Chromatographic Analysis of Opium (*Papaver somniferium*) Cultured on ZnO NPs Stress

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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 "Chromatographic Analysis of Opium (*Papaver somniferum*) Cultured on ZnO NPs Stress" submitted by Beenish Aslam in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in Biotechnology.

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Dedicated to my dear parents

And

Husband.

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Beenish Aslam

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List of Abbreviations

ZnO NPs	Zinc oxide Nanoparticles
NPs	Nanoparticles
NMs	Nanomaterials
ENPs	Engineered Nanoparticles
MNPs	Metallic Nanoparticles
AAE	Ascorbic acid equivalent
GAE	Gallic acid equivalent
TPC	Total phenolic content
TFC	Total flavonoid content
DPPH	2,2-diphenyl-1-picrylhydrazyl
TRP	Total reducing power
TAC	Total antioxidant capacity
FW	Fresh Weight
FC	Follin Ciocalteu
SD	Standard deviation
UV	Ultra-violet

Abstract

Nanotechnology, the emerging discipline of conjugative science, has tremendous applications in medical, agriculture, environmental and many other fields. However, beside the beneficial effects, there are many concerns regarding use of nanomaterials. The most common alert is toxicity especially regarding metallic nanoparticles as their release in water, soil and air may affect all life from microbial to plant and animals. To disclose negative effect of nanoparticles on plant growth, the work was carried on ZnO nanoparticles and *Papaver somniferum.* Initially seed germination efficiency was analysed followed by plant physiological characteristics. Furthermore, to analyse toxic effect of NPs on plant metabolic pathways, different phytochemical analysis were performed. The results show that low to moderate concentration of ZnO NPs has nonsignificant effect on seed germination however, on plant architecture, length and weight parameters, significant difference is observed though the difference was concentration dependent. The results also reveal that NPs presence caused oxidative stress on plants therefore increased level of total antioxidative activity, total reducing power potential and DPPH based free radical scavenging activity were observed elevated with increase of ZnO concentration in the media. It was further observed that to combat oxidative stress, non-enzymatic antioxidants, phenolics and flavonoids concentrations increase in stressed plants. HPLC analyses on plants extracts shows that gallic acid and caffeic acid concentrations increased in the *P. somniferum* plants presuming that these phenolic molecules play major role to minimize oxidative stress caused by the nanoparticles.

1 Introduction and Review of Literature

1.1 Nanotechnology

Nanotechnology can be defined as (Lou, Archer and Yang 2008)

"Nanotechnology is the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometre scale". NPs are engineered materials with at least one dimension less than 100 nm. They are elementary unit for nanotechnology. NPs can be of three dimensions on the nanoscale i.e. one dimension (e.g., Nano layers), two dimensions (nanowires and nanotubes), or three dimensions (NPs, quantum dots, metal NPs and fullerenes) (Ju-Nam and Lead 2008). Nanotechnology and NPs turned up as a fast growing and provoking area of research. It has gained the attention of scientific society globally (Feynman 1960). Nanotechnology is a field with an extraordinary potential to make new items fusing unmistakable properties and enhancing the items for different applications. Many nanotechnology-based products are now accessible in the business sector, including wearing products, gadgets, individual consideration, and car parts (Kang, Mackey and El-Sayed 2010). It was evaluated that by 2010, $11 trillion business sector will be spoken to by nanotechnology (Pitkethly 2004).

Materials at Nano-scale have properties not the same as their mass reciprocals and this uniqueness is the reason that the worldwide consideration is now on nanotechnology. Properties of materials change perceptibly when their size decrease to nanometres. Because of noteworthy uses of Nano-particles in different mechanical fields, the physical and synthetic properties of metal oxide nanomaterials have been incredibly illustrated and portrayed (Devan et al. 2012). Nanotechnology is gradually moving out from the experimental into the practical regime and is making its presence felt in agriculture and the food processing industry. Nanotechnology applications in agriculture are gradually moving from the theoretical possibilities into the applicable realms (Baruah and Dutta 2009)

The prefix "Nano" is gotten from the old Greek "Nanos", signifying "minor person". Today, "Nano" is utilized as a prefix that signifies "billionth" or a component of 10^{-9}. Coupling "Nano" with the unit "meter" brings the expression "nanometre", which shows a unit of spatial estimation that is one billionth of a meter. In view of this, nanotechnology should be characterized as the science, building, and innovation led at the scale that reaches between 1 to 100 nanometres. (Peng et al. 2009). Nano scaled particles are thought to be the one with size under 100nm no less than one measurement (Tambe and Bhushan 2004). This to a great degree little size of NPs, gives special physical and concoction properties that outcomes in higher reactivity with expansive surface region, making NPs attainable to use in countless for instance paints, beautifying agents, prescriptions, sustenance and suntan salves, and additionally applications which straightforwardly discharge NPs into nature, for example, remediation of contaminated situations (Maynard et al. 2006).

1.2Nanoparticles

The term Nano is gotten from Greek word, which signifies "predominate". Width of a DNA particle is 2.5 nm, while that of a protein is 50nm. An influenza infection was measured having width of roughly 100 nm and human hair thickness is around 10,000 nm. Infinitesimal molecule with no less than one measurement, having size under 100nm, is considered as nanoparticle. NPs are spanning up the hole between mass materials and nuclear or sub-atomic structures. Not at all like NPs, mass materials have consistent physical properties paying little mind to their size. The Nano-scale investigations of mass materials (well characterized) prompted the revelation of intriguing properties in them (Thakkar, Mhatre and Parikh 2010). Numerous extensive concerns have been raised with quick advancement and potential arrival of engineered NPs (ENPs), by considering the specific properties of NPs. To comprehend the interaction of ENPs with plants (base part of biological system), is an essential part of hazard evaluation of ENPs. Measure, piece, fixation and critical physical and substance properties of ENPs and plant species, decides the degree of effect of ENPs on plant. Reports have been recorded demonstrating the impacts of ENPs (both enhancive and inhibitive impacts) on plant development at various phases of improvement. The size, quantity, shape of ENPs and plant life systems significantly impact the uptake of ENPs by plant roots and its potential transport to shoots through vascular framework (Liu et al. 2010).

1.2.1 Types of Nanoparticles

There are different types of nanoparticles. Some of them are given as below.

Silver: Silver nanoparticles are known as one of the best having antimicrobial efficacy against virus, bacteria and eukaryotic micro-organisms (Gong et al. 2007). Silver nanoparticles are widely used nanoparticles in different fields like textile industry, antimicrobial agents and water treatments etc. (Rai and Ingle 2012, Rai, Yadav and Gade 2009).

Gold: These nanoparticles are commonly used in the diagnostic studies and in the field of immunochemistry to examine the protein interactions with different chemicals. These nanoparticles are widely used as tracers in the lab of DNA fingerprinting where these are being used to detect presence of DNA in the test samples. In the medical field these nanoparticles are used in the detection of aminoglycoside antibiotics. Moreover, Gold nanoparticles are also used in the field of oncology where these are used to detect the cancer cells. In the field of microbiology these are used to identify the types and classes of bacteria (Baban and Seymour 1998).

Alloy: These nanoparticles depicts different structural properties as compare to its bulk samples (Yun et al. 2008). One example of alloy nanoparticles is Ag nanoparticles and these have highest electrical conductivity and because of this property alloy nanoparticle are being widely used as well in the research field. The properties of alloy nanoparticles are under influence of metallic nanoparticles but have more advantages as compare to ordinary metallic nanoparticle (Goyal et al. 2011).

Magnetic: The examples of magnetic nanoparticles are Fe3O4 (magnetite) and Fe2O3 (maghemite) and these two are known as biocompatible with each other. These types of nanoparticles are widely used in the treatment of cancer, drug delivery systems, stem cell sorting and manipulation. These are also used in the field of DNA analysis, gene therapy and resonance imaging (Fan, Chow and Zhang 2009).

1.2.2 Characterization of Nanoparticles

i. Normal NPs; Natural nanoparticles have existed from the earliest starting point of the earth' history and still happen in the earth (volcanic dust, lunar dust, mineral composites)

ii. Accidental NPs; likewise characterized as waste or anthropogenic particles, occur as the after effect of artificial mechanical procedures (diesel fumes, coal burning, welding vapour, and so on.)

iii. Engineered NPs; are further gathered into four classifications;

a) carbon based NPs which incorporate fullerene, single walled carbon nanotube (SWCNT) and multiwall carbon nanotubes (MWCNT);

b) metal based NPs including quantum dabs, Nano gold, Nano zinc, nonaluminum and nanoscales metal oxides like TiO2, ZnO and Al2O3;

c) dendrimers are Nano-sized polymers worked from fanned units, which are skilled to be intended to perform particular compound capacity;
d) composites which consolidate nanoparticles with different nanoparticles or with bigger mass sort materials (Lin and Xing 2008) and present distinctive morphologies, for example, circles, tubes, bars and crystals (Ju-Nam and Lead 2008).

ENPs have picked up a ton of significance because of their extensive uses in industry and enhancing the economy parts, for example, shopper items, pharmaceutics, cosmetics, transportation, vitality and farming and so on. (Roco 2003). Metallic nanoparticles (MNPs) have a central position in the classification of ENPs because of their one of a kind element, for example, electronic, optical, mechanical, attractive and compound properties that can be essentially not quite the same as those of mass materials (Boddu et al. 2011).

Nanoparticles occur in a number shapes including, spherical, cube, rods, wires, tetrapod, and octahedron. Both kinetic and thermodynamic factors control the shape of nanoparticles. It has been demonstrated that nanoparticles show shape dependent properties as well.

1.3 Metal nanoparticles

MNPs union is a dynamic area in research and more information is required about nanotechnology. Union of metallic NPs utilizes various physical and synthetic methodologies. However, the restrictions being used of these techniques incorporate the utilization of solvents of dangerous nature, high energy utilization, and arrangement of dangerous side effects. Improvement of environment inviting strategies for combination of metallic NPs is the need of present age. Abusing the variety of organic assets introduce in nature is one approach to accomplish this target. The act of creation of minimal effort, nontoxic and vitality proficient NPs utilizing plants, microscopic organisms, infections, green growth and parasites, has been accounted for in the course of recent years (Thakkar et al. 2010).

