ORIGINAL ARTICLE

An *in vitro* evaluation of anti-oxidant properties of novel nano-composite material containing titanium oxide, zinc oxide and green tea extract

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ABSTRACT

Introduction: Green tea is a medicinal beverage extracted from the plant Camellia sinensis. Antioxidants that exist naturally can be extracted as pure compounds from their parent materials for nutraceutical and medicinal applications.
The present study aims to assess the antioxidant activity of Zinc oxide-titanium dioxide nano-composites (ZnO-TiO2 NCs) containing green tea extract.

Materials and Methods: The antioxidant activity was tested by Hydrogen Peroxide [H2O2] assay, Fluorescence recovery after photo-bleaching [FRAP] assay and 2, 2-diphenyl-1-picrylhydrazyl[DPPH] assay. All tests have shown very good results for the ZnO-TiO2 NCs.

Results: In this study, we present a straightforward, eco-friendly alternative for producing non-toxic zinc oxide and titanium oxide nano-composite material. This study could make a valuable contribution and create new opportunities in the market such as biological and pharmaceutical applications.

Conclusion: The in vitro tests concluded that the novel nanocomposite containing ZnO-TiO2 and green tea extract has good anti-oxidant properties and it is non-toxic to the biological systems.

KEYWORDS:
Antioxidant activity, Green tea, Nano-composites.

INTRODUCTION

Nano-composite materials are made of amorphous or one, two, or three-dimensional structures blended at the nano-scale. These are made of clay, carbon, polymer, or a mixture of these components.[1] Green tea is a medicinal beverage extracted from the plant *Camellia sinensis* with a total polyphenol content of20% to 35% by weight, 60–80% of which are catechins.[2] It has various health benefits like prevention of cancer, and cardiovascular disease, the anti-inflammatory, anti-arthritic, antibacterial, anti-angiogenic, anti-oxidative, antiviral, neuro-protective and anti-cholesterol effects.[3,4] It has therapeutic effects in liver diseases, type 2 diabetes, Alzheimer's disease, obesity and many other systemic conditions.[5] It acts as an antioxidant by inducing glutathione-S-transferases and by inhibiting 'pro-oxidant' enzymes like inducible nitric oxide synthase, lipo-oxygenase and xanthine oxidase.[6] Natural antioxidants obtained from medicinal plants boost the plasma's antioxidant capacity and lower the risk of developing certain diseases.[7,8] Secondary metabolites like phenolics and flavonoids are effective free radical scavengers.[9] The undesirable effects of synthetic antioxidants include liver damage, cancer etc.[10,11] FDA permits the use of TiO_2 and biosynthesized Zinc Oxide nano-particles in human food, medication, and cosmetics.[12,13] The present study aims to assess the antioxidant activity of a nano-composite containing TiO_2, ZnO and Green tea extract.

MATERIALS AND METHODS

Preparation of plant extract

Distilled water (100Ml) and green tea powder (2g) were mixed and boiled for 10 min. And the extract was filtered through muslin cloth. After stirring thoroughly in a heating mantle at 50-60 0C for 30 min, it is filtered using Whatman no. 1 filter paper. Of the 100mL filtered green tea extract, 60 mL of plant extract was used for the preparation of nano-particles. The remaining 30-40 mL of plant extract was concentrated into 3-4 mL of plant extract using a heating mantle, and could also used as the control. Sixty millilitres of the extract were used to synthesize the nano-particles.

Preparation of nanoparticles

Titanium oxide (0.35 g)was dissolved in 70 mL of distilled water, added to 30 mL of green tea extract, and stirred in an orbital shaker for 48 hours at 700-750 rpm. The mixture was centrifuged to synthesize titanium dioxide nano-particles.

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Pellets were collected and stored for characterization and biological analyses.

