, surface covering, reactivity, and most importantly the dose at which they are effective (Khodakovskaya et al., 2012). There are large number studies have been done showing both beneficial and negative effects on plant growth and development (Monica and Cremonini, 2009). Many studies have proved that zinc oxide NPs (ZnONPs) in peanut, soyabean, wheat and onion at low concentrations exhibiting beneficial effect on germination of seed (Prasad

39**et al., 2012;** Sedghi **et al.,** 2013; Ramesh **et al., 2014;** Raskar and Laware, **2014)**

. Carbon nanotubes have also gained importance due to unique properties in plant development by influencing the seed germination in various studies (Villagarcia et al., 2012; Tiwari et al., 2014). Silver nanoparticles have great impact on plant growth. A study was carried out to check the effect of biologically synthesized AgNPs on Bacopa monnieri, and results have revealed

15**a significant effect on seed germination** and **induced the**

15**synthesis of protein** and **carbohydrate and decreased the total phenol** contents and **catalase and peroxidase activities (Krishnaraj et al., 2012).**

In another study, the effects of nanoscale titanium oxide (TiO2) and silicon oxide (SiO2) on Soyabean plant were observed and these NPs improved the activity of nitrate reductase in Soyabean, and germination and growth was apparently accelerated (Lu et al., 2002). Nanoparticles also elicit the production of secondary metabolites that are produced by plants under stress conditions and these are bioactive compounds play important role in host immune system. Aloe vera L. is of the important medicinal plant due to its secondary metabolites, is treated with TiO2 NPs and AgNPs to increase the production of secondary metabolites and it was concluded that NPs elicit the production of aloin in Aloe vera. (Mona et al., 2014) Copper oxide nanoparticles (CuONPs) have gained special attention due to its simplicity and various physical properties including superconductivity, electron correlation effects, and spin dynamics. CuONPs are progressively

52**used in various applications such as** in **catalysis, batteries, gas sensors, heat transfer fluids,**

and solar energy (Maqusood et al., 2014).

54**CuO crystal structures possess a narrowband gap, giving useful photocatalytic** and **photovoltaic properties**

(Chang et al., 2012). Although nanotechnology have huge number of beneficial applications, but technology always comes with a price i.e. there are environmental and healthcare concerns related to nanoparticles (Maynard et al., 2006). Nanoparticles enter into the environment by intentional or unintentional means including waste streams from manufacture facilities, volcanic emissions, industrial processes and transportation (Klaine et al., 2008; Dutschk et al., 2014). Toxicity studies of NPs have been carried out aiming on biological and ecological effects but there are still gaps due to lack of knowledge release rates into the environment (Hansen et al., 2008). NPs kill pathogenic bacteria effectively but on the other hand they have deleterious effects on helpful microbes in the environment such as plant growth promoting microbes, pollutants degrading and element cycling. Industrial wastes with NPs move into waters thus polluting oceans by accumulating on the surface of oceans and pose risk to marine life, birds and mammals (Kennedy et al., 2004; Nowack and Bucheli, 2007).

1**Fenugreek is one of the oldest plants in**

the world, commonly known as “methi”.

45**It is a self- pollinating,** leguminous **crop native** to **the Indian subcontinent and the Eastern Mediterranean region.**

Nearly 260 different varieties of fenugreek were suggested by Linnaeus but

5**only 18 species of fenugreek have been traced.**

It is currently widely cultivated in central Asia, central

35**Europe, northern Africa, North America and parts of Australia,**

with India being the leading fenugreek producer in the world. (Vani et al., 2014)

> 5**Most popular species of this genus is Trigonella foenum-graecum**

L. Trigonella, is a Latin word meaning “little triangle”, due to the triangular shape of its flowers and “foenum-graecum” stands for “Greek hay” which refers to its use as forage crop. It is also known as ‘ox horn’ or ‘goat horn’ because its two seed pods project in opposite directions from the nodes of the stem base and resemble an ox or goat horns. It is grown mainly as a spice crop (Morcos et al., 1981; Patil et al., 1997). Fenugreek plant exhibits antioxidant activity. It

> 43**is due to the presence of** certain **phytochemicals** in the plant **including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin,** saponins **etc.**

Different studies have proved that fenugreek extracts have scavenging activity against free hydroxyl radicals that are associated with diseases like diabetes mellitus, atherosclerosis, cataract, rheumatism, and other auto immune diseases. Studies have also shown anti-bacterial activity possessed by fenugreek. Extracts of fenugreek have shown to inhibit different bacterial species;

> 25**Pseudomonas spp., E. coli, Shigella dysentiriae,** Saccharomyces bayanus **and Salmonella typhi.**

(Akbari et al., 2012) Objectives of the study

> 20**Objectives of this study were;** 1. **To** evaluate **the effect of**

copper oxide nanoparticles in different concentrations on seed germination, root and shoot elongation as well as on callus of Trigonella foenum graecum L. 2. To determine the antioxidant activity Chapter 2 Review of Literature Nanotechnology is a developing field with a great potential to make new products incorporating distinctive properties and improving the products for various applications. There are many products of nanotechnology that are already

> 34**Many nanotechnology-based products are already available in the** market, **including sporting goods, electronics, personal care,** and **automotive parts (Kang, 2010).**

It was estimated by 2010, $11 trillion market will be represented by nanotechnology (Pitkethly, 2003). According to another estimate the product sale incorporating nanotechnology will rise from

> 31**<0.1% of global manufacturing output in 2004 to 15% in 2014, totaling $2.6 trillion (Lux research 2004).**

The term nanotechnology is a combination of two words; “nano” is a Greek word denoting a billionth and the word technology. Therefore nano scaled particles are considered to be the one with size less than 100nm at least one dimension (Bhushan, 2004; Sattler, 2010). This extremely small size of NPs, confers unique physical and chemical properties that results in higher reactivity with large surface area, making NPs feasible to utilize in a huge number of products for example

17**paints, cosmetics, medicines, food and suntan lotions, as well as applications which directly release NPs into the environment, such as remediation of polluted environments (Aitken et al., 2006).**

Classification of Nanoparticles Nanoparticles are classified into following three types: i. Natural NPs;

3**Natural nanoparticles have existed from the beginning of the earth' history and still occur in the environment (volcanic dust, lunar dust, mineral composites)** ii. **Incidental** NPs; **also defined as** waste or **anthropogenic particles, take place as the result of manmade industrial processes (diesel exhaust, coal combustion, welding fumes,** etc.) iii. **Engineered** NPs; are **further grouped into four** categories; a) **carbon based** NPs which include **fullerene, single walled carbon nanotube (SWCNT) and multiwalled carbon nanotubes (MWCNT);** b) **metal based** NPs including **quantum dots, nanogold, nanozinc,** nanoaluminum **and nanoscales metal oxides like TiO2, ZnO and Al2O3;** c) **dendrimers**

are nano-sized polymers built from branched units, which are capable to be designed to perform specific chemical function; d) composites which combine nanoparticles with other nanoparticles or with larger bulk-type materials (Lin and Xing, 2007) and present different morphologies such as spheres, tubes, rods and prisms (Yu-Nam and Lead, 2008). Engineered NPs have gained a lot of importance due to their wide range of applications in industry and improving the economy sectors such as