MNPs are presently routinely utilized as a part of ventures for variety of uses. Metal nanoparticles have a place for the most part with the engineered nanoparticles and have exceptional physical and synthetic properties as well as various organic activities. In the previous decade, research endeavours in nanoscience and nanotechnology have become dangerously worldwide (Lukačová et al. 2013). In any case, their discharge in environment makes them an undermining specialist to living creatures as it were of harmfulness. The toxic impacts are because of size, surface area proportion, morphology, nature, piece, reactivity, and others (Zaka et al. 2016). Opium Poppy: Botany, Chemistry, and Pharmacology. Joined States: CRC Press. p. 164. ISBN 1-56024-923-4.) It might be utilized straightforwardly or artificially changed to deliver manufactured opioids, for example, heroin. It might be utilized specifically or artificially adjusted to deliver manufactured opioids, for example, heroin.

Table 1.1: Classification and characteristics of nanomaterials

Classifications of nanomaterials	Description
Nanoparticles	Submicron or even ultra-micron size particles possible as elite brilliant resistant materials, magnetic materials, sun based battery materials, bundling materials, and attractive liquid materials.
Nanotubes and nanofibers	Nanometre estimate long linear material, optical materials, micro conductors, microfibers, nanotubes of PEEK, PET, and PTFE
Nano films	Films used as gas impetus materials
Nano block	Nanometre crystalline materials created by considerable accuracy, developing controlled crystallization or nanoparticles
Nanocomposites	Composite nanomaterials, which use Nano size fortifications rather than ordinary filaments or particulates

Nanocrystal line solids	Polycrystals with the extent of 1 to 10 nm and half or more of strong comprises of inborn interface amongst precious stones and diverse orientations. The groups that shaped through homogenous (Aslani et al., 2014) nucleation and develop by blend and consolidation of iotas.

(Aslani et al., 2014).

Metal oxide nanoparticles are as of now made in substantial scale for both contemporary and domestic component use, yet hold significantly more noteworthy guarantee for future applications. For instance, titanium dioxide nanoparticles are as of now utilized as UV-blocking agents as a part of sunscreens (Popov et al. 2005), in photocatalytic water cleansing (Hagfeldt, Björkstén and Grätzel 1996) and will probably be utilized as a part of the new era of sun oriented cells (Wang 2004). Zinc oxide nanoparticles are a starting material for hardware applications, straightforward UV assurance movies and synthetic sensors (Meulenkamp 1998) and additionally UV channels in sunscreens (Serpone, Dondi and Albini 2007). Through assembling and family squander the metal oxide nanoparticles are prone to wind up in characteristic water bodies. While the novel properties of nanoparticles are increasingly concentrated, little is known of their cooperation with (Heinlaan et al. 2008). In agro-nanotechnology, analysts effectively presented different Nano stages under invitro conditions. Protected and controlled arrival of agro chemicals and site specific targeting of different macromolecules required for the enhanced plant disease resistance, expanded capability of supplement usage and increased plant development, are advantages which could be accomplished through this innovation. More productive utilize and more secure treatment of pesticides guarantees the Eco-assurance by less introduction to the earth, is one of the liven of Nano encapsulation procedures. Plants demonstrate distinction in take-up capability of NPs furthermore the impact of NPs on plant development and metabolic capacities varies from plant to plant. Change in plant species is presently conceivable through hereditary alteration by abusing the NP mediated change strategies. Focusing on particular agrarian issues in plant-pathogen interaction and new strategies for harvest insurance, are created by applying nanoparticle innovation in plant pathology field (Nair et al. 2010).

The capacity of materials for supercapacitors relies on upon vast surface range per weight or film thickness. The one of a kind size related physical properties are not subject to this geometrical thought. Tenable light emanating gadgets consolidates the utilization of size reliance of the iridescence wavelength of the ZnO NPs. Estimate subordinate properties and high surface region assume critical part in catalysis applications. Molecule estimate has noteworthy significance in surface vitality and vitality of surface destinations, because of which the reactant movement might be improved by changing the size related physical properties. High surface zone sensors additionally misuse similar idea, and further research is required to completely comprehend the clarification of components in such applications. The controlled amalgamation of NPs with moderately little size circulation in the creation related systems of high surface zone materials, has distinctive preferences among which regularly known are control over film dispersion and film properties. For instance, thin, permeable films of TiO_2 NPs are straightforward to obvious light. ZnO films are utilized as a part of curative industry like in manufacture of sun square cream. The sun piece cream is straightforward rather than white glue due to the use of ZnO NPs manufactured in controlled way (despite the fact that not quantum-sized). NPs of variety of materials blended in controlled way can bring about better direct leading oxides, electrochromic or savvy windows, and so on. Colour sharpened sun oriented cells are developed by utilizing permeable, nanostructured metal oxide films sharpened with a colour and they require high surface area for adsorption of substantial number of colour particles.

The proficiency of these sunlight based cells is significantly enhanced by utilizing TiO_2 NPs blended as a part of controlled way. Field of use and utilization of metal oxide NPs blended just on the size reliance of the physical properties is not yet completely investigated. To find the maximum capacity of size-ward metal oxide NPs and their applications in new skylines needs assist logical research (Oskam 2006). Ranges where metallic NPs are connected incorporate cosmetics, bundling, covering, biotechnology and gadgets. Single strands of DNA are covered with metallic NPs with no harm to the single stranded biomolecule. The medicinal symptomatic applications broadly utilize this covering system of DNA. To limit any objective organ, NPs are presented in the living body as these NPs can cross through the vasculature. This can prompt revelation of new helpful, biomedical and imaging applications (Thakkar et al. 2010).

Metal oxide NPs are extensively used as a part of food materials, chemicals, and organic sciences (Maynard et al. 2006). Recently, ENPs are being utilized as a part of channels and films for water filtration and diverse other natural purposes. An extensive variety of NPs are utilized for the creation of lighter yet more intense and element weapons furthermore utilized as a part of in chemical– natural arms indicators. NPs can likewise be utilized as a part of merchandise, for example, golf, tennis in the manufacturing of superior tires, in new helpful medications and in pharmaceutical items (Terrones et al. 2010). Recently, a few analysts have been pulled in to variety of doped NPs because of their improved proficiency for various applications. It has been observed that surface covering of ENPs gives better results as contrast with their uncovered frame. ENPs doped with reasonable material, can accomplish particular surface attributes which can make them an ideal contender for a specific application. Then again, sometimes doped NPs have been seen to be more poisonous than uncovered NPs. NPs have come up as another class of ecological contaminations that may extensively and proficiently impact the earth and human wellbeing in view of the dynamic advancement and use of nanotechnology. ENPs discharge into air, earthbound, and amphibian situations because of their expanding applications in business items and modern applications that in the long run result in their spreading (Roy and Srivastava 2015). In the third sort, the introduction to NPs can be through water and soil when the whole environment being presented to ENPs through the water and soil (Oberdörster, Oberdörster and Oberdörster 2005). The procedure of nanotechnology started with the era, control, and organization of nanomaterials, speaking to an area holding huge guarantee for an extensive variety of utilizations. Nanotechnology has turned into a powerfully creating industry, with different applications in vitality, materials, PC chips, producing, medicinal services, and therapeutic conclusion (Safari and Zarnegar 2014).

1.4 Applications of nanoparticles

The procedure of nanotechnology started with the era, control, and organization of nanomaterials, speaking to a territory holding huge guarantee for an extensive variety of utilizations. Nanotechnology has turned into a powerfully creating industry, with different applications in vitality, materials, PC chips, medicinal services, and therapeutic conclusion (Safari and Zarnegar 2014).

The wide range of centre materials accessible, combined with tuneable surface properties, make nanoparticles an incredible stage for an expansive scope of natural and biomedical applications. The utilization of nanomaterials in biotechnology consolidates the fields of material science and science. Nanoparticles give an especially (De, Ghosh and Rotello 2008). The one of a kind properties and utility of nanoparticles emerge from an assortment of qualities, including the comparative size of nanoparticles. (De et al. 2008).

It is trusted that there are more than 800 nanomaterial items right now accessible in the business sector, and it is required to increment throughout the following couple of years. Tus, through 2014, it likewise approximated that an abundance of 15% of all items on the overall business sector would have some kind of nanotechnology incorporated inside their generation forms. Nanomaterials are presently being made and utilized as a part of numerous items. Nonetheless, our insight into the human wellbeing impacts and ecological convergences of built nanomaterials or nanoparticles is incomplete. Humans are now presented to a scope of characteristic and man-made nanoparticles noticeable all around, and introduction by means of the evolved way of life, water supply, and therapeutic applications is likely. (Handy, Owen and Valsami-Jones 2008). The potential advantages of these new nanomaterials to the life sciences and human wellbeing is wonderful, and they incorporate sensors for ecological checking, Nano-drug-conveyance frameworks, bio robotics, Nano arrays, and nanoscale inserts in drug.

1.4.1 Diagnostics and Therapeutics

Nanotechnology is an interdisciplinary field coordinating building, science, science and pharmaceutical, along these lines making it valuable for demonstrative and helpful purposes. It has been effectively utilized the analysis of growth as well as to regard tumour by utilizing it as a drug carrier (Laroui et al. 2013). Recent advances have demonstrated that NPs have bio affinity property making it a test for atomic and cell imaging, focused on NP drugs for growth treatment, and coordinated Nano gadgets for early screening and discovery of tumour. These improvements offer energizing open doors for the advancement of customized treatment, in which the sub-atomic profiles of an individual's hereditary and protein biomarkers might be utilized to analyse and treat the patient's malignancy (Chen et al. 2008).