574g (20mM) of Zinc sulphate was dissolved in 70 mL of distilled water, added to 30 mL of green tea extract, and stirred in an orbital shaker for 48 hours at 700-750 rpm. The mixture was centrifuged to synthesize zinc oxide nano-particles. The pellets were collected and stored for further characterization and research. [Figure 1]

Preparation of Zinc oxide and Titanium dioxide nano-composites
The titanium dioxide and zinc oxide nano-particle pellets were collected and dissolved in distilled water. Equal volumes of the solutions were placed separately in a sterile centrifuge and centrifuged at 8000 rpm for 10 min. The mixture was then added to the green tea extract and dried in a hot air oven. The powder was collected and stored for further research .

Characterization of nanocomposite
In this study, green tea was used as a reducing and stabilizing agent to synthesize Titanium dioxide nano-particles, ZnO nano-particles, and Titanium dioxide-Zinc oxide nano-composite. The green tea-mediated nano-particles and nano-composites were subjected to characterization techniques, such as Scanning Electron Microscopy (SEM) and Elemental dispersive analysis (EDX) to analyze their shape, and elemental and functional composition.

Scanning electron microscopy
Scanning Electron Microscopy (SEM) analysis of the green-synthesized TiO_2-ZnO nano-composite revealed its distinctive morphology [Figure 2]. This intriguing observation suggests a complex and heterogeneous structure within the nano-composite material, with two distinct particle shapes. The nano-composite exhibited a combination of both rhomboid-shaped TiO_2 nano-particles and spherical ZnO nano-particles. The particles had a certain degree of uniformity and regularity. This size-dependent activity suggests that the synthesized nano-particles can be tailored for specific applications by controlling their size. The coexistence of rhomboid and spherical particles in the TiO_2-ZnO nano-composite may have implications for its unique properties and potential applications, making it a promising candidate for various biomedical applications. The unique properties and morphologies of these nano-particles offer opportunities for application in the fields of biomedicine and antimicrobial coatings for prosthetic devices.

Elemental dispersive analysis
Energy dispersive X-ray spectroscopy (EDX) analysis of the green-synthesized titanium dioxide, zinc Oxide (TiO2-ZnO) nanocomposite provided insights into its elemental composition as depicted in [Figure 3]. The EDX spectra revealed the presence of several elements including titanium, zinc, and oxygen confirming the presence of both TiO_2 and ZnO components. in the nano-composite.

The significant carbon content suggests the persistence of organic residues from the green synthesis process, which may contribute to the nano-composite's unique properties. These findings underscore the versatility of green tea-mediated synthesis for tailoring nano-particle morphologies and offer potential applications in various fields of nanotechnology and materials science.

Evaluation of antioxidant activity by the DPPH method
The antioxidant activities of the biogenically synthesized zinc oxide and titanium dioxide nano-composites were evaluated using the DPPH assay. Different quantities (2–10µg/mL) of green tea extract containing the ZnO-TiO_2 nanocomposite, were added to 450 µL of 50 mM Tris HCl buffer (pH 7.4) and 1 ml of DPPH(0.1 Mm) in methanol and incubated for 30 min. Subsequently, based on the absorbance at 517 nm, the decrease in the number of DPPH free radicals was evaluated. Butylated hydroxytoluene (BHT) was used as control. The following equation was used to calculate the percentage inhibition:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control- Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Evaluation of antioxidant activity by hydroxyl radical scavenging assay
Modified Halliwell method was used to perform the assay.[14] 1.0 mL of freshly prepared reaction mixture containing 100 mL of 28 mM 2-deoxy-2-ribose, which was dissolved in phosphate buffer at pH 7.4. 500 mL of a solution containing different concentrations of green tea with ZnO-TiO2 nano-composite (10 to 50 µg/mL), 200 mL of 200 mM FeCl3 and 1.04 mM EDTA (1:1 v/v), 100mL H_2O_2(1.0mM) and 100µL(1.0mM) ascorbic was used. The TBA (Thiobarbituric acid) reaction was used to measure the degree of deoxyribose breakdown after incubating for one hour at 37°C. The absorbance at 532 nm was calculated in a blank solution, and the positive control, vitamin C, was used.