63**consumer products, pharmaceutics, cosmetics, transportation, energy and agriculture etc.** (Roco, 2003; Novak **and**

Bucheli, 2007). Metal nanoparticles (MNPs) belong to the category of engineered NPs, have been in focus

28**due to their unique** features such as **electronic, optical, mechanical, magnetic and chemical properties that** can be **significantly different from those of bulk materials** (Boddu **et al.,**

2011). Metal NPs are synthesized employing various methods that confer different properties for desired purposes. Mostly methods that are used for synthesis of metal NPs are sol-gel, hydrosol/magnetic fluid, vacuum deposition and ball milling method (Lue, 2007). Applications The process of nanotechnology began with the

7**generation, manipulation, and** deployment **of** nanomaterials, representing **an area holding significant promise for a wide range of applications. Nanotechnology has become a dynamically developing industry, with multiple applications in energy, materials, computer chips, manufacturing, health care, and medical diagnosis**

(Safari and Zarnegar, 2014; Neto, 2014; Dutschk et al., 2014). Diagnostics and Therapeutics

6**Nanotechnology is an interdisciplinary field** integrating engineering, **chemistry, biology and medicine,** thus making it **useful**

for diagnostic and therapeutic purposes. It has been successfully used not only the diagnosis of cancer but also to treat cancer by using it as a drug delivery vehicle (Larouia et al., 2013). Recent advances have shown that NPs have bioaffinity property making it a probe

6**for molecular and cellular imaging, targeted NP drugs for cancer therapy, and integrated** nano devices **for early screening and detection of cancer. These developments offer exciting opportunities for the development of personalized therapy, in which the molecular profiles of an individual's genetic and protein biomarkers may be used to diagnose and treat the patient's cancer**

(Cai et al., 2008). Nanometrology Nanometrology deals with the

36**measurement of functionally important, mostly dimensional parameters** and **components with at least one critical dimension which is smaller**<100 **nm.**

Success in nanomanufacturing of devices will rely on new nanometrologies needed to measure basic materials properties including their sensitivities to environmental conditions and their variations, to control the nanofabrication processes and materials functionalities, and to explore failure mechanisms (Nomura et al., 2004). Nano and Energy Energy comes highest on the list of priorities in human needs (Ghoniem, 2011). World's

40**primary energy demand will increase by 36% between 2008 and 2035 according to International Energy Agency**

(IEA). Due to carbon dioxide emission and climate changes that are influencing life and health, renewable energy resources needs to play vital role in developing more reliable and sustainable energy path. Solar energy is the most abundant, infinite and pure renewable energy source to date and it can be harnessed using photovoltaic cells. Although inorganic semiconductors including silicon, gallium arsenide and sulfide salts are primarily used but organic materials with nanostructures are more advantageous because of low cost and large scale manufacturing processes (Yu et al., 1995; Forrest, 2004). Nanoparticles and Agriculture Engineered NPs act as a smart tool

50**for the efficient delivery of fertilizers, herbicides, pesticides and plant growth regulators etc.**

(Johnston, 2010; Ditta, 2012). Applications of NPs has influenced the earlier plant germination as well as enhanced the yield of plants for food, fuel, and other uses (figure). Nanoparticles have been employed in numerous researches to show crop improvement. Carbon nanotubes (CNT) have increased the seed germination in tomato (Khodakovskaya, 2009). Other than CNT, metal nanoparticles including gold (Au), silver (Ag), copper (Cu), zinc (Zn), aluminum (Al), silica (Si), zinc oxide (ZnO), cesium oxide (Ce_2O_3), titanium dioxide (TiO_2) and magnetized iron (Fe) have also found to improve crop yield (Zhang and Webster, 2008). Figure 2.1: Applications of engineered nanoparticles in agriculture (Aslani et al., 2014) In a study effect of Au-NPs on Brassica juncea was checked at different concentrations and it was found that Au-NPs decreased overall growth of plant but increased the free radical stress supporting increase in biomarker antioxidative enzymes, proline and hydrogenperoxide (Gunjan et al., 2014). In another study zinc and silver nanoparticles were employed on two important crops; Zea mays L. and Brassica oleracea var. capitata L. and both plants showed lower nanoparticle toxicity as compared to the free ions (Pokhrel

and Dubey, 2013). Copper (Cu) is an essential nutrient for the growth of plants and

29 **plays an important role in the photosynthetic reactions.**

Copper

29 **activates several enzymes and contributes in RNA synthesis and** improve **the performance of photosystems.**

But at higher level of Cu, seed germination and plant growth may be adversely affected. Both soybean and chickpea are sensitive to Cu. Copper oxide NPs (CuONPs),

11 **one of the major and frequently used engineered oxide NPs with significant industrial applications**

like catalyst, superconducting materials, thermoelectric materials, sensing materials, glass, ceramics etc.

11 **Thus significant industrial production and applications of**

CuO

11 **NPs may lead to environmental exposure. The**

11 **acquired results reflect the toxicity of**

engineered nano particles

11 **to bacteria, nematodes, and other organisms.**

26 **Studies on the toxicity of** nano materials **are still emerging and basically evidence several negative effects on growth and development of plants** (Adhikari **et al.,**

2012). CuNPs at various concentrations have been shown to improve the growth and yield of wheat as compared to control by improving chlorophyll content, leaf area, number of spikes and number of grains when applied to soil pots (Hafeez et al., 2015). Nanotoxicology Increased production of NPs has raised the concern of its negative impact on the environment and interaction with the living organisms (Ye et al., 2012; Lok and Pokhrel, 2014). NPs enter living systems

42 **through intentional and unintentional releases such as solid/liquid waste streams from** manufacture **facilities and atmospheric emissions**

Nanomaterials can come into contact with living organisms via multiple routes (Figure), such as

7**incidental release, direct release from industrial products or processes, as well as commercial products during intended uses that in turn** enter **the sewer-to-wastewater treatment plants**

(Grieger et al., 2009; Zhang and Fang, 2010). Figure 2.2: Nanomaterials in living systems Fenugreek (Trigonella-foenum gracecum L.)