1.4.2 Nanoparticles and Agriculture

Designed NPs go about as a shrewd device for the productive delivery of composts, herbicides, pesticides and plant development controllers and so on. (Johnston, 2010). Uses of NPs has affected the before plant germination and upgraded the yield of plants for nourishment, fuel, and different uses (Ditta 2012). Nanoparticles have been utilized in various examines to show crop change. Carbon nanotubes (CNT) have expanded the seed germination in tomato (Khodakovskaya et al. 2009). Other than CNT, metal nanoparticles including gold (Au), silver (Ag), copper (Cu), zinc (Zn), aluminium (Al), silica (Si), zinc oxide (ZnO), cesium oxide (Ce2O3), titanium dioxide (TiO2) and polarized iron (Fe) have likewise found to enhance crop yield(Zhang and Webster 2009).In a study impact of Au-NPs on Brassica juncea was checked at various focuses and it was found that Au-NPs diminished general development of plant however expanded the free radical anxiety supporting increment in biomarker antioxidative catalysts, proline and hydrogen peroxide (Srinet, Kumar and Sajal 2014). In another study zinc and silver nanoparticles were utilized on two critical yields; *Zea mays* L. what's more, *Brassica oleracea* var. *capitata* L. what's more, both plants indicated lower nanoparticle poisonous quality when contrasted with the free particles (Pokhrel and Dubey 2013).

1.4.3 Nano and Energy

Energy comes most elevated on the deprived of needs in human needs (Zinkle and Ghoniem 2011). World's essential vitality interest will increase by 36% somewhere around 2008 and 2035 as indicated by International Energy Agency (IEA). Because of carbon dioxide discharge and atmosphere changes that are affecting life, renewable vitality assets need to assume indispensable part in growing more dependable and manageable vitality way. Sun oriented vitality is the most bounteous, interminable and unadulterated renewable vitality source to date and it can be bridled utilizing photovoltaic cells. Albeit inorganic semiconductors including silicon, gallium arsenide and sulphide salts are principally utilized yet natural materials with nanostructures are more invaluable as a result of ease and expansive scale fabricating forms (Zhang, Anninos and Norman 1995).

Nano metrology manages the estimation of practically essential, for the most part dimensional parameters and segments with no less than one basic measurement which is littler <100 nm. Accomplishment in Nano manufacturing of gadgets will depend on new Nano metrologies expected to gauge essential materials properties including their sensitivities to ecological conditions and their varieties, to control the nanofabrication procedures and materials functionalities, and to investigate disappointment components (Nomura et al. 2004).

1.5 Danger and toxicity of nanoparticles

In spite of the fact that nanotechnology has enormous number of valuable applications, yet innovation dependably accompanies a cost i.e. there are ecological and social insurance concerns identified with nanoparticles (Maynard et al. 2006). Nanoparticles go into the earth by purposeful or accidental means including waste streams from fabricate offices, volcanic emanations, modern procedures and transportation (Klaine et al. 2008). Nanotechnology and the use of nanoscale materials is a relatively new area of science and technology, and inevitably, the potential benefits of these new materials must be weighed against possible adverse effects and the public perception (Handy et al. 2008). Metal oxide nanoparticles are already manufactured in large scale for both industrial and household use, yet hold even greater promise for future applications. For example, titanium dioxide nanoparticles are already used as UV-blocking agent in sunscreens (Popov et al. 2005)), in photocatalytic water purification (Hagfeldt et al. 1996). Zinc oxide nanoparticles are a starting material for electronics applications, transparent UVprotection films and chemical sensors (Meulenkamp 1998). In the form of manufacturing and household waste the metal oxide nanoparticles are likely to end up in natural water bodies. While the novel properties of nanoparticles are increasingly being studied, little is known of their interactions with aquatic organisms (Heinlaan et al. 2008). In aquatic risk assessment algal growth inhibition assay is widely used (Kamo et al. 1982). Nanomaterials are now being manufactured and used in many products. However, our knowledge of the human health effects and environmental concentrations of engineered nanomaterials or nanoparticles is incomplete. Humans are already exposed to a range of natural and man-made nanoparticles in the air, and exposure via the food chain, water supply, and medical applications is likely (Handy & Shaw, 2007).

Nano toxicology can be characterizing as the examination of the collaboration amongst nanostructures and organic frameworks. It likewise clarifies the relationship between the physical and substance properties of nanostructures i.e. arrangement, estimate, shape, surface science, and conglomeration with acceptance of dangerous natural reactions (Maynard et al. 2006).

Nano-toxicology is the new zone of research which manages filling the crevices in information and to investigate and address the unfriendly wellbeing impacts identified with utilization of NPs (Donaldson et al. 2004). Nano toxicology is included in finding strong, solid and information guaranteed test strategies for nanomaterials in human and environment hazard evaluation (Lewinski, Colvin and Drezek 2008). Cytotoxicity identified with the nanomaterial is presently a noteworthy wellbeing concern as a result of the expanded utilization of the nanomaterials in field of organic applications. CuO NPs are accounted for to have higher cytotoxicity when contrasted with their mass partner and to other metal oxides NPs (Oberdörster et al. 2005). NPs enter living frameworks through purposeful and accidental discharges, for example, strong/fluid waste streams from assembling offices and environmental emanations. Nanomaterials can come into contact with living creatures by means of numerous courses (figure 2.2, for example, coincidental discharge, direct discharge from mechanical items or procedures, and in addition business items amid planned uses that thus enter the sewer-to-wastewater treatment plants (Zhang and Webster 2009).

Nanoparticles are available in the environment actually however there is a little work done on natural framework and arrival of NPs noticeable all around. They collect into the environment through diesel and it is worried because of a worldwide temperature alteration perspective. The key species in the air are sulphuric corrosive, nitric corrosive and natural gasses and creating optional NPs that are unpredictable. Consequently, expanding molecule number in the earth (Klaine et al. 2008).

1.6 Zinc oxide (ZnO)

Zinc oxide (ZnO) is an inorganic compound broadly utilized as a part of ordinary applications. ZnO is at present recorded as a for the most part perceived as sheltered (GRAS) material by the Food and Drug Administration and is utilized as sustenance additive. The approach of nanotechnology has driven the improvement of materials with new properties for use as antimicrobial specialists. Hence, ZnO in nanoscale has indicated antimicrobial properties and potential applications in nourishment safeguarding. ZnO nanoparticles have been fused in polymeric frameworks so as to give hostile to microbial movement to the bundling material and progress bundling properties. Among different metals, zinc (Zn) assumes indispensable part in biochemical, physiological and anatomical reactions yet underneath to edge level. Zinc oxide (ZnO) NPs are generally utilized as a part of paints, covering materials, therapeutic and individual consideration items, and some more. ZnO NPs are additionally utilized as UV defender and safeguard material. Beside their potential use, it has expanded natural and wellbeing dangers because of their association with organic and substance materials

(Chithrani et al., 2006). The creation of ZnO NPs is up to 528 tons/year and there is increment underway and usage with time (Zhang and Webster 2009).

There are limited studies investigated phytotoxicity of ZnO nanoparticles on plants. Some plant species, i.e., assault, corn, lettuce, radish, ryegrass, cucumber (Lin and Xing 2008), From a natural point of view, comprehension the ZnO NPs lethality toward earth significant plant species is of incredible significance. Nanoparticles are blessed with an assortment of properties because of the proximity of substantial surface range. ZnO nanoparticles are one of the generally contemplated classes of nanoparticles for different applications. Zinc oxides were equally toxic in bulk and nano formulations. The other studied metal oxides, TiO2 and CuO, were remarkably more toxic to algae as nanoparticles. The most toxic of the nano metal oxides was nano ZnO followed by nano CuO and nano TiO2, nano CuO and ZnO being toxic to algae already at sub mg/l concentrations. The shading of light by nanoparticles was not contributing to the overall toxic effect. Toxicity of ZnO and CuO was attributable to soluble metal ions originating from the metal oxide particles. At these low concentrations, both zinc oxides were totally soluble (Aruoja et al. 2009).

Zinc (Zn) and zinc oxide (ZnO) are categorized as commonly used metal/metal oxide engineered nanomaterials. Zn is an essential micronutrient for humans, animals, and plants [259–263]. ZnO is mostly utilized in a range of applications such as sunscreens and other personal care products, solar cells, and photocatalysis, biosensors, and electrodes [261]. According to the analysis of 289 soil samples collected from different countries in the world, Zn/ZnO deficiency was found to be the most widespread micronutrient deficiency and the fourth most important yield-limiting nutrient after nitrogen, phosphorus, and potassium. Due to its increasing utilization in consumer products, it is quite possible that through both accidental release and deliberate application, Zn/ZnO might find their way into atmospheric environments, whether terrestrial or aquatic. It induces noticeable effect on many organisms, especially on plants, which are an essential base component to all ecosystems.

A number of researchers described the key role of Zn/ZnO nanomaterials for plant growths and yield. For example, higher plant mostly absorbs Zn as a divalent cation (Zn^{+2}), which acts either as a functional, structural, or as the metal component of enzymes or a regulatory cofactor of numerous enzymes [266]. Zn nanomaterials are needed for chlorophyll production, fertilization, pollen function, and germination. Among the micronutrients, Zn affects the susceptibility of plants via drought stress. The germination rate of the plant may be affected in the presence of Zn and ZnO. ZnO nanomaterials are hazardous and affect both the chromosomal and the cellular facets. Clear root germination effects, due to the presence of ZnO, were observed for the species of Buckwheat (*Fagopyrum esculentum*). Furthermore, the presence of the ZnO nanoparticles also promoted the permeation of onion(*Alliumcepa*) roots and effected the roots elongation, genetic materials, and metabolisms. The ZnO suspension meaningfully inhibited root growth of corn, with the termination of root development.

The toxicity of ZnO nanoparticle and Zn^{+2} could be derived by two theories; a chemical toxicity based on chemical composition and the stress or stimuli caused by the size, shape, and surface of the ZnO nanoparticles. Both theories significantly affected the cell culture response of the plants. A number of mechanisms underlined the efficiency of Zn/ZnO. Depending on the plant species and the experimental conditions, the most important mechanism may be Zn/ZnO utilization in tissues, called the internal efficiency, or Zn/ZnO uptake, called the external efficiency. This, in turn, helped ZnO nanoparticle enter the root cells and inhibit seedling growth (Aslani et al. 2014).