Evaluation of antioxidant activity by FRAP assay

Reagents for FRAP assay
a. To prepare an acetate buffer with 300 mM of pH 3.6, 3.1g of sodium acetate trihydrate was added to 16 ml of glacial acetic acid, and then mixed with distilled water to make a volume of 1L. b. TPTZ (2, 4, 6-tripyridyl-s-triazine), 10 mM in 40 m M H Cl (M.W. 312.34) (M.W. 36.46). c. FeCl3.6 H2O (20mM; M.W. 270.30). Immediately before testing, the FRAP reagent was prepared by combining components a, b, and c in a ratio 10:1:1. FeSO4. 7 H2O: 0.1 to 1.5 mM in methanol served as the standard. All reagents were prepared by the Merck Company in Germany.

Procedure for FRAP assay
The FRAP solution (3.6 mL) to distilled water (0.4 mL) and incubated for 5 min at 37 °C. This solution was mixed with a specific concentration of green tea nano-composites containing ZnO-TiO2 nano-particles (10 to 50 µg/mL) and incubated for 10 min at 37°C.The absorbance of the reaction mixture was measured at 593 nm. Five concentrations of FeSO4 and 7H2O (0.1, 0.4, 0.8, 1, 1.12, and 1.5 m M) were used to create a calibration curve, and the absorbance values were calculated for the sample solutions.

RESULTS

Visual observation

In the presence of the ZnO-TiO2 nano-composite, the color of the solution gradually changed to pale yellow.

Scanning electron microscopy

SEM of the nano-composite suggested a complex heterogeneous structure with two distinct particle shapes. Upon examination, a combination of both rhomboid-shaped particles of TiO_2 and spherical particles of ZnO of uniform shape and size was observed [Figure 1]. The coexistence of rhomboid and spherical particles in the TiO_2-ZnO nano-composite may have implications for its unique properties and potential applications, making it a promising candidate for various biomedical applications.

EDX Spectrography

The elemental compositions of TiO_2 and ZnO , namely titanium, zinc, oxygen and carbon, in the nanocomposite was confirmed by EDX Spectrography [Figure 2].

DPPH assay

In the DPPH assay, the higher the concentration of green tea in the ZnO-TiO_2 nano-composite higher was the % of inhibition. At the concentration of nano-composites 10µg/ml, inhibition was 64%, 20 µg/mL has a 76%, 30 µg/mL has an 82%, 40 µg/mL has 64% of inhibition and 50 µg/mL has an 89%, respectively [Figure 3].

H_2O_2 assay

In the H_2O_2 assay, the higher the concentration of green tea in the ZnO-TiO2 nano-composite higher was the inhibition rate rate. A concentration of nano-composites with 10µg/ml inhibited 51.3% of inhibition, 20 µg/ml inhibited 56.1% , 30 µg/ml 62.8% of inhibition,40 µg/ml inhibited 78% and 50 µg/ml inhibited 87.5% [Figure 3].

FRAP assay

In the FRAP assay, the % of inhibition increased in a dose-dependent manner. At a concentration of 10µg/mL the nano-composites showed 64.8% inhibition, 20 µg/mL inhihibited 74.3% , 30 µg/mL has an 82.6%, 40 µg/mL has an 84.7%, and 50 µg/mL has a 90.1%. (Figure 4) The plant-mediated nano-particles were added to the zinc sulphate solution and titanium oxide solution. Zinc sulfate was used to synthesize ZnO nano-particles and titanium oxide was used to synthesize titanium dioxide nano-particles. After one hour a color change was observed, which indicate the initiation of a chemical reaction in the solution. After 24 h, the color changed completely, indicating the synthesis of the nano-particles [Figure 3].