53**Fenugreek (Trigonella foenum-graecum L.) is one of the oldest medicinal plants**

commonly known as “methi”, cultivated all over the world (Passano, 1995; Basch et al., 2003). Fenugreek has been used as both food and medicine since ancient times in different regions of the world. This crop is native to Indian subcontinent and Eastern Mediterranean region but also cultivated in central Asia, central

35**Europe, northern Africa, North America and parts of Australia**

(Petropoulos, 2002). T. foenum graecum L. is annual dicotyledonous herb belonging to family Leguminosae (Table 1) and its main producers are India, Pakistan, Bangladesh, Iran, Nepal, Egypt, Morocco, China, France, Spain, Turkey and Argentina (Basu et al., 2004; Sheikhlar, 2013). Its height is up to 60cm. The characteristic features of this plant are trifoliate leaves, white flowers of triangular shape and roots bearing conspicuous root nodules (Figure 1). Its seeds are of sickle pod shape and are of brown color with 3×4mm in dimensions (Morton, 1990; Acharya et al., 2006). Figure 2.3: Fenugreek (T.foenum graecum L.) plant (Akbari et al., 2103) Table 1: Classification of fenugreek (T.foenum graecum L.) plant

5**Kingdom Plantae Phylum Anthophyta Class** Dicotyledonae **Order Fabales Family Fabaceae Genus Trigonella Species Trigonella foenum graecum Binomial name Trigonella foenum graecum L.** Active Compounds **Fenugreek**

5**has** also **been used as a medicinal plant since**

ancient times. The active constituents of fenugreek seed consist of carbohydrates (45-60%), proteins (20-30%) and lipids (5-10%). The carbohyrdates mainly contains galactomanans (mucilaginous fiber) that helps in decreasing the consumption

5**of glucose and other calorie rich foods, thus** plays **an effective** role to **control blood glucose levels in diabetic patients.**

Glycosides produce steroidal sapogenins through hydrolysis (diosgenin, yamogenin, tigogenin, neotigogenin) that aid in the production of synthetic sex steroids as well as used in the treatment of hypercholesterolemia. Other chemical compunds that are produced include spirostanol saponins,

5**triterpenoids, trigonelline, flavonoids (e.g. atroside, quercetin, etc.)**

with presumed therapeutic effects. Apart from these pharmacological effects leaf extracts of fenugreek are used to treat Pediculus humanus capitis (head lice) in human beings (Al Asadi, 2014). Figure 2.4: Galactomanan Figure 2.5: Diosgenin Medicinal Importance Fenugreek is a medicinal plant and it has therapeutic effects against various diseases like

57**atherosclerosis, rheumatism, sugar lowering, blood lipids lowering** and **appetizer.** It **also contain antioxidant activity**

(Akbari et al., 2012). Hypoglycemic effects Hypoglycemia refers to abnormal decrease in the blood sugar level in human body (Meghwal and Goswami, 2012). The seeds of fenugreek stimulate the glucose dependent insulin secretion from pancreatic beta cells thereby, exerting hypoglycemic effects in humans. It also inhibits the activity of two intestinal enzymes, alpha amylase and sucrase involved in metabolism of carbohydrate (Amin et al., 1987; Ajabnoor and Tilmisany, 1988). Hypocholesterolemic activity Hypocholesterolemia is condition with abnormal deficiency of cholesterol level in blood. In a study it has been shown that administration of fenugreek seed extracts lower the

46**serum cholesterol, triglyceride and low-density lipoprotein in hypercholesterolemia suffering patients and experimental models**

(Singhal et al., 1982). It also reduces accumulation of triglyceride in the liver without interfering with plasma insulin in rats suffering obesity (Basch et al., 2003). Gastroprotective effect Fenugreek is effective against gastric ulcer and it is used for this purpose since old times. In a study

1**ulcer protective effect of fenugreek** seed was **compared to omeprazole on ethanol-induced gastric ulcer in rats and**

1**seeds showed significant ulcer protective effects compared to those on omeprazole.** Scientists have discovered **that** seeds **of**

fenugreek has cytoprotective effect

1**due to the anti-secretory action and to the effects on mucosal glycoproteins**

(Pandian et al., 2002). Antimicrobial activity Antimicrobial activity of plant extracts is due to variety of compounds including aldehyde and phenolics. Antibacterial effects of fenugreek have been reported in numerous studies. Researhers

1**reported strong activity of T. foenum-graecum against 26 bacterial pathogens**

(Omolosa and Vagi, 2001). In another study

1**a high antimicrobial activity against peptic ulcer-linked Helicobacter pylori in the fenugreek sprout extract**

was observed (Randhir et al., 2004).

41**Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties** (Hugo **and**

Bloomfield, 1971). Methanol

> 25**extract of fenugreek and coriander** have shown increased **antimicrobial** activity **against Pseudomonas spp.,** Escherichia **coli, Shigella dysentiriae** and **Salmonella typhi**

(Dash et al., 2011).

> 1**Fatty oil of fenugreek seeds showed very significant antimycotic activity against Aspergillus niger and A. fumigates**

(Wagh et al., 2007). Tissue culture studies of T. foenum graecum L.

> 8**Plant tissue cultures provide propagation of plants which are rare or economically important and can be used to induce quantitative and qualitative modifications on the production of plant secondary metabolites by changing nutrient and hormonal media culture conditions (Collin, 2001).**

Plant tissue cultures, is an important tool to understand physiological, biochemical, and morphological reactions taking place in cell under controlled conditions to specified factors in order to gain insight into the intact plant life to its natural environment (Neuman et al., 2009).Fenugreek is an important medicinal plant producing several secondary metabolites that help in treating various disease conditions thus several studies have been conducted for the production of secondary metabolites from cell suspension cultures, callus and protoplast culture as well as organogenesis and genetic transformation. In 1945, Cerdon et al. carried out first tissue culture on fenugreek to evaluate the effects of diniconazole (broad spectrum triazole-type fungicide) on cell suspension cultures of fenugreek and found that cell growth decreased by addition of fungicide as compared to control after 21 days.. Trisonthi et al. (1980) found effects of mevalonic acid on steroidal sapogenins synthesis in suspension cultures of fenugreek tissue and it should positive promoting effects. In another study cultures treated with nicotinic acid had increased trigonelline concentration (37%) (Ramesh et al., 2010). Leguminous plants produce medicarpin,

> 12**in response to biotic or abiotic elicitation from either their glycosidic conjugate pools or by de novo synthesis.**

Seedlings of fenugreek were treated with CuCl2 to find out the origin of isoflavonoid pterocarpans, like medicarpin, medicarpin