1.6.1 Effect of ZnONPs on Plants

Up till now it has been observed that some types of nanoparticles are toxic to nature, environment and living organisms (Oberdörster et al. 2005). There are approximately 800 research articles on the toxicology of nanoparticles and these are about the cytotoxic effects of nanoparticles in the cell culture system based on the in vitro studies (RCEP, 2008). The toxicity of nanoparticles depends upon the intrinsic nature of nanoparticles and these characteristics are size, density and shape of nanoparticles (Grazyna Bystrzejewska-Piotrowska). Nanoparticles are different from the salts of metals; elements of nanoparticles consist of non – ionized form moreover these are also present in the state of zero oxidation. But mainly the nanoparticles possess non ionized compounds and these compounds mainly dissociate from the nanoparticles. So it is known that how cells of organisms' uptake the nanoparticles inside the cells after dissociation from the main compound in soluble state. So the toxic effects of nanoparticles are different from the bulk material of nanoparticles. One of most hazardous effect of nanoparticles is due to their release in the open environment (Aitken, Creely and Tran 2004). In most part of developed countries, the nanoparticles like Ag, AlO, FeO, SiO2, TiO and ZnO are being widely used in the industrial sector ((Schmid and Riediker 2008). The recent studies show that exogenous nanoparticles that contain zinc and aluminium have toxic effects on the root growth and seed germination of many plants (Lin and Xing 2008).

1.7 *Papaver Somniferum*

Papver somniferum commonly known as opium plant and this is specie of flowering plant in the family Papaveraceous. It is the species of plant from which opium and poppy seeds are derived and is a valuable ornamental plant, grown in gardens. Its native range is probably the eastern Mediterranean, but is now obscured by ancient introductions and cultivation. The opium poppy is the only species of papaveraceous that is grown as an agricultural crop on a large scale. Other poppy species, such as *Papaver rhoeas* and *Papaver argemone*, are important agricultural weeds, and may be mistaken for the crop.

Fig. 1.1 *P. somniferum* Plant

[Source: Simon, J.E., A.F. Chadwick and L.E. Craker. 1984. Herbs: An Indexed Bibliography. 1971-1980. The Scientific Literature on Selected Herbs, and Aromatic and Medicinal Plants of the Temperate Zone. Archon Books, 770 pp., Hamden, CT.]

It is the types of plant from which opium and poppy seeds are determined and is a profitable elaborate plant. The opium poppy is the main types of papaveraceous. Poppy, *Papaver somniferous* L., is a yearly herb local to South-eastern Europe and western Asia. Otherwise called opium poppy, the species is developed broadly in numerous nations, including Iran, Turkey, Holland, Poland, Romania, Czechoslovakia, Yugoslavia, India, Canada, and numerous Asian and Central and South American nations. Achieving a tallness of 1.2 meters, the erect plant can have white, pink, red, or purple blooms. Seeds range in shading from white to a slate shade that is called blue in business arrangements. The reported life zone of poppy is 7 to 23 degrees centigrade with a yearly precipitation of 0.3 to 1.7 meters and a dirt pH of 4.5 to 8.3. The plants develop best in rich, wet soil and have a tendency to be ice delicate. A latex containing a few vital alkaloids is acquired from juvenile seed cases one to three weeks subsequent to blossoming. Entry points are made in the dividers of the green seed units, and the smooth exudation is gathered and dried. Opium and the isoquinoline alkaloids morphine, codeine, noscapine, papaverine, and thebaine are derived from dried material. The poppy seeds and altered oil that can be communicated from the seed are not opiate, since they create after the container has lost the opium-yielding potential. All out yield of alkaloids is subject to light, temperature, the plant species, and the season of harvest.

In opium poppy, morphine is the real alkaloid in the latex (Ziegler et al. 2009). Heroin is made from opium delivered by the opium poppy (Farrell and Thorne 2005). Poppy seeds are utilized as a fixing with heated merchandise and cakes for their nutty scent and flavour. Poppy oil is broadly utilized as an eatable cooking oil. The oil is likewise utilized as a part of the assembling of paints, varnishes, and cleansers. Opium is utilized as a part of the creation of morphine, codeine, different alkaloids, and freshened up types of opium. Morphine is the crude material from which heroin is gotten. Poppy plants are vital as fancy plants in blossom gardens. Poppy is a standout amongst the most essential therapeutic plants. Generally, the dry opium was viewed as an astringent, antispasmodic, love potion, diaphoretic, expectorant, entrancing, opiate, and narcotic. Poppy has been utilized against toothaches and hacks. The capacity of opium from poppy to serve as a pain relieving is notable. Opium and subordinates of opium are utilized as a part of the pharmaceutical business as opiate analgesics, hypnotics, and tranquilizers. These mixes are additionally utilized as antidiarrheal, antispasmodics, and antitussives. Opium and the medications got from opium are addictive and can have toxicological impacts

1.8 Phytochemical Assays

Plants are the potential wellspring of normal cancer prevention agents. Common cell reinforcements or phytochemical cancer prevention agents are the optional metabolites of plants. Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic corrosive, ascorbic corrosive, tocopherols, tocotrienols and so forth., are a portion of the cancer prevention agents delivered by the plant for their sustenance. Beta-carotene, ascorbic corrosive and alpha tocopherol are the generally utilized cancer prevention agents (McCall and Frei, 1999).

Engineered NPs showed an assortment of natural applications, including poisonous quality. There is confirmation of a variety of toxic impacts created by fabricated NPs, including both the information from writing and the new information of the writers (Ostroumov and Poklonov 2009). The toxicity of nanomaterials was demonstrated both to prokaryotic and eukaryotic life forms. Concerning eukaryotic creatures, poisonous quality was found in bioassays with both creature and plant test frameworks. In further studies, it is important to proceed with the investigations of different parts of toxicity of NPs, and to augment the scope of creatures and test frameworks that are being utilized for surveying the natural impacts of nanomaterials. The techniques that were created to study phytotoxic of chemicals will be valuable to produce new information on toxicology of nanomaterials (Ostroumov and Poklonov 2009).

1.8.1 DPPH Radical Scavenging Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH), is a sort of stable natural radical. The limit of organic reagents to rummage DPPH radical, can be communicated as its size of antioxidation capacity. The DPPH oxidative test (Peng et al., 2000) is utilized worldwide as a part of the evaluation of radical searching limit. The cancer prevention agent exercises of plant concentrates and the standard were evaluated on the premise of the free radical searching impact of the stable DPPH free radical action (Braca et al., 2002). Because of expanded enthusiasm for cell reinforcements, particularly in those which keep from injurious impacts of free radicals in the human body and to keep the disintegration of fats and different constituents of foodstuffs. In both cases normal wellsprings of cell reinforcements are favoured. Along these lines different techniques have additionally enhanced for the estimation of cell reinforcements including DPPH. The atom of DPPH (2,2-diphenyl-1-picrylhydrazyl) is portrayed as a steady free radical including delocalization of the additional electron over the particle in general, counteracting dimerization of particles, as would be the situation with most other free radicals. The delocalization likewise offers ascend to the profound violet shading, portrayed by an assimilation band in ethanol arrangement focused at around 520 nm (Molyneux, 2004).

1.8.2 Total Antioxidant Activity (TAC)

The cell reinforcement action of Fe_3O_4 and NiO NPs and discovered 80% and 90% cancer prevention agent movement separately (Saikia and Parthasarathy 2010). Metallic nanoparticle have the significance because of their applications as antimicrobials, gas sensors, catalysis, batteries, high temperature super conveyors, sunlight based vitality change devices, and so on. A late study recommends that (Ahamed, AlSalhi and Siddiqui 2010) oxidative push might be the reason for the cytotoxic impact of ZnONPs in human aviation route epithelial cells. (Fahmy and Cormier 2009) reported that in human aviation route smooth muscle cells, the weakened cell reasonability and diminished in cell contractility happened because of introduction of CuO NPs. In this study, an endeavour has been made to investigate the cell reinforcement and antibacterial movement of ZnONPs. Das et al. (2013) have effectively integrated CuO NPs and assessed their cancer prevention agent and additionally antibacterial movement. Metallic nanoparticles demonstrate free radical rummaging movement up to 85% in 1h which is moderately higher in contrast with other metal oxide NPs.

1.8.3 Total Reducing Power (TRP)

Reducing power test measures the electron-giving limit of a cell reinforcement (Yen and Chen 1995). Nearness of reducers causes the transformation of the Fe^{+3}/ferricyanide complex to the ferrous shape which serves as a critical marker of its cancer prevention agent limit (Gülçin et al. 2002). (Koksal et al. 2011) assessed the decreasing force and radical rummaging exercises of water and ethanol removes from Rhus coriarial. They found that the reducing power of the concentrates and standard cancer prevention agents was diminished in the request of BhA > trolox > BhT > tocopherol > water remove > ethanol separate, in proximity of 30 μg/ml test. They watched that both reduction power and aggregate phenolic substance of water concentrate were higher than those of the ethanol separate. (Ebrahimzadeh and Bahramian 2009) inspected the cancer prevention agent movement of Crataegus pentaegyna sub sp. Elburensis (cp) and saw that methanolic and watery concentrates displayed a frail decreasing force at 25-800 μg/ml.

1.8.4 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Phenolic contents are known to be capable chain breaking cancer prevention agents and are imperative constituents of plants. Phenolic contents may contribute straightforwardly to antioxidative activity. It is proposed that phenolic contents affect mutagenesis and carcinogenesis in people, when ingested up to 1.0 gm every day from an eating regimen rich in products of the soil. The aggregate phenolic substance of the MEGA measured by Folin-Ciocalteu reagent as far as gallic acid equivalent (GAE) was 356 ± 1.4 mg/g (Patil et al. 2009).