Examination of ZnO-TiO2 nano-composites using UV-visible spectroscopy

UV-visible spectroscopy was used for preliminary confirmation of the nano-particle synthesis. The absorbance of the UV-visible spectrum exhibited the highest peak at 310 nm. As the concentration of the ZnO-TiO_2 nanocomposite increases from 10 mg to 100 mg, the absorbance at 310 nm decreased in intensity. The peak refers to the synthesis of nanoparticles.[15] The reduction of titanium ions and generation of TiO_2 NPs were completed after overnight incubation at room temperature. The reduction of titanium ions is indicated by the formation of a light green color. The absorption spectra of the TiO_2 NPs formed in the solution had absorbance peaks at approximately 280 nm.[16] The synthesis of nano-particles was observed in the color changes of the nanoparticle solution. The synthesis of ZnO nano-particles was confirmed by UV-visible spectroscopy, which showed the highest peak absorbance at 360 nm. After 24h of reaction, the color change was stopped and precipitation was observed, indicating the completion of nano-particle synthesis.[17] The formation of ZnO NPs was confirmed by the observation of maximal absorbance at 480 nm, which is the typical wavelength for this formation. The observed UV spectrum around 450 nm indicates that in the reduction process, the semiconducting property of ZnO has not been lost[18] [Figure 4]. In the presence of the ZnO-TiO_2 nanocomposite, the color of the solution gradually changed to pale yellow. The absorption intensity was confirmed and localized by UV-visible spectroscopy at 310 nm. [Figure 4]

The antioxidant activity of green synthesized ZnO-TiO_2 nano-composite using green tea extract was measured by DPPH, H_2O_2, and FRAP assays to evaluate their radical scavenging activity. The solution was kept undisturbed for two hours to evaluate the stability of the nano-composites. The color change of the solution to pale yellow indicates an increase in free radical scavenging activity with an increase in the concentration of the ZnO-TiO_2 nano-composite. When the antioxidant activity was tested, green tea in the ZnO-TiO_2 nano-composites had good antioxidant properties.

In the DPPH assay, the higher the concentration of green tea in the ZnO-TiO_2 nano-composite higher was the % of inhibition. The antioxidant activity of green tea containing ZnO-TiO_2 nano-composites was comparable to that of the standard. When DPPH was inhibited, the color changed from purple to brown, indicating that hydrogen was donated and, demonstrating high scavenging activity. The high redox potential of ZnO breaks the water molecules into hydroxyl and hydrogen radicals, stabilizing DPPH free radicals and inhibiting the DPPH effect. The Antioxidant activities of different percentages of inhibition of oxidation were 52%, 63%, 71%, 85%, and 90% in a dose-dependent manner. Plant extracts mediated by titanium dioxide nano-particles at 40µL and 50µL concentrations exhibited a high antioxidant activity of 90%.

In the H_2O_2 assay, the higher the concentration of green tea in the ZnO-TiO_2 nano-composite higher was the % of inhibition. The % of inhibition shown by the green tea containing nano-composites was similar to the standard values. Previous studies have shown that green tea-mediated TiO_2 NPs exhibit 57–72% scavenging at 100 µg/mL concentration, while at 10 µg/mL, scavenging was 4–9%, indicating ineffectiveness at lower concentrations.

In FRAP assay, the % of inhibition increased in a dose-dependent manner. All values were nearly the same as those the standard. In FRAP assay, the maximum concentration of antioxidant activity of 8.99 ± 0.21 µM/mL in 1000 µg/mL, and the minimum concentration of antioxidant activity of 0.59 ± 0.01 µM/mL in 60 µg/mL of ZnO un-doped ZnO nano-

Table I: Antioxidant activity of green tea TiO2 ZnO nano-composite

Concentration	Antioxidant activity of green teaTiO2ZnO nanocomposite					
	DPPH % of inhibition		H2O2 % of inhibition		FRAP % of inhibition	
	GT, TiO2 ZnO	Standard	GT, TiO2 ZnO	Standard	GT, TiO2 ZnO	Standard
10 µL	64%	66	51.3	53	64.8	67
20 µL	76	78	56.1	58	74.3	78
30 µL	82	83	62.8	64	82.6	87
40 µL	64	66	78	80	84.7	88
50 µL	89	91	87.5	90	90.1	93.