> 12**in response to copper elicitation.** Accumulation **of**

> 12**isoflavonoid aglycones and their glycosides were** measured **by** using **High Performance Liquid** Chromatograpghy **(HPLC)** and their **results**

showed the clear relationship between copper and de novo synthesis of medicarpin (Tsiri et al., 2009). Callus Culture Plants can be regenerated through micropropagation, in vitro culture techniques including callus culture from shoot tips and axillary bud explants. Callus is an unorganized mass of cells or tissue derived from explants with the help of plant

> 4**growth regulators. Plant regeneration from calli is possible by de novo**

organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the in vitro applied environmental stimuli, including growth factors added to the cultured cells

(Larkin and Scowcraft, 1981; Flick et al., 1983; Raghavan, 1986). Callus cultures of

44**T. foenum graecum L.** in **the** presence **of** Adenosine triphosphate (ATP **and**

MgCl2 have showed

10**conversion of nicotinic acid and S.** adenosylmethionine into **trigonelline**

10**3 to 4 times** higher **than seeds and 12-** 13 **times** higher **than**

root and shoot cultures and successive stable subculturing was also obtained (Joshi and Handler, 1960). Khanna and Jain (1975), induced higher steroidal contents from callus culture compared to seeds and produced diosgenin,

10**gitogenin and tigogenin with other sterols**

of spirostane derivatives on agar solidified MS medium containing 1 mg/l 2, 4-D. They noted that six week old cultures were more prone to the

10**production of diosgenin, gitogenin** and **tigogenin,** other **sterols,**

high growth index and the total steroidal content compared to the seeds. Effect of Polyethylene glycol (PEG) and Polyvinylpyrrolidone (PVP) on Plants Plants continue to grow and develop under variable environment and adapt to the conditions. Water deficiency can result into limited growth of plant often. It affects the shoot growth more than the root. Actually

13**even under mild water deficit shoots may stop growing completely while roots continue to grow. Continued root growth allows the plant to plumb the soil for water and can be especially important for seedling establishment**

(Corine et al., 2000). Polyethylene glycol is often used to induce drought stress in seed germination studies and also influence growth of plant negatively as the concentration of PEG increases by reducing the oxygen availability (Hardegree and Emmerich, 1994).

22**Polyvinylpyrrolidone (PVP), is a versatile polymer, water soluble with excellent** colloidal, **stabilizing and complexing properties, being at the same time metabolic and** physiologically **inert** (Magnunsan **et al., 1996).**

Previous studies have shown that PVP has positive effect on plant growth at various concentrations. Phytochemical Assays Total flavonoid and phenolic content Flavonoids and phenolics are important compounds of T. foenum graecum L. and they accumulate at different stages of growth.

2**Dietary flavonoids are usually glycosylated and can be classified as anthocyanidins, flavanols (catechins), flavones, flavanones, and flavonols which responsible for the orange, red and blue colors in fruits and vegetables (Merken and Beecher, 2000). Traditionally, deep colored fruits, vegetables or foods are recognized as more healthy to human body, especially in the oriental countries. There has been a growing interest in pigment components of fruits and vegetables, which may promote human health or lower the risk for disease**

(Lin and Tang, 2006). Total antioxidant capacity Antioxidants are present naturally in plants as well as supplements are also available. Antioxidants of natural origin have benefits over synthetic because they do not

37**side effects while synthetic were found to have genotoxic effects (Chen et al., 1992; Kahl and Kappus, 1993;**

Zheng and Wang, 2001; Rohman et al., 2010). Hence determination of

38**biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous.**

Generation of excessive reactive oxygen species (ROS) lead to the oxidative stress which plays an important

30**role in development of** development of **chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular and** neurodegenerative **diseases.** (Mandal **et al.,**

2009; Ebrahimzadeh et al., 2010). Antioxidants inhibit and scavenge radicals and protecting against infections and degenerative diseases. Chapter 3 MATERIALS AND METHODS Nanoparticles Copper oxide nanoparticles (CuONPs) were used

15**to evaluate the effect of** these **nanoparticles on** seed **germination**

frequency as well as for biochemical screening of Trigonella foenum graecum L. in the experiment. Three types of CuONPs were tested; simple CuONPs and two others were capped with PEG and PVP (CuONP-PEG and CuONP-PVP) respectively. The NPs were provided by Miss Rabia Javed, PhD

51**scholar at Department of** Biotechnology **Quaid I Azam University Islamabad. The** NPs **were** synthesized **by** chemical method **in**

Plant Transformation Laboratory while characterization including

62X-ray diffraction, UV-visible spectroscopy and Fourier transform infrared spectroscopy

(FTIR) was performed at Physics Department and Microbiology Department Quaid I Azam University Islamabad. The size of all NPs that are employed was <50nm (Table 3.1). Table 3.1: Size of test nanoparticles Sr. No. Nanoparticles Size 1 CuONP 2 CuONP-PEG 3 CuONP-PVP 46nm 47nm 40nm Preparation of Nanoparticles, Salt, PEG and PVP Dilutions The test NPs

24were suspended directly in distilled water and dispersed by ultrasonic vibration (100W, 40 kHz) for 30 min.

Different doses of NP suspensions 50, 100, 200 and 400 mg/L, were prepared for the seed germination experiment. PEG and PVP solutions were also prepared with concentrations; 10, 20, 40 and 80 mg/L. Along with these, salt solutions of copper acetate, 0.5 and 1% were also prepared.

59Plant material Seeds of Trigonella foenum graecum L. were purchased from

National Agricultural Research Centre (NARC). Seeds were identified and authenticated by the Medicinal and Aromatic plants Research Institute, NARC. Selection of the seeds was based on ethno botanical, traditional medicinal importance and least exploration. Seeds were cleaned, freed from dust and foreign material and then kept in dark and dry place before use. Seed Germination Media was prepared for the germination of seeds containing 2.2g/L (half-strength) of Murashige and Skoog medium (MS0), 3% sucrose and 0.44% gellan.