Polyphenols speak to the aromatic mixes shaped in auxiliary digestion system in plants. These phenolic mixes, in some occasion, influence the key plant procedures, for example, photosynthesis, chlorophyll blend, water relations, protein amalgamation, breath, layer porousness and so on. The phenols and their oxidative items are likewise known for inhibitory activity on different chemical frameworks e.g. Indole acetic acidoxidase (Shekhawat, Jain and Arya 1980). Phenolic mixes are a vast gathering of the secondary metabolites far reaching in plant kingdom. They are ordered into classes relying upon their structure and subcategorized inside every class as indicated by the number and position of hydroxyl group and the nearness of different substituents. The broadest and differing group of the polyphenols are the flavonoids which are based upon C6–C3–C6 flavone skeleton. Also, other phenolic mixes, for example, benzoic acid or cinnamic acid subordinates have been distinguished in products of the soil (Aherne and O'Brien 2002). Phenolic mixes, particularly flavonoids, have diverse natural applications, yet the most imperative are cancer prevention agent movement, narrow defensive impact, and inhibitory impact evoked in different phases of tumour (Czeczot 2000). Phenolics can search reactive oxygen species because of their electron donating properties. Their cell reinforcement adequacy relies on upon the steadiness in various frameworks, and in addition number and area of hydroxyl groups. In numerous in vitro studies, phenolic mixes showed higher cell reinforcement action than cancer prevention agent vitamins and carotenoids (Re et al. 1999). Flavonoids and phenolics are essential mixes of *T. foenum graecum* L. also, they accumulate at various phases of development. Phenolic mixes are critical plant constituents showing antioxiodant movement by inactivating lipid free radicals, or by keeping the decay of hydroperoxides into free radicals (Maisuthisakul, Suttajit and Pongsawatmanit 2007). Dietary flavonoids are normally glycosylated and can be delegated anthocyanidins, flavanols (catechins), flavones, flavanones, and flavonols which in charge of the orange, red and blue hues in foods grown from the ground (Merken and Beecher 2000). Customarily, profound shaded organic products, vegetables or nourishments are perceived as more beneficial to human body, particularly in the oriental nations. There has been a developing enthusiasm for shade segments of products of the soil, which may advance human wellbeing or lower the danger for infection (Lin and Xing 2007).

Objectives

The objectives of present work were:

1.To find toxicological effect of ZnO NPs on *P. somniferum* seed germination and plant physiological response.

2.To disclose oxidative stress caused by ZnO NPs concentrations on grown plants.

3.HPLC fingerprinting to find signature antioxidative molecules in response to NPs stress.

2 Materials and Methods

2.1 ZnO Nanoparticles

ZnO NPs (Zinc oxide Nanoparticles) were employed to know the effect of these nanoparticles on seed germination frequency of *Papaver somniferum*, the analysis of biochemical screening of plants were done and shoot, root length were also taken in care during study.

Five concentrations 50,100,200, 400 and 800 mg/L of ZnO NPs were employed in the study. The ZnO NPs were synthesized by coprecipitation method and characterized accordingly (Javed et al., 2016). ZnO NPs were prepared by Attard Ali, a PhD scholar in Quaid-i-Azam university Islamabad (QAU). ZnO NPs were of size between 45 to 50 nm.

2.2 Collection of Plant Seeds

The seeds were collected from National Agriculture Research Centre (NARC), Islamabad, Pakistan. The selection of seeds was done on the basis of least exploration Ethno botanical character traditional medicinal importance seeds were rinsed with water to get rid of dust and foreign particles and kept in Dark and dry place

Chemical Supplies and Instruments

Ethanol, mercuric chloride, Whatmann filter paper, vacuum oven, Eppendorf tubes, pipettes, petri dishes, beakers and sonicator. Flasks, Cutters, scalpels and forceps were autoclaved before use and before inoculation, these instruments were placed under the UV light inside the laminar flow for 20 minutes.

Seed Germination

Media having half strength i.e. 2.2 g/L of MS media (Murashige and Skooog medium) and this strength also contained 50, 100, 200, 400 and 800 mg/L ZnO NPs and Half MS media was prepared without ZnO NPs so that it can be used as a control.

The media was enriched with 3 % sucrose. For the solidification of media, 0.44 % gelrite was used as solidifying agent at the pH 5.7. Sonication was done for 30 minutes and media was heated to dissolve the gelrite. Shaked the media for harmonisation before pouring it in 100 ml flask. Cotton plugs were used to close the flask's mouth and then covered the cotton plugged mouth with aluminium foil so that autoclavation can be done at 121°C while maintained the pressure at 15 psi for 20 minutes. UV lamps kept switched on for 20 minutes to get rid of unwanted microbes inside the laminar flow.

Then brought the seeds in aseptic condition for surface sterilization and these seeds were soaked in freshly prepared solution of 0.1 % mercuric chloride for 1-2 minutes and washed with distilled water to get rid of mercuric chloride so that seeds remained viable for germination. For inoculation in laminar flow 5 seeds were picked and washing of hands was done with spirit and gloves were used to avoid contamination. Inoculation was done near the spirit lamp so that entry of foreign particles in the flasks can be avoided. UV lamps were switched off before the inoculation. Five flasks per one concentration of nanoparticles were selected to inoculate the seeds. Total 25 flasks having nanoparticles and 5 were without the nanoparticles so that analysis of germination can be done both in control and with nanoparticles. All the 30 flasks were kept in the growth room after inoculation and dark effect was imposed for a week at 25°C.

2.3Seedling growth parameters

2.3.1 Root and shoot length

Plantlets were taken out from MS media after 4 weeks. The length of root and shoot was measured with the help of ruler in centimetre (cm).

2.3.2 Fresh and dry weight of plants

From each flask, 5 seedlings were collected after 4 weeks to record the fresh weight with the help of very sensitive analytical weighing balance so finally average weight was taken as final reading. Plants were freeze dried to record the dry weight and this process was done by lyophilisation.

2.4 Phytochemical Screening

Chemical and apparatus

Quercetin, Aluminium chloride, Folin-Ciocalteu reagent (FC), Potassium acetate, Gallic acid, Methanol, Ascorbic acid, Sulfuric acid, Sodium Phosphate, Ammonium molybdite, Phosphate buffer, Ammonium molybdite, Trichloroacetic acid, Ferric chloride, DMSO (Dimethyl sulfoxide), Incubators, 96 well plates, micropipette and microplate reader were among the chemicals and apparatus.

2.4.1 Extract preparation

Dry extract of *P. somniferum* were selected for applying biological assays for which the results were analysed by different parameters. The dried powder of plant material (shoot and root) was dipped in ethanol. Mixture was filtered after 24 hr by repeating this process thrice. To get the final extract, all filtrates were combined after the evaporation of ethanol. 20 mg of the dried weight extract dissolved in 1 ml of dimethyl sulfoxide (DMSO) to make the 20 mg/ml of extracts.

2.5 Antioxidant Assay

Following assays were performed on plant extracts.

- DPPH free radical scavenging assay.
- Determination of total antioxidant capacity (TAC).
- Reducing power assay (TRP).

2.5.1 DPPH free radical scavenging assay

The reaction for scavenging activity between DDPH and an antioxidant H-A can be described as below.

(DPPH) + (H-A) → DPPH-H + (A)

(Purple) (Yellow)

Antioxidant which is stable free radical reacts with DPPH and this DPPH is reduced to DPPH-H and because of this reduction absorbance decreases. The discoloration depicts and the scavenging ability of the antioxidant compounds is due to hydrogen donating ability (Oktay, Gülçin and Küfrevioğlu 2003).

Procedure

190 μL of DPPH reagent was mixed after pipetting the blank, standard and test sample 10 μL each in 96 well microplate. All this reaction mixture was exposed to incubation for 1 hour at 37^0C. Microplate reader was used to measure the OD (optical density) at 517nm wavelength.

2.5.2 Determination of Total Antioxidant Capacity (TAC)

TAC (Total antioxidant activity) was measured with the help of phosphomolybdenum method as per the procedure defined by (Prieto, Pineda and Aguilar 1999).

Procedure

100 μL stock solution which consist of extract mixed with 900 μL solutions of reagent and all this was mixed in Eppendorf tubes. Samples were, incubated at 95^0C for 90 min

in water bath and cooled the reaction mixture at room temperature then 200 μL of this mixture was pipetted out in microplate to measure the OD (Optical Density). With the help of microplate reader, the OD (Optical density) was measured at 630 nm. The resultant was expressed as μg AAE/mg DW extract.

2.5.3Total Reducing Power Assay (TRP)

Chemical entities that have the ability of reduction when react with Potassium ferricyanide form Potassium ferricyanide and this Potassium ferricyanide react with ferric chloride. This reaction step results in the formation of ferric ferrous complex. The absorption of this complex can be measure maximum at 730nm. The reduction potential of this extract was analysed as pet the procedure of Hemalatha et al. (2010).

Potassium ferricyanide + Ferric chloride ⇨ Potassium ferricyanide + ferrous chloride

Procedure

Collected 100 μL of the test samples in Eppendorf tube then phosphate buffer (200 μL, 0.2 M, pH 6.6) was mixed. All this mixture was incubated in water bath at 50^0C for 20 minutes. Followed by incubation, this mixture was mixed with 200 μL of 10% trichloroacetic acid so the resultant mixture was employed centrifugation at 3000 rpm for 10 minutes. 150 μL of supernatant layer was transferred in 96 well microplate and mixed with 50μL of 0.1% ferric chloride from each centrifuged mixture. So by using microplate reader the readings were taken at 630nm. All this result was expressed in the equation as μg AAE/mg DW extract.

2.6 Phytochemical Screening

Total phenolic content (TPC) and Total Flavonoid content (TFC) was carried out to do the phytochemical analysis of *P. somniferum*.

2.6.1 Determination of Total Phenolic Contents (TPC)

Folin-Ciocalteu was employed to measure total polyphenol in the extract of plant. Folin-Ciocalteu reagent is composed of yellow acidic solution that contains complex polymeric ions which is formed from phospmolybdic and phosphotungsticheteropoly acid. The oxidation of phenolates results in the formation of complex molybdenum-tungsten blue (Singleton and Rossi, 1965). TPC was measured by following the method of (Macdonald et al. 2001).

Procedure

Gallic acid as positive control, negative control as DMSO and 20 µL of test sample were taken in 96 well microplate and after this added 90 µL of Folin-Ciocalteu reagent followed by incubation for 5 minutes at room temperature. Sodium carbonate was added followed by incubation and this was added into the plate. and reading was taken at 630nm wavelength by using microplate reader.