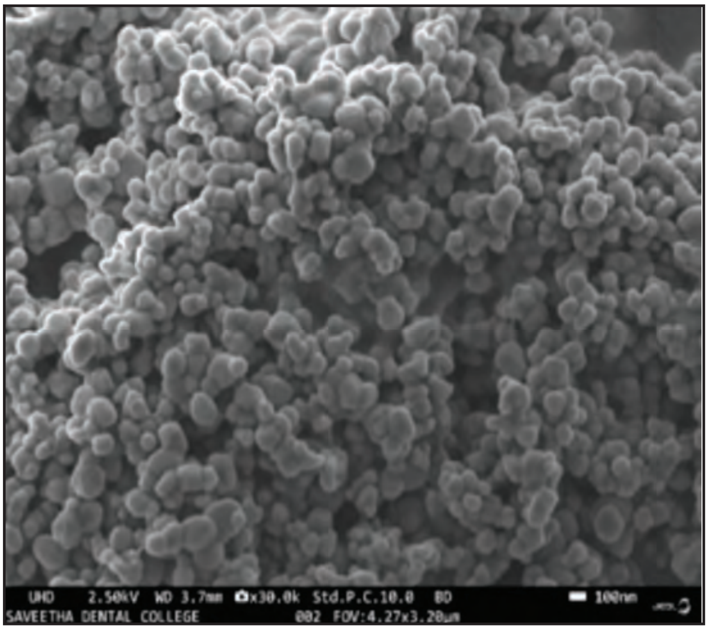

Fig. 1: SEM image of green synthesized TiO2-ZnO nano-composite

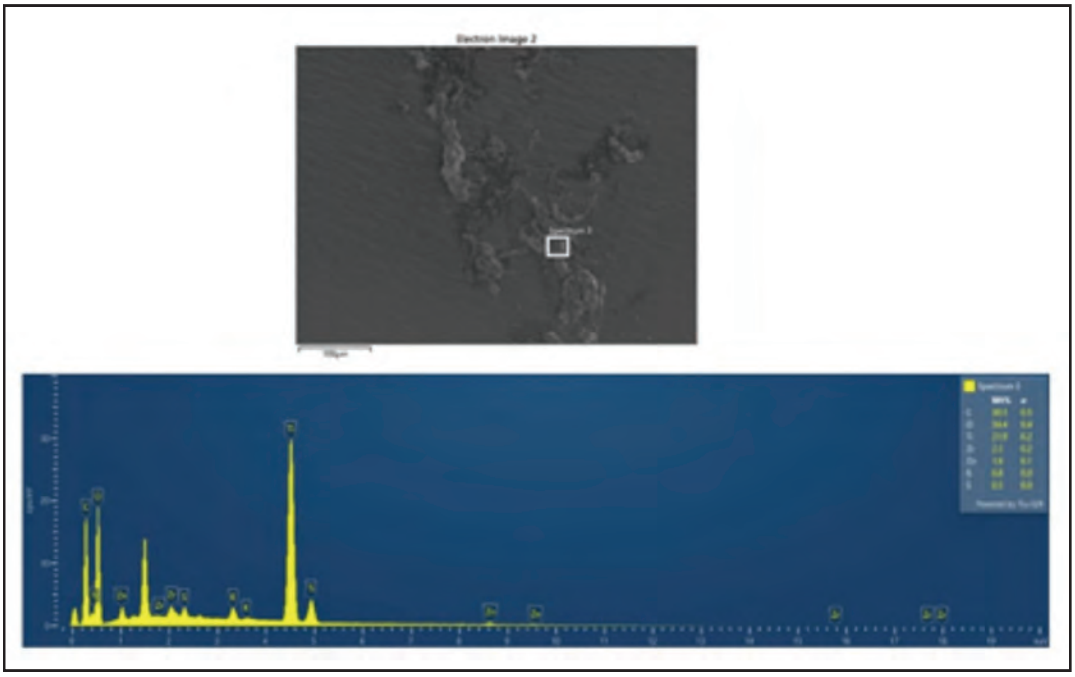

Fig. 2: EDX spectra of green synthesized TiO2-ZnO nanocomposite

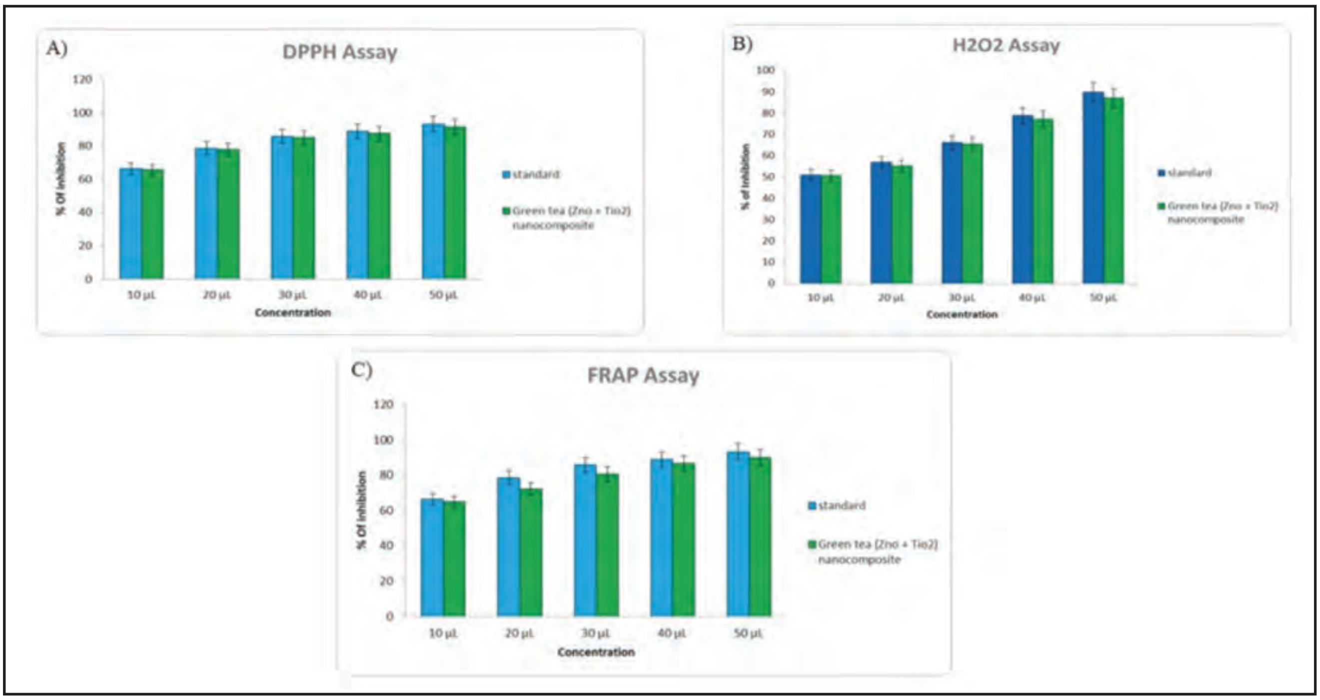

Fig. 3: A) DPPH assay B)H2O2 Assay C) FRAP assay

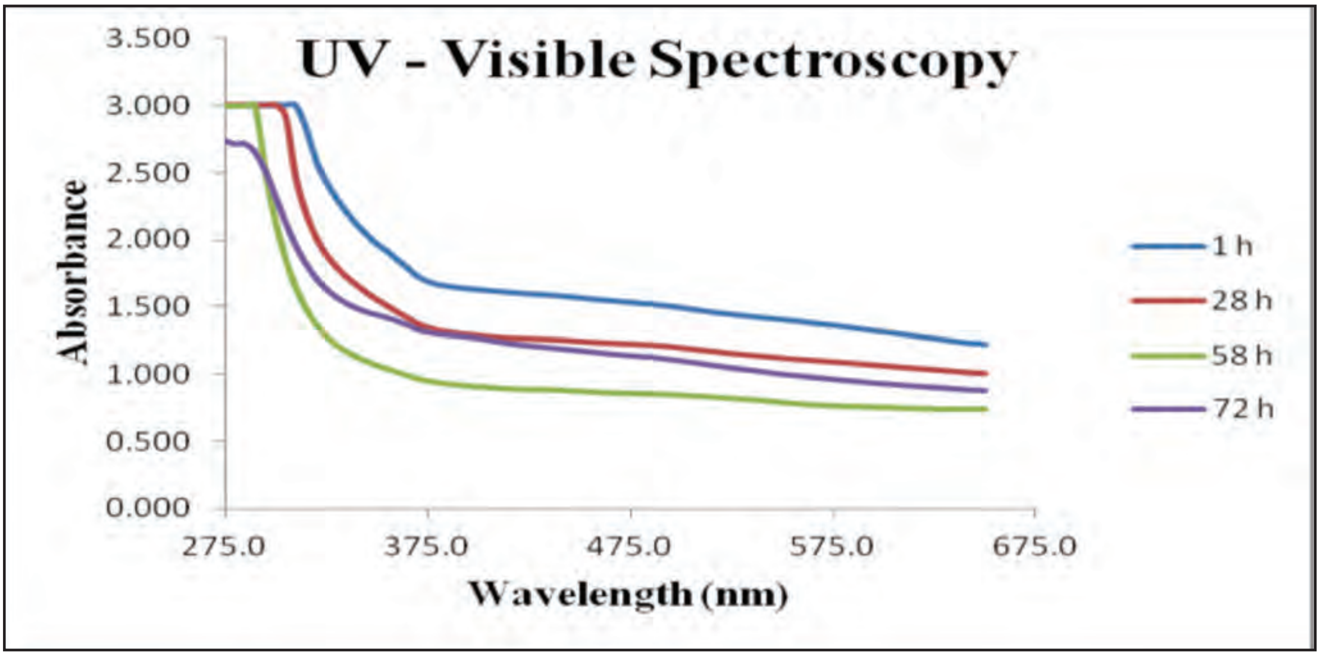

Fig. 4: Graphical representation of the UV-visible spectroscopy is ZnO-TiO2 nano-composite for green tea extract

particles. The antioxidant activity of Mn-doped ZnO nano-particles varied from 0.79±0.11 to 10.9 ± 0.11 µM/mL. Mn-doped ZnO nano-particles exhibited a higher FRAP than un-doped ZnO nano-particles.