61In order to study the effect of CuO NPs on seed germination, solutions of nanoparticles were

also added after sonication to the medium at concentrations 0 (control), 50, 100, 200 and 400mg/L in duplicates. Solutions of PEG and PVP, 10, 20, 40 and 80mg/L. into the media. In addition to these, media was also supplemented with solutions of Cu-acetate, 0.5 and 1%. The

8pH of media was adjusted to 5.7 and then plugged with

cotton buds followed by aluminum foil cover to seal the flasks. Media was autoclaved at 121°C for 20 minutes. Seeds were immersed in freshly prepared 0.1% mercuric chloride solution for 3-4 minutes for surface sterilization. Subsequently washed with distilled water thrice. Five seeds per flask (100ml) containing 25ml medium were inoculated, sealed and shifted to growth room. The temperature of growth room was maintained at 25°C with 16 hours of photoperiod. Seed Germination Parameters Percentage germination frequency Final percentage (%) germination was calculated after five days using formula;

64 (%) = × 100

16Rate of germination (RG) = ∑ / Where; N= daily increase in seedling number D= number of days from seed placement

Mean period of final germination (MPFG) = ∑()/

16**Where; N= daily increase in seedling number D= number of days from seed placement**

S= total number of seeds germinated Germination index (GI) () = % % Percentage inhibition (%) % h (%) = 1 − { % } × 100 Seedling growth parameters Length of root and shoot After 5 days of inoculation plantlets were separated from the medium. Root and shoot lengths of plantlets were measured using a ruler in centimeter (cm) and average was taken. Fresh and dry weight Five seedlings were taken from each flask after 5 days and their fresh weight was recorded using analytical weighing balance and average fresh weight was calculated For dry weight plantlets were dried in open air for overnight time period and then weighed. Callus Induction For callus induction, seeds of Trigonella foenum graecum L. were germinated and plantlets were collected grown over a period of 10 days under controlled conditions on MS0 media. Callus was initiated by cutting stem of a plantlet into pieces of 8mm ±1mm as well as leaf was also cut into size of 5mm ±1 using a sterilized scalpel. Later, two explants of stem and leaf each were placed in separate flasks of 100ml containing 20ml MS media supplemented with 2mg/L 1-Naphthaleneacetic acid (NAA),

48**0.5mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D)** and **0.** 5mg **/L** 6-benzylaminopurine **(BA).**

10**In addition to plant growth regulators**

(PGRs) CuONPs; CuONP, CuONP-PEG and CuONP-PVP were also added into the medium at concentrations of 0 (control), 2.5mg/L, 5mg/L and 10mg/L in duplicates. The media was autoclaved prior to induction at 121°C for 20 minutes. After inoculation the flasks were transferred to growth room where temperature is maintained at 25°C with 16 hours of photoperiod. The calli generated after the time period four weeks from both stem and leaf explant were collected and fresh weight was calculated using analytical balance. The callus was then subjected to drying in a vacuum oven at 45°C and -0.06 Pa for three days. After drying, callus was once again weighed and readings were noted. Biochemical Screening of Fresh Weight Extract Extract preparation Fresh weight extract of shoot and root were prepared in ethanol and air dried. Later 20mg/ml extracts were made in DMSO i.e. 20mg of the desired fresh weight extract dissolved in 1ml of dimethyl sulfoxide (DMSO). Dry weight callus extracts were prepared at concentration of 100mg/ml i.e. 100mg of the dry weight extract dissolved in 1ml of DMSO. Phytochemical Screening Chemicals and apparatus Quercetin, Potassium acetate, Aluminium chloride, Folin–Ciocalteu reagent, (Riedel-da Haen, Germany), Gallic acid, Methanol, 2, 2-diphenyl-1-picryhydrazyl (DPPH), Ascorbic acid, Sulfuric acid, Ammonium molybdate, Sodium phosphate, Phosphate buffer (Riedel-de-Haen, Germany),

58**Potassium ferricyanide, Trichloroacetic acid (TCA),** Ferric chloride **and Dimethyl** sulfoxide **(DMSO) were purchased from**

Sigma (Sigma Aldrich, USA), Incubator IC83 (Yomato, Japan), 96 well plate (SPL life science, Korea), Microplate reader (Biotek USA, Elx 800) and Micropipette (Sartorius, France).

19**Determination of total** flavonoids **contents** (TFC) **Total** flavonoid **contents of the test** samples **were**

identified

10**according to the method** previously **described by** Almajano **et al.,**

(2008). 20µL test sample, standard and blank were taken in to 96 well microplate, followed by adding 10µL of aluminum chloride solution and then 10 µL of potassium acetate (1 M). 160µL of distilled was added to attain final volume of 200 µL. The plate was then

49**incubated for 30 min at room temperature.** The **absorbance was measured** by using **microplate reader** (Bioteck, USA) **at**

415

19**nm. Determination of total phenolic contents** (TPC) **Total phenolic contents of the test** samples waere **determined by** using **method** described by Astill **et al.,**

2001. 20µL of the test sample, positive control (gallic acid) and DMSO (negative control) were added to

27**96 well** microplate, **followed by addition of 90µL of Folin-Ciocalteu**

reagent and

27**incubated for 5 minutes** at room temperature. **After** incubation **90µL of sodium carbonate was added** into **the**

plate. Readings were taken at 630nm wavelength of microplate reader. Antioxidant Assays The antioxidant capacities of

32**the test samples were determined by**

three

60**assays; DPPH free radical scavenging assay, total antioxidant capacity** and **reducing power.**

32**DPPH free radical scavenging assay** The **free radical scavenging activity of the test samples**

against 2, 2-diphenyl-1-picrylhydrazyl reagent (DPPH)

14**was determined according to the** protocol **described by** Clarke **et al.,** 2013 In **a**

96 well microplate, 10µL of test sample, standard (ascorbic acid) and blank were added. Then 190µL of DPPH reagent was added and incubated for 1 hour at 37°C. Further readings were noted at 517nm wavelength on microplate reader

56**Total antioxidant capacity** determination **The total antioxidant capacity of**

the fresh weight extracts **was determined by**

Clarke et al., 2013. In appendorf tubes, 100µL of test sample, standard and blank was taken and mixed by adding 900µL of antioxidant reagent. The

21**tubes were** then **incubated at** 95 **°C for 90** minutes. After incubation **the** reaction **mixture was cooled to room temperature and** 200µL **of** sample **was**

transferred to microplate. Optical density was measured at 630nm on microplate reader. Total

55**reducing power determination The reducing power of the** test samples **was** investigated **according to the**

protocol described by Jafri et al. (2014). 100µL of test sample, positive control (ascorbic acid) and negative control (DMSO) were taken and then 200µL of phosphate buffer

44**was added, followed by addition of** 250µL **of 1%**

potassium ferricyanide

14**into the** eppendorf **tubes. The mixture was** then **incubated at 50°C for 20** minutes. **After incubation,** 200µL **of** 10% **TCA was added. The** reaction mixture was **centrifuged at** 3000rpm **for 10**

minutes. Carefully supernatant layer, 150µL was picked and poured into microplate well and then 50µL of 0.1% ferric chloride was added. Readings were taken at 630nm using microplate reader.