2.6.2 Determination of Total Flavonoids Contents (TFC)

Most widely and distributed plant phenolic group are flavonoids. These are further characterized by benzo-y-pyrone structure and these are present in vegetables and fruits (Kanner *et al.,* 1994). TFC (Total flavonoid contents) were measured by adapting the method described by Kanner *et al.* (1994)

Procedure

Standard, blank and 20 µL were collected in 96 microplates after the addition of 10 µL of aluminium chloride solution after this added 10 µL of potassium acetate (1M). To attain the final volume of 200 µL distilled water was added in 160 µL volume. Absorbance was calculated at wavelength 415 nm by using microplate reader.

2.7 HPLC-DAD Analysis

2.7.1 Extract Preparation

Agilent Chem station Rev.B.02-01-SR1(260) and Agilent 1200 series connected with diode array detector (DAD; Agilent technologies, Germany) was used to perform High performance liquid chromatography. Zorbex-C8 analytical column (Agilent, USA).

2.7.2 Preparation of Standard solution for HPLC analysis

Gallic acid, Caffeic acid and Rutin were used as standards to perform HPLC and stock solution of these references was diluted by pouring methanol so that final concentration could be achieved as 50 µg/ml. So this fresh solution used for HPLC analysis. Sartolon polyamide membrane filter was used to filter the solution.

2.7.3 Preparation of Sample Stock Solution

Test samples at 10 mg/ml concentration were dissolved in methanol to perform HPLC. After this the vials with samples were placed in sonicator for sonication for 30 minutes. For HPLC analysis fresh samples were used and remaining samples were stored at 4°C if not consumed in one hour.

2.7.4 Chromatographic conditions

Chromatographic analysis protocol used for analysis of extracts of *P. somniferum* adapted from the method of (Saleem et al. 2014). HPLC method comprises of DAD detector which is coupled with a C-8 analytical column. Two mobile phases were prepared to analyse the polyphenols and these phases were mobile phase A and B. Mobile Phase A consisted of acetic acid, methanol, l water and acetonitritile acid 1:10:85:5 while mobile phase B comprising of acetic acid, methanol and acetonitrile following the proportion 1:60:40. The unit to measure the flow rate of both the phases was 1ml/min. 20 μl of sample was injected into the column by injection port and this was used to analyse analytically. Before the next analysis reconditioning of column was done for 5 minutes. In the first 0-20 minutes, mobile phase B gradient volume 0-50% was achieved and 50100% in 20-25 minutes and 100% in 25-30 minutes. Wavelength 279nm was used to measure the absorbance for Gallic acid, 257nm for Rutin and 325 nm was used for Caffeic acid.

2.8 Statistical Analysis

Completely randomized design was used for analysis and all the experiment was performed in triplicate. To analyse the effect of ZnO NPs on seed germination. The results are presented as mean with standard deviation. The means were further analysed by Analysis of Variance (ANOVA) and Least Significant Difference (LSD) at 0.05 probability.

3 Results

ZnO NPs (Zinc oxide nanoparticles) were used in the study to analyse the effect on *Papaver somniferum* seed germination, root elongation and metabolic profile. The frequency of seed germination was measured after 5-7 days after inoculation while shoot and root length was measured after 4 weeks. Phytochemical assays were performed to know the antioxidant activities variability in effected plants.

3.1 Effect of ZnO NPs on seed germination of *P. somniferum*

Seeds were germinated in the media containing five different concentrations of ZnO NPs and these concentrations were from 50-800 mg/L. The seed germination was compared with the control MS media and it was observed that there was 100 % frequency of seed germination in control MS media while germination frequency was different in rest of media flasks containing nanoparticles (Table 3.1). Due to presence of ZnO NPs in the media, seed germination efficiency affected mildly by increasing the concentration. So it was observed that seed germination had a minimum effect on the germination of seeds. It was also observed that seed germination dropped up to 73% when concentration of ZnO NPs was gradually increased up to 800 mg/L.

Table 3.1: Seed germination frequency of *Papaver somniferum* L in presence of ZnO NPs

Sr. no	Concentration (mg/L)	No of plants	Germination (%)
1	50	14	93_{ab}
2	100	13	86_{b}
3	200	13	86_{b}
4	400	13	86_{b}
5	800	11	73_{c}
6	No NPs	15	100_{a}

3.2 Effect of ZnO NPs on Root and Shoot Length of *P. somniferum*

After 3 weeks of seed inoculation, root and shoot average length was measured. The data collected shows that average root length is 1.54 cm and average shoot length is 2.04 cm of *P. somniferum plants* when they allowed to grow in the control MS media (Table 3.2). Physiological characteristics of plant are affected by the presence of nanoparticles in the media. It is also observed that all parameters are on decreasing side with the increase in the concentration of nanoparticles. The average root length 1.5 cm was measured on the 50 mg/L ZnO NPs which was decreased with the increase in the concentration of nanoparticles. There is least effect on shoot length. The average shoot length 1.25 cm was observed in presence of 50 mg/L NPs. The maximum inhibition is observed in presence of 100 mg/L that resulted in shoot length 1.17 cm. Overall there is a non-significant difference on shoot length parameter.

3.3 Effect of ZnO NPs on Fresh and Dry Weight of *P. somniferum*

To find the effect of ZnO NPs on *P. somniferum*, fresh and dry weight was observed. It is observed that fresh and dry weight are prominently influenced by the presence of nanoparticles in the media when compare to control MS media. Average fresh weight was measured 20.61 mg on 50 mg/L ZnO NPs and it decreased upto11.23 mg with the increase in the concentration of nanoparticles (800 mg/L). Same effect is observed on dry weight, because nanoparticles significantly affected the dry mass of plant and this affect is also concentration dependent. The minimum dry weight 3.9 mg is observed when NPs concentration is raised up to 800 mg/L while in presence of 50 mg/L NPs average dry weight is8.4 mg.

Table 3.2: Growth characteristics of *Papaver somniferum L.* plantlets grown in presence of ZnONPs concentrations

mg/L	Root Length (cm)	Shoot Length (cm)	Fresh Weight (mg)	Dry Weight (mg)
50	1.5 ± 0.18^{b}	1.25 ± 0.13^{b}	$20.61\pm.2^{b}$	8.4 ± 0.15^{b}
100	1.16 ± 0.11^{c}	1.14 ± 0.09^{c}	20.09 ± 1.1^{b}	7.6 ± 0.11^{c}
200	1.13 ± 0.11^{c}	1.3 ± 0.10^{ab}	16.75 ± 1.4^{bc}	4.6 ± 0.06^{d}
400	1.03 ± 0.12^{cd}	1.2 ± 0.08^{b}	14.17 ± 1.6^{d}	4.1 ± 0.09^{d}
800	0.73 ± 0.11^{d}	1.2 ± 0.07^{b}	11.23 ± 2.0^{de}	3.9 ± 0.08^{e}
No NPs	2.04 ± 0.24^{a}	1.54 ± 0.11^{a}	27.8 ± 1.4^{a}	13.4 ± 0.12^{a}

3.4 DPPH Radical scavenging activity

The free radical scavenging activity is observed higher in ZnO NPs treated plants as compared with control. The minimum activity 4.37% is observed in plants treated with 50 mg/L that tend to increase by increasing NPs concentration. It was observed that there is significant difference among the percent radical scavenging activity in plants except treated with 400 and 800 mg/L ZnO NPs. In control plants, untreated with NPs 31.26% DPPH based free radical scavenging activity was observed (fig. 3.1).

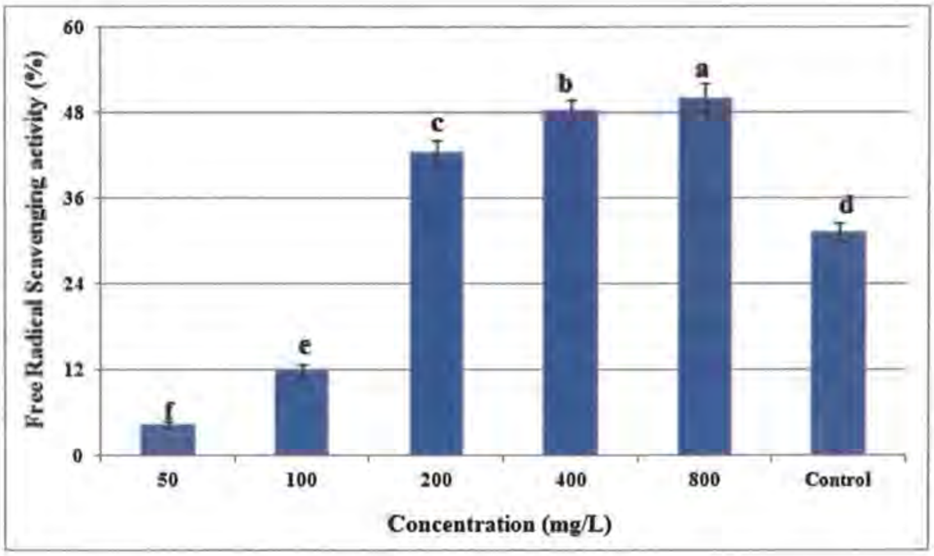

Fig. 3.1 DPPH free radical scavenging activity of *P. somniferum* grown in presence of different concentrations of ZnO NPs. Different alphabetical letters marked on the bars show significant difference among the mean values at P< 0.05 using LSD test.

3.5 TAC and TRP

Total antioxidant activity 0.28 µg AAE/mg DW is observed in control plants. Presence of ZnO NPs in the media significantly increase total antioxidant activity that rose up to

0.37 µg AAE/mg DW in 50 mg/L treated plant to 1.75 µg AAE/mg DW in 800 mg/L NPs treated plants. Maximum TAC is observed in plants grown in presence of 100 mg/L ZnO NPs (fig. 3.2). However total reducing power potential varied in control and treated plants. 2.04 and 3.20 µg AE/mg DW is observed in 50 and 100 mg/L treated plants, respectively that is lower than control plants (4.13 µg AAE/mg DW). Increase in NPs concentration up to 100 mg/L increase TRP activity many time (10.48 µg AAE/mg DW) however further increase in NPs concentration decreased TRP activity (fig. 3.3).