In the DPPH assay, the higher the concentration of green tea in ZnO-TiO2 nano-composite, higher the% inhibition. At the concentration of nano-composites 10µg/mL, 64%, 20 µg/mL has a 76%, 30 µg/mL has an 82%, 40 µg/mL has 64% of inhibition and 50 µg/mL has an 89%. [Table I]

In the H2O2 assay, the higher the concentration of green tea in theZnO-TiO2 nano-composite higher was the % of inhibition. Nano-composites at 10µg/ml has, 51.3% inhibition, 20 µg/mL has a 56.1% inhibition, 30 µg/mL has, 62.8% inhibition,40 µg/mL has a 78% inhibition and 50 µg/mL has an 87.5% inhibition, respectively. [Table I]

In the FRAP assay, the % of inhibition increased in a dose-dependent manner. At a concentration of 10µg/mL the nano-composites showed 64.8% inhibition, 20 µg/mL inhibited

74.3%, 30 µg/mL has an 82.6%, 40 µg/mL has an 84.7%, and 50 µg/mL has a 90.1%. [Table I]

Plant-mediated nano-particles were added to the zinc sulfate and titanium oxide solutions. Zinc sulfate was used to synthesize ZnO nano-particles, and titanium oxide was used to synthesize titanium dioxide nano-particles. After one hour a color change was observed which indicated the initiation of a chemical reaction in the solution. After 24 h the color changed completely, indicating synthesis of the nano-particles.