33**Statistical Analysis Each treatment was conducted in** duplicate **and the results were presented as mean values with respective standard deviations.**

Chapter 4 RESULTS Objectives of this were to determine the effect of CuONPs on seed germination frequency of medicinally important plant Trigonella foenum graecum L. as well as on growth parameters including shoot and root elongation. CuONPs were used as elicitors to predict either they increase or decrease seed germination. Three different types of CuONPs were used to test the effect; CuONP, CuONP-PEG and CuONP-PVP at four different concentrations. The data was collected on 5th day after inoculation of seeds and it showed significant effect on seed germination frequency, root and shoot elongation along with biochemical profile showing variations at different concentrations. Effect of CuONPs on seed germination Seeds were germinated on four different concentrations of CuONP, CuONP-PEG and CuONP- PVP i.e.50, 100, 200 and 400mg/L, PEG and PVP; 10, 20, 40 and 80mg/L, two different salt concentrations of copper acetate (Cu-acetate); 0.5 and 1% and control 0 (simple MS medium). After 5 days of inoculation final germination percentage (%) was 100% in control, CuONP-PEG, PEG, PVP and Cu-acetate at all concentrations. Whereas it was 90 and 80% in CuONP at concentrations 50, 100 and 400mg/L respectively and 100% at 200mg/L. Seed germination frequency of CuONP-PVP was 90% at 50 and 400mg/L while 100% at 100 and 200mg/L (Table 4.1). No significant difference was found on seed germination frequency. Table 4.1: Seed germination frequency of Trigonella foenum graecum L. on CuONPs Treatment Concentration Final RG MPFG Germination (mg/L) germination (%) index % inhibition 50 CuONP 100 200 400 90 1.8 80 1.6 100 2.0 80 1.6 0.5 0.9 0.5 0.8 0.5 1.0 0.5 0.8 10 20 0 20 CuONP-PEG 50 100 200 400 100 2.0 0.5 1.0 0 100 2.0 0.5 1.0 0 100 2.0 0.5 1.0 0 100 2.0 0.5 1.0 0 CuONP-PVP 50 100 200 400 90 100 100 90 1.8 2.0 2.0 1.8 0.5 0.5 0.5 0.5 0.9 1.0 1.0 0.9 10 0 0 10 100 2.0 0.5 1.0 0 100 2.0 0.5 1.0 0 100 2.0 0.5 1.0 0 10 PEG 20 40 80 100 2.0 0.5 1.0 0 10 PVP 20 40 80 90 1.8 100

2.0 100 2.0 100 2.0 0.5 0.9 0.5 1.0 0.5 1.0 0.5 1.0 10 0 0 0 Cu-acetate 0.5% 1% 100 100 2.0 2.0 0.5 1.0 0 0.5 1.0 0 Control 0 100 2.0 0.5 1.0 0

9**Root and Shoot Length** Average **root and shoot length of** plantlets **were recorded after**

5 days of inoculation on all the concentrations. In control average shoot length of plantlet was 7.2 cm and root length 4.1 cm (Table 4.2). Maximum shoot length was found at CuONP, 50mg/L i.e. 7.7cm and gradual decrease as the concentration of nanoparticles increased. Data has shown the significant decrease in shoot length treated with Cu-acetate at 0.5%, 4.9cm and 1%, 4.3cm as well as significant decrease in root length i.e. 2.3cm at 0.5% and 1.6cm at 1% (figure 4.1). Whereas maximum root length was 6.8cm at 50mg/L of CuONP-PEG and trend gradually decreased as concentration of nanoparticles increased (figure 4.2) and same results were found for CuONP-PVP (figure 4.3). Table 4.2: Average shoot and root length of Trigonella foenum graecum L. plantlets Treatment Concentration Average shoot length Average root length (mg/L) (cm) (cm) CuONP 50 7.7 100 6.5 200 6.4 400 6.3 6.6 5.1 4.4 4.3 CuONP-PEG 50 7.0 6.8 100 6.9 6.1 200 6.8 6.0 400 5.4 3.0 CuONP-PVP 50 100 200 400 7.0 6.7 6.6 6.6 5.9 5.8 4.6 3.4 10 6.0 3.3 20 6.1 4.2 40 7.6 2.2 80 7.6 5.8 PEG 10 PVP 20 40 80 6.6 3.7 6.5 3.6 6.2 3.6 5.3 3.5 Cu-acetate 0.5% 1% 4.9 2.3 4.3 1.6 Control 0 7.2 4.1 Figure 4.1: Effect of CuONP and Cu-acetate on root and shoot length of T. foenum graecum L. at different concentrations Figure 4.2: Effect of CuONP-PEG and PEG on root and shoot length of T. foenum graecum L. at different concentrations Figure 4.3: Effect of CuONP-PVP and PVP on root and shoot length of T. foenum graecum L. at different concentrations Average

9**fresh and dry weight** Average fresh weight **of whole plantlet was calculated after** 5 days **of inoculation**

and dry weight was calculated after overnight period of drying in open air. Fresh and dry weight of control was 0.28g and 0.04g. Fresh weight was recorded maximum at CuONP 50mg/L, 0.47g as well as maximum dry weight, 0.05g (Table 3). Table 4.3: Average fresh and dry weight of Trigonella foenum graecum L. plantlets Treatment Concentration Average fresh weight Average dry weight (mg/L) (g) (g) CuONP 50 0.47 100 0.42 200 0.13 400 0.11 0.05 0.03 0.01 0.01 CuONP-PEG 50 0.34 0.04 100 0.32 0.03 200 0.20 0.02 400 0.31 0.03 CuONP-PVP 50 100 200 400 0.43 0.31 0.32 0.31 0.04 0.03 0.03 0.02 PEG 10 0.28 0.04 20 0.26 0.03 40 0.24 0.03 80 0.34 0.03 PVP 10 20 40 80 0.32 0.29 0.45 0.13 0.04 0.04 0.03 0.01 Cu-acetate 0.5% 1% 0.19 0.02 0.25 0.03 Control 0 0.28 0.04 Biochemical screening

9**Total flavonoid and phenolic content According to the results obtained after performing total flavonoid and** phenolic **activities,** the **trend** has shown **maximum** flavonoids **and** phenolic accumulation **in** Cu-acetate treated **plantlets**

as compared to control and CuONPs treatment (figure 4.4 and 4.5). Whereas in plantlets treated with PEG have shown significantly higher flavonoid and phenolic content in comparison with CuONP-PEG and control (figure 4.6 and 4.7). In addition to these, CuONP-PVP and control have shown much lower contents of flavonoid and phenolic as compared to PVP treatment (figure 4.8 and 4.9). Figure 4.4: Effect of CuONP and Cu-acetate on total flavonoid content derived from shoot and root extracts Figure 4.5: Effect of CuONP and Cu-acetate on total phenolic content derived from shoot and root extracts Figure 4.6: Effect of CuONP-PEG and PEG on total flavonoid content derived from shoot and root extracts Figure 4.7: Effect of CuONP-PEG and PEG on total phenolic content derived from shoot and root extracts Figure 4.8: Effect of CuONP-PVP and PVP on total flavonoid content derived from shoot and root extracts of T. foenum graecum L. Figure 4.9: Effect of CuONP-PVP and PVP on total phenolic content derived from shoot and root extracts

20**DPPH radical scavenging activity DPPH activity was determined**

in plantlets treated with nanoparticles, PEG, PVP and compared with control. The results have shown that Cu-acetate at concentration of 1% has maximum radical scavenging activity in both root and shoot extracts as compared to CuONP and control (figure 4.10). But in case of CuONP-PEG, PEG and control, CuONP-PEG has shown maximum scavenging activity at 50mg/L and trend gradually decreased as concentration of nanoparticles is increased (figure 4.11). The free radical scavenging activity of CuONP-PVP was found to be maximum in root extracts at concentration 100mg/L whereas seedlings grown on PVP have also shown increased DPPH radical scavenging activity as compared to control (figure 4.12).