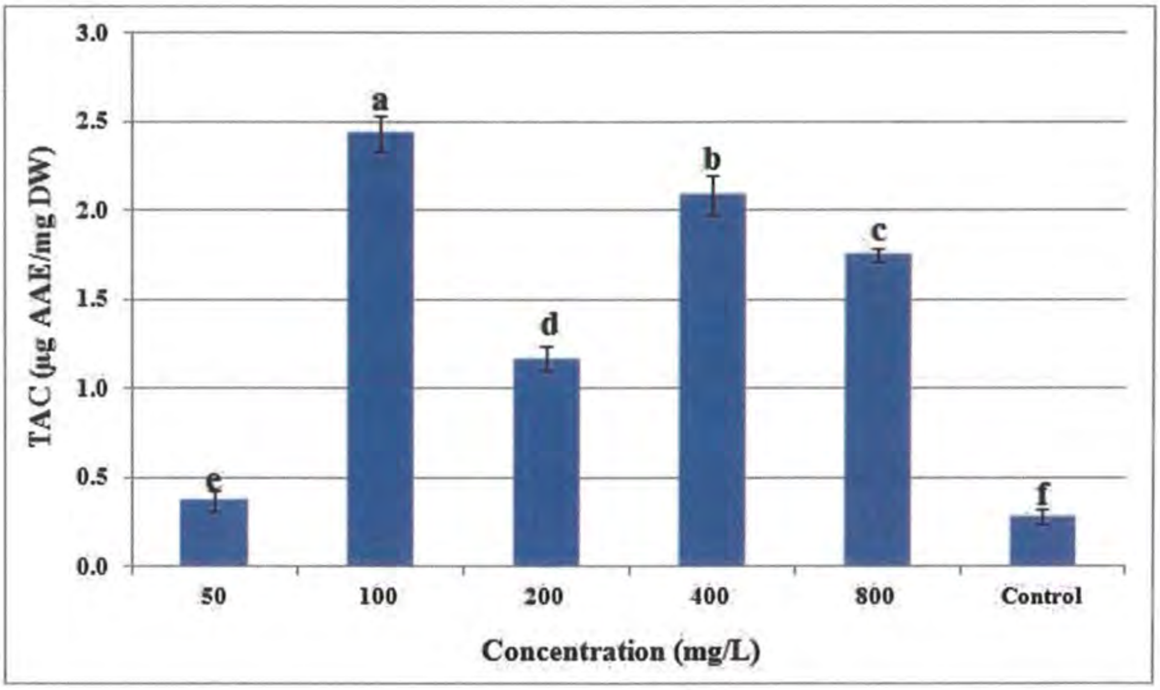

Fig. 3.2 Total antioxidant of *P. somniferum* grown in presence of different concentrations of ZnO NPs. Different alphabetical letters marked on the bars show significant difference among the mean values at P< 0.05 using LSD test.

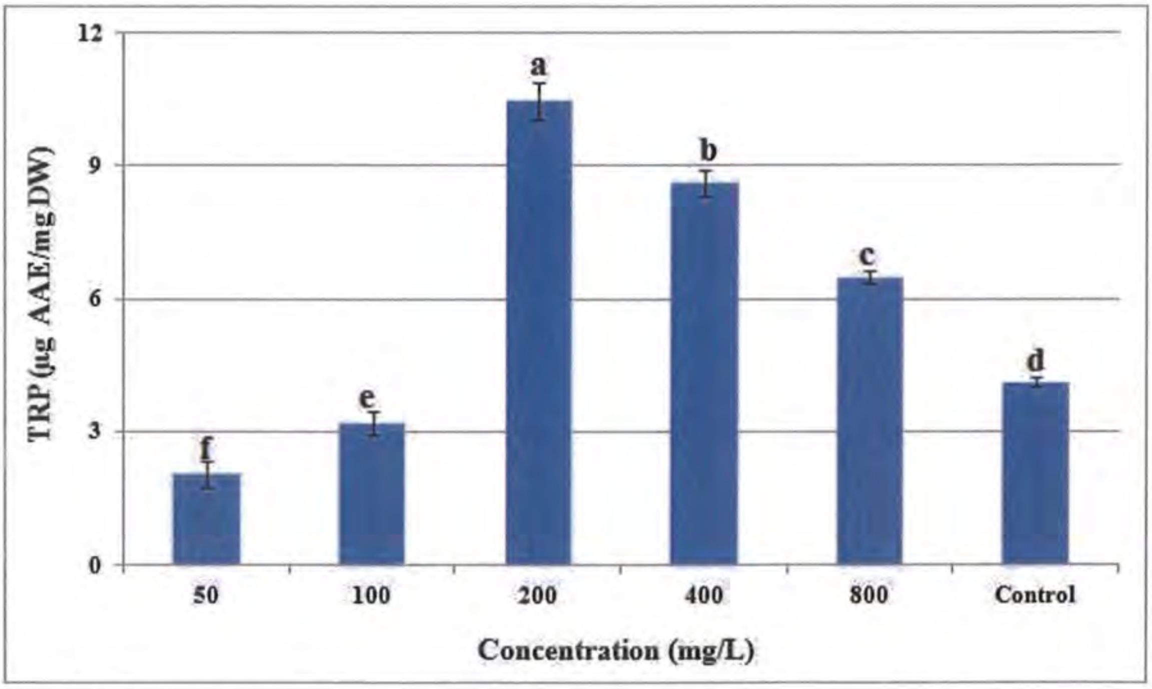

Fig. 3.3 Total reducing potential of *P. somniferum* grown in presence of different concentrations of ZnO NPs. Different alphabetical letters marked on the bars show significant difference among the mean values at P< 0.05 using LSD test.

3.6 TPC and TFC

With a specific end goal to break down grouping of non-enzymatic oxidative stress preventing agents, add up to phenolic substance (TPC) and aggregate flavonoid substance (TFC) assurance is performed. it is watched that TPC substance gradually increases (1.56-7.11 μg GAE/mg DW) up to 800 mg/L concentration of ZnO NPs. The TPC contents gradually increased while increasing the NPs concentration (fig. 3.5). TFC tend to increase by increase in NPs concentration up to 400 mg/L except at convergence of 50 mg/L. Highest TFC 1.34 and 1.42 μg QE/mg DW is seen at 100 and 200 mg/L NPs treated plants, respectively. By further increase in NPs focus unusual reduction in TFC is watched (1.19 and 0.98 μg QE/mg DW in 600 and 800 mg/L treated plants, respectively) (fig. 3.4).

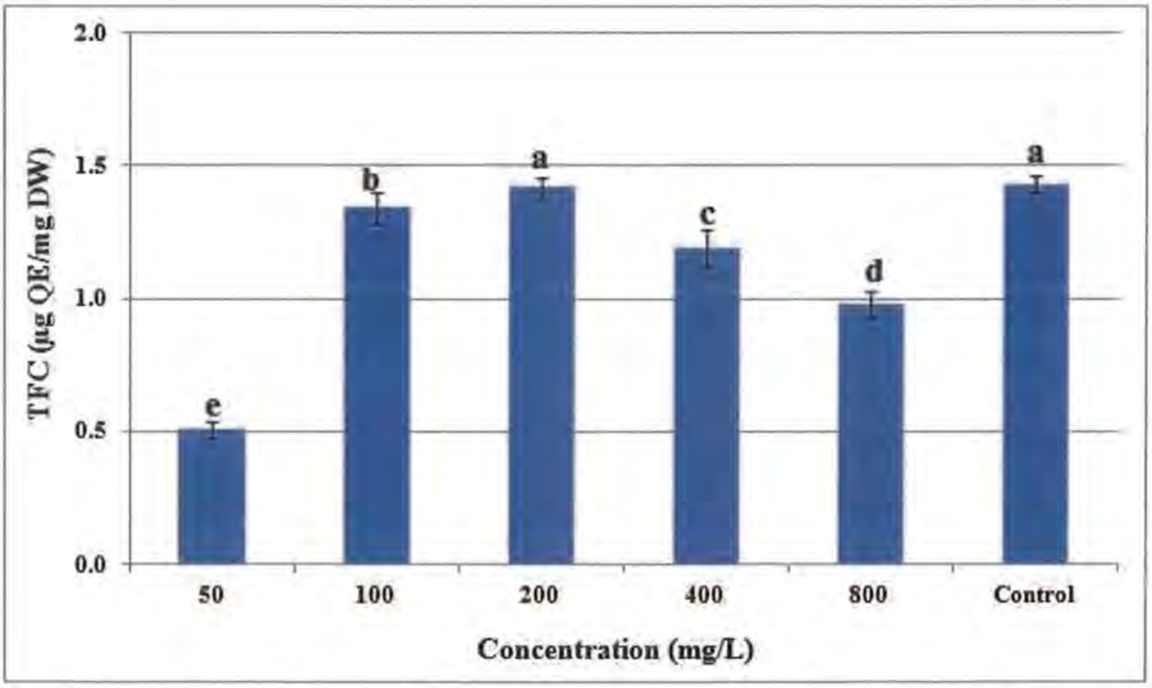

Fig. 3.4 Total flavonoid contents of *P. somniferum* grown in presence of different concentrations of ZnO NPs. Different alphabetical letters marked on the bars show significant difference among the mean values at P< 0.05 using LSD test.