23**Figure 4.** 10: **DPPH radical scavenging activity of** root **and**

shoot extracts of T. foenum graecum L. against different concentrations of CuONP and Cu-acetate

23**Figure 4.** 11: **DPPH radical scavenging activity of** root **and**

shoot extracts of T. foenum graecum L. against different concentrations of CuONP-PEG and PEG

23**Figure 4.** 12: **DPPH radical scavenging activity of** root **and**

shoot extracts of T. foenum graecum L. against

21**different concentrations of** CuONP-PVP **and** PVP **Total antioxidant capacity The**

total antioxidant assay results have shown maximum activity in plantlets tested on Cu- acetate as compared to control and CuONP derived from shoot extracts but roots extract data revealed maximum antioxidant activity in CuONP and Cu-acetate as compared to control (figure 4.13). CuONP-PEG tested root and shoot extracts have shown maximum antioxidant activity as compared to PEG and control (figure 4.14). Maximum TAC activity was observed in PVP treated plantlets as compared to CuONP-PVP and control (figure 4.15). Figure 4.13: Total antioxidant activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP and Cu-acetate Figure 4.14: Total antioxidant activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP-PEG and PEG Figure 4.15: Total antioxidant activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP-PVP and PVP Total reducing power According to the results to total reducing power assay, maximum activity of shoot extracts was found in Cu-acetate and maximum reducing power of root shown by CuONP as compared to control (figure 4.16). CuONP-PEG have shown maximum total reducing power as compared to PEG and control (figure 4.17). Whereas, in case of CuONP-PVP, PVP and control, PVP have increased reducing power activity as compared to others (figure 4.18). Figure 4.16: Total reducing power activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP and Cu-acetate Figure 4.17: Total reducing power activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP-PEG and PEG Figure 4.18: Total reducing power activity of root and shoot extracts of T. foenum graecum L. against different concentrations of CuONP-PVP and PVP Callus Induction Effect of CuONP, CuONP-PEG and CuONP-PVP was determined at three concentrations; 2.5, 5 and 10mg/L on callus induction from shoot and leaf explant. The experiment was conducted over a time period of 30 days. After 30 days, calli were collected and their average fresh weight and dry weight were calculated as well as biochemical profiling was performed. Average fresh and dry weight of explant Average fresh weight and dry weight were calculated for both stem and leaf explant. According to the calculated values average fresh weight of control both in stem, 1.65 g and leaf, 1.33g explants was higher as compared to the explants treated with different concentrations of nanoparticles. Whereas dry weight was also found to be higher in control i.e. 0.10g for stem explant and 0.09g for leaf explant (Table 4). Table 4.4: Average fresh and dry weight of T. foenum graecum L. plantlets treated with CuONPs Treatment Concentration Average fresh Average dry weight/explant (mg/L) weight/explant (g) (g) Stem Leaf Stem Leaf CuONP 2.5 5 10 0.67 0.25 1.33 0.85 0.12 0.13 0.05 0.02 0.07 0.07 0.01 0.01

CuONP-PEG 2.5 5 10 1.25 0.99 0.82 0.11 0.33 0.21 0.08 0.05 0.06 0.01 0.02 0.02 CuONP-PVP 2.5 5 10 1.13 1.31 1.42 1.24 0.14 0.12 0.07 0.08 0.09 0.08 0.02 0.01 Control 0 1.65 1.33 0.10 0.09 Biochemical Screening Total flavonoid content According to the data, total flavonoid contents was found to be maximum in shoot and leaf explants treated with CuONP-PVP at all three concentrations 2.5, 5 and 10mg/L as compared to CuONP, CuONP-PEG and control (figure 4.19 and 4.20). Figure 4.19: Effect of CuONP, CuONP-PEG and CuONP-PVP on total flavonoid content derived from shoot explant of T. foenum graecum L. Figure 4.20: Effect of CuONP, CuONP-PEG and CuONP-PVP on total flavonoid content derived from leaf explant of T. foenum graecum L. Total phenolic content Results have shown maximum phenolic contents accumulation in CuONP-PVP treated expalnts of shoot as compared to CuONP, CuONP-PEG and control (figure 4.21). Whereas in leaf explants maximum phenolic contents found at concentration 2.5mg/L of CuONP-PEG i.e. equivalent to control (figure 4.22). Figure 4.21: Effect of CuONP, CuONP-PEG and CuONP-PVP on total phenolic content derived from shoot explant of T. foenum graecum L. Figure 4.22: Effect of CuONP, CuONP-PEG and CuONP-PVP on total phenolic content derived from leaf explant of T. foenum graecum L. Total antioxidant capacity Total antioxidant capacity was found maximum in control and CuONP-PVP as compared to other nanoparticles treatment derived from shoot explant (figure 4.23). The trend of total antioxidant capacity derived from leaf explant was found maximum in CuONP and CuONP-PEG treated explants as compared to CuONP-PVP and control (figure 4.24). Figure 4.23: Effect of CuONP, CuONP-PEG and CuONP-PVP on total antioxidant capacity derived from shoot explant of T. foenum graecum L. Figure 4.24: Effect of CuONP, CuONP-PEG and CuONP-PVP on total antioxidant capacity derived from leaf explant of T. foenum graecum L. Total reducing power Total reducing power assay results have shown maximum activity in shoot explants treated with CuONP-PVP as compared to control, CuONP and CuONP-PEG (figure 4.25). In case of leaf explants, maximum reducing power was found in CuONP treated explants (figure 4.26). Figure 4.25: Effect of CuONP, CuONP-PEG and CuONP-PVP on total reducing power derived from shoot explant of T. foenum graecum L. Figure 4.26: Effect of CuONP, CuONP-PEG and CuONP-PVP on total reducing power derived from leaf explant of T. foenum graecum L.

20**DPPH radical scavenging activity DPPH radical scavenging activity was**

found maximum in shoot explants treated with CuONP- PEG, CuONP-PVP and control and decreased activity has been shown by CuONP (figure 4.27). Whereas maximum scavenging activity was shown by CuONP-PEG as compared to others in leaf explant (figure 4.28). Figure 4.27: Effect of CuONP, CuONP-PEG and CuONP-PVP on DPPH radical scavenging activity derived from shoot explant of T. foenum graecum L. Figure 4.28: Effect of CuONP, CuONP-PEG and CuONP-PVP on DPPH radical scavenging activity derived from leaf explant of T. foenum graecum L. Chapter 5 Discussion Nanoscience has expanded greatly in the past decade due to unique properties exhibited by nanoparticles. Thus, nanoparticles are becoming main source of revolutionizing the industry and playing significant role in economy (Rosei, 2004). Nanotechnology has also permitted advanced research in areas including biotechnology and agriculture to improve crop yield, thus using nanoparticles as nanofertilizers. Nanoparticles possess features that are beneficial to crops, released the nutrients on-demand, controlled release of chemicals fertilizers that regulate plant growth and enhanced target activity (DeRosa et al., 2010; Nair et al., 2010). Nonetheless, technology always comes with price, due to widespread use of nanoparticles there are concerns related to environment and health i.e. taken up by living organisms especially plants and entering into food chain (Leacoanet et al., 2004; Auffan et al., 2011). Many researches have shown the effect of engineered nanoparticles, especially metal nanoparticles on the plants including morphology and physiology depending on the properties of nanoparticles. The objectives of current study were to investigate the effect copper oxide nanoparticles

47**on seed germination,** root **and** shoot elongation **of** T. **foenum** graecum **L.**

at various concentrations using tissue culture technique and also evaluating antioxidant activity of plantlets treated with CuONP, CuONP-PEG, CuONP-PVP, PVP and PEG. Almost all treatments have shown 100% seed germination and no adverse effect was observed. This data was in accordance with the study conducted by Adhikari et al. (2012), showing 100% germination of seeds of soyabean and chickpea grown on CuONP. In addition to this in another experiment, fenugreek seeds were treated with AgNPs and results have shown increased seed germination enhancing the seed potential by increasing the

characteristics of seed germination (Hojjat, 2015). Seed germination is a physiological process and widely used as phytotoxicity test because it is sensitive, simple and low cost and it depends on nanoparticle-plant physical interactions (Wang et al., 2001; Munzuroglu and Geckil, 2002). It also depends on size of the nanoparticles. CuONP-PEG, 47nm and showed no inhibitory effects on seed germination (Table 4.1). Germination starts

24**with water** imbibitions **by seeds and** ending with **the emergence of rootlet.**

Polyethylene glycol (PEG) is used to control the water potential and induce drought stress in seed germination studies. The results of this study have shown seed germination frequency was not affected by PEG treatment. But shoot and root elongation results were not the same as seed germination and showed variations at different concentrations of nanoparticles. CuONP has shown maximum shoot elongation i.e. 7.7cm at 50mg/L as compared to control but trend gradually decreased as concentration of nanoparticles have increased and trend was followed in plantlets treated with CuONP-PEG and CuONP-PVP. But significant shoot inhibition has been observed in seedlings treated with Cu-acetate salt i.e. 4.3cm at 1% concentration. Thus indicating minimal toxicity of copper oxide nanoparticles on shoot growth with increase in concentration in contrast to copper acetate salt. Whereas in case of PEG, average shoot length have shown to be increased at higher concentrations in contrast to previous study in which PEG has significantly reduced the shoot growth in two populations of Anthxanthum odoratum performed by Anwer et al. (2004). Average shoot elongation was decreased from lower to higher concentrations of PVP. Root elongation has not been adversely affected by CuONPs and other two forms as compared to control but copper acetate salt has inhibited the root elongation significantly. At lower concentrations CuONPs have shown maximum root elongation but it was inhibited with increase in concentrations of nanopartilcles in the media showing phytoxicity and evidently terminated root development. PEG and PVP also inhibited root growth due to osmotic stress induced by PEG and in accordance with the results of Anwer et al. (2004). Thus phytoxoicity should be investigated in higher plants for comprehensive toxicity profile of nanoparticles. Average fresh and dry weight was found to be maximum in CuONP at lowest concentration as compared to control but gradual decreasing trend was

18**observed. The increase in biomass at certain concentration suggests optimum dose limit for the growth of**

fenugreek.

18**However the decrease in biomass beyond this concentration** suggested **the toxic** effects **of**

CuONPs. Total flavonoid and phenolic contents were determined and results have shown that ethanolic extracts of root have accumulated more flavonoids and phenols as compared to control root extracts while plantlets treated with Cu-acetate salt has accumulated maximum phenolic contents indicating the elicitation of secondary metabolites production in the presence of abiotic stress. The same trend was seen in the shoot extracts and CuONPs have shown variations in the flavonoid and phenolic content accumulation. Reactive oxygen species are proposed to be responsible for negative effects of nanoparticles (Nel et al., 2006) but toxicity mechanism of nanoparticles has not yet been clearly understood. Antioxidant activity of T. foenum graecum L. treated with CuONPs was determined in the current study. According to results obtained have shown less significant difference on exposure to CuONP, CuONP-PEG and CuONP-PVP as well as reducing power. Callus Induction Micropropagation is an interesting method to that can be economically exploited for medicinal as well as ornamental purposes. Callus culture, a type of plant tissue culture consisting of undifferentiated tissues, constitutes an important tool in plant biotechnology. It can be used in numerous ways, for example, for organogenesis1, indirect somatic embryogenesis, and generation of somaclonal variation (Jattana et al., 2008; Homhuan et al., 2008). Fenugreek is a plant with medicinal properties and callus culture is an alternative method to enhance the production of secondary metabolites like flavonoids, phenolics and antioxidants. The best growth of callus was obtained from stem and leaf explants grown on MS0 medium and it was determined by fresh weight, 1.65g (stem explant) and 0.10g

(leaf explant) as compared to callus grown on CuONP after 30 days of growth. The phytochemical assays results of this study have shown more flavonoid and phenolic content accumulation in explants of stem and leaf treated with capped CuONP i.e. CuONP-PVP as compared to other two forms of nanoparticles and control. While DPPH radical scavenging activity and antioxidant activity was evidently higher in explants treated with CuONP-PEG. These results indicate that capped nanoparticles have more phytotoxic effects on explants as compared to simple CuONP. Overall experimental results depicted that presence of CuO nanoparticles affects the

47 **growth of T. foenum graecum** L. seedlings **at** different **concentrations**

and it depends on plant-nanoparticle interaction as well as depend on the size and type of nanoparticles and species of plant.