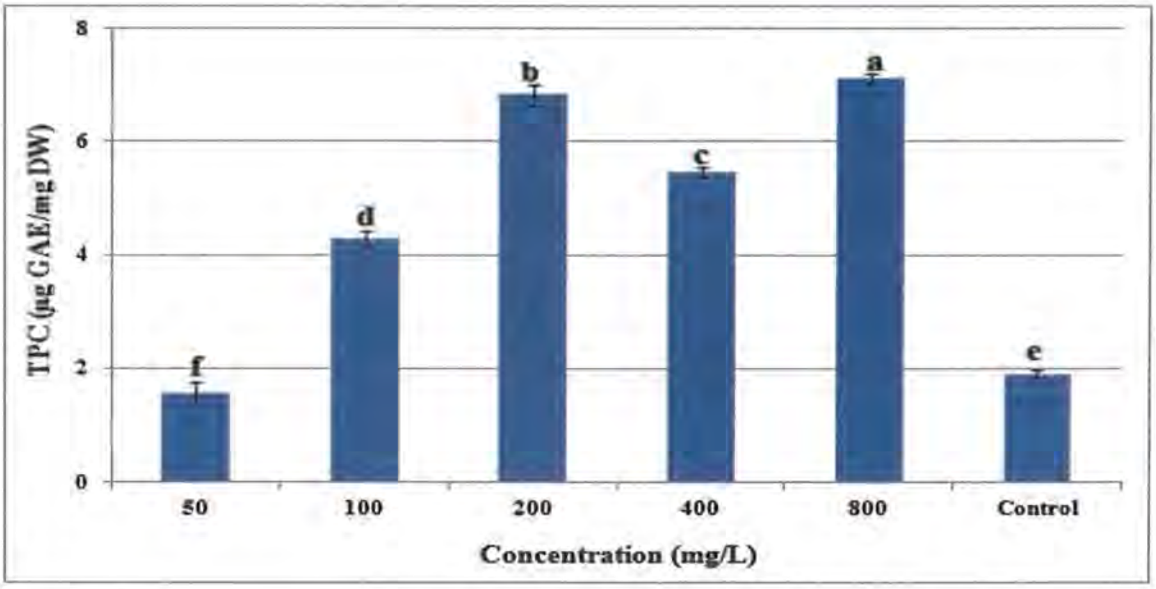

Fig. 3.5 Total phenolic contents (TPC) and total flavonoid contents (TFC) of *P. somniferum* grown in presence of different concentrations of ZnO NPs. Different alphabetical letters marked on the bars show significantly different among the mean values at P< 0.05.

3.7 HPLC-DAD analysis

Turn around stage HPLC strategy is utilized to play out the quantitative examination of *P. somniferum* L removes. UV ingestion is utilized to look at the pinnacles and standard/reference mixes maintenance time of various critical mixes i.e. rutin, caffeic acid and gallic acid. Quantitative profiling by HPLC exhibits that 445.05 µg/100 mg DW gallic acid is found in the *P. somniferum* L separates treated with ZnONPs 800 mg/L which is most astounding as contrast with different concentrates. In 50, 100, 200 and 400 mg/L ZnO NPs treated plants separates 262.25, 261.22, 297.21, 428.25 and 445.05 µg/100mg DW gallic acid, respectively. While Caffeic acid is found in most elevated quantity 176.29 µg/100mg DW in the concentrates of *P. somniferum* L that were treated with the nanoparticle ZnO NPs 800 mg/L. While caffeic was found in various quantity in the concentrates that were treated with 50mg/L, 100 mg/L, 400 mg/L and 800 mg/L and this quantity is 57.76 µg/100mg DW, 129.16 µg/100mg DW, 140.55 µg/100mg DW and 152.73 µg/100mg DW, respectively. To the extent rutin is concerned this is not found in the HPLC investigation.

4 Discussion

Nanotechnology is now creating a growing sense of excitement in the life sciences especially biomedical devices and biotechnology. NPs exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology (Savithramma, Rao and Suhrulatha 2011). The development of techniques for the controlled synthesis of NPs of well-defined size, shape and composition, to be used in the biomedical field and areas such as optics and electronics, is a big challenge (Gericke and Pinches 2006).

ZnO NPs (Zinc oxide nanoparticles) were used in the study to analyse the effect on *Papaver somniferum* seed germination, root elongation and metabolic profile. The frequency of seed germination was measured after 5-7 days after inoculation while shoot and root length was measured after 15 days. Phytochemical assays were performed to know the antioxidant activities variability in effected plants.

4.1 Effect of ZnO NPs on seed germination and physiological characteristics

Seeds were germinated in the media containing five different concentrations of ZnO NPs and these concentrations were from 50-800 mg/L. In each flask 5 seeds were placed for germination and three flasks were prepared having each concentration. The seed germination was compared with the control MS media and it was observed that there is 100 % frequency of seed germination in control MS media while germination frequency is different in rest of media flasks containing nanoparticles. Due to presence of ZnO NPs in the media, seed germination efficiency affected mildly by increasing the concentration. Seed germination is a physiological process starts with water imbibition by seeds and ending with the emergence of rootlet 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. 2012). It is probably due to the seed coat which acts as protector for the embryo and plays important role in selective permeability.

After 3 weeks of seed inoculation, root and shoot average length was measured. The data collected shows that average root length is 1.54 cm and average shoot length is 2.04 cm of *P. somniferum* plants when they allowed to grow in the control MS media. Physiological characteristics of plant are effected by the presence of nanoparticles in the media. It is also observed that all parameters are on decreasing side with the increase in the concentration of nanoparticles. The average root length 1.5 cm is measured on the 50 mg/L ZnO NPs which decreases with the increase in the concentration of nanoparticles. There is least effect on shoot length. The average shoot length 1.25 cm is observed in presence of 50 mg/L NPs. The maximum inhibition is observed in presence of 100 mg/L that resulted in shoot length 1.17 cm. Overall there is non-significant difference on shoot length parameter.

Exposure to excess ZnO NPs has detrimental effects on plant growth. Zinc ions released from ZnO may tend to accumulate in the root tissues with little translocation to the shoot, thus principle effect of Zn ion or ZnO 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 metallic ions in seedlings of rice, directly co-related with the toxicity in the plant.

To find the effect of ZnO NPs on *P. somniferum*, fresh and dry weight was observed. It was observed that fresh and dry weight are prominently influenced by the presence of nanoparticles in the media when compare to control MS media. Average fresh weight is measured 20.61 mg on 50 mg/L ZnO NPs and it decreased up to 11.23 mg with the increase in the concentration of nanoparticles (800 mg/L). It is observed also that same affect was on dry weight, because nanoparticles significantly affected the dry mass of plant and this affect is also concentration dependent. The minimum dry weight 3.9 mg is observed when NPs concentration is raised up to 800 mg/L while in presence of 50 mg/L NPs average dry weight is 8.4 mg.

4.2 Antioxidative potential of *P. somiferum* plants

A single assay does not describe complete mechanism of toxicity. Therefore, comprehensive phytotoxicity profile should be investigated in higher plants for nanoparticles (Lin and Xing 2008). The free radical scavenging activity is observed higher in ZnO NPs treated plants as compared with control. The minimum activity 4.37% is observed in plants treated with 50 mg/L that tend to increase by increasing NPs concentration. It is observed that there is significant difference among the percent radical scavenging activity in plants except treated with 400 and 800 mg/L ZnO NPs. While total antioxidant activity 0.28 μg AAE/mg DW is observed in control plants. Presence of ZnO NPs in the media significantly increase total antioxidant activity that rose up to 0.37 μg AAE/mg DW in 50 mg/L treated plant to 1.75 μg AAE/mg DW in 800 mg/L NPs treated plants. Increase in NPs concentration up to 100 mg/L increase TRP activity many time (10.48 µg AAE/mg DW) however further increase in NPs concentration decrease TRP activity. This is clearly pointed out by the powerful DPPH scavenging activity and other anti-oxidant activities of *P. somniferum* extracts. But once threshold level is achieved and still plant cells are prone to continued addition of ZnO nanoparticles, imbalance of plant growth regulators and the release and accumulation of free radicals or reactive oxygen species (ROS) take place to a larger extent resulting in degradation of few cells (Choi and Hu 2008). ZnO NPs implicate an oxidative stress by the release of metal ions or free radicals into MS culture medium (Lee et al. 2013, Dimkpa et al. 2012)).

With a specific end goal to break down grouping of non-enzymatic oxidative stress preventing agents, total phenolic content (TPC) and total flavonoid content (TFC) test is performed. It is watched that TPC substance are essentially increased (1.56-7.11 µg GAE/mg DW) up to 800 mg/L concentration of ZnO NPs. The TPC contents gradually increase while increasing the NPs concentration. TFC tend to increase by increase in NPs concentration up to 400 mg/L except at convergence of 50 mg/L. Greatest TFC 1.34 and 1.42 µg QE/mg DW is seen at 100 and 200 mg/L NPs treated plants, respectively. By further increase in NPs concentration extraordinary reduction in TFC is observed (1.19 and 0.98 µg QE/mg DW in 600 and 800 mg/L treated plants, respectively).

Flavonoids and phenolics play an important role in detoxification of ROS. Total flavonoid and phenolic contents were determined and results show that extracts of plant accumulated more flavonoids and phenols as compared to control plant extracts' are proposed to be responsible for negative effects of NPs but toxicity mechanism of NPs has not yet been clearly understood {Applerot, 2012 #312}. NPs induce oxidative stress that damages cell (Hossain *et al.*, 2015). DPPH results depict that ZnO NPs are responsible for oxidative stress and interfere in normal growth through length, fresh weight and dry weight of the plants. Therefore, plants activate their defence mechanism to protect themselves from damage. And failure of defence mechanism leads to lipid peroxidation, mitochondrial perturbation, DNA damage and eventually apoptosis of cell {Li, 2008 #51}.

Turn around stage HPLC strategy is utilized to play out the quantitative examination of *P. somniferum* L removes. UV absorption is utilized to look at the peaks and standard/reference mixtures maintenance time of various critical mixes i.e. rutin, caffeic acid and gallic acid. Quantitative profiling by HPLC exhibits that 445.05 µg/100 mg DW gallic acid is found in the *P. somniferum* L separates treated with ZnO NPs 800 mg/L which is most astounding as contrast with different concentrates. While Caffeic acid is found in most elevated quantity 176.29 µg/100mg DW in the concentrates of *P. somniferum* L that are treated with the ZnO NPs 800 mg/L. To the extent rutin is concerned this is not decided in the HPLC investigation.

Conclusions

The present study concludes that ZnO nanoparticles mildly effect *Papaver somniferum* seed germination but significantly effect on plant growth, however these all are concentration dependent. Furthermore, presence of ZnO NPs in the media creates oxidative stress in poppy plants that is shown by elevated levels of oxidative activity and free radical scavenging activity. Though phenolics and flavonoids produce in response of NPs stress but at lesser extent. Among the phenolics, gallic acid and caffic acid produce at higher quantities in *P. somniferum* plant on increase of NPs concentration in the media. This shows that plants have natural mechanism to mitigate oxidative stress caused by the nanoparticles.

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