DISCUSSION

The antioxidant activity of green synthesized ZnO-TiO_2 nano-composite using green tea extract was measured by DPPH, H2O2 and FRAP assays to evaluate their radical scavenging activity. The solution was kept undisturbed for two hours to evaluate the stability of the nano-composites. The color change of the solution to pale yellow indicated an increase in free radical scavenging activity with an increase in the concentration of the ZnO-TiO_2 nano-composite. When the antioxidant activity was tested, green tea in the ZnO-TiO_2 nano-composites had good antioxidant properties.

The DPPH assay is used to measure the ability of compounds to scavenge free radicals, particularly DPPH radicals which are stable free radicals. Antioxidants react with DPPH and donate hydrogen atoms or electrons to neutralize it, turning the solution from purple to yellow. It is widely used to predict the antioxidant capacity of substances and understand the mechanisms of antioxidant action against lipid oxidation. In the DPPH assay, the higher the concentration of green tea in the ZnO-TiO_2 nano-composite higher was the % of inhibition. The antioxidant activity of green tea containing ZnO-TiO_2 nano-composites was comparable to that of the standard. When DPPH was inhibited, the colour changed from purple to brown, indicating that hydrogen was donated, demonstrating high scavenging activity.[19] The high redox potential of the ZnO breaks the water molecules into hydroxyl and hydrogen radicals, stabilizing the DPPH free radicals and inhibiting the DPPH effect.[20,21] Antioxidant activity of different percentages of inhibition of oxidation were 52%, 63%, 71%, 85%, and 90% in a dose-dependent manner. Plant extracts mediated by titanium dioxide nano-particles at 40µL and 50µL concentrations exhibited a high antioxidant activity of 90%.

The H2O2 assay evaluated the ability of the samples to scavenge hydrogen peroxide (H_2O_2). Antioxidants, particularly phenolics, can donate electrons to H_2O_2 and convert it into water (H2O). It helps to understand how substances can mitigate oxidative stress by neutralizing H_2O_2. In H_2O_2 assay, the higher the concentration of green tea in ZnO-TiO_2 nano-composite higher the % of inhibition. The % of inhibition shown by the green tea containing nano-composites was similar to the standard values. Previous studies have shown that green tea-mediated TiO_2 NPs exhibit 57–72% scavenging at 100 µg/mL, while at 10 µg/mL, scavenging was 4–9%, indicating ineffectiveness at lower concentrations.[22]

The FRAP assay measures the total antioxidant activity of a sample based on its ability to reduce ferric ions (Fe^3+) to ferrous ions (Fe^2+). Antioxidants in the sample reduce Fe^3+ to Fe^2+ in a colored complex, which can be quantified spectrophotometrically. The FRAP assay is commonly used in the food, beverage, and nutritional supplement industries to assess the overall antioxidant capacity, often using Trolox (a vitamin E analog) as a standard for calibration. In the FRAP assay, the % of inhibition increased in a dose-dependent manner. All values were nearly the same as those of the standard. In FRAP assay, the maximum concentration of antioxidant activity of 8.99 ± 0.21 µM/mL in 1000 µg/mL, and the minimum concentration of antioxidant activity of 0.59 ± 0.01 µM/mL in 60 µg/mL of ZnO un-doped ZnO nano-particles. The antioxidant activity of Mn- doped ZnO nano-particles varied from 0.79±0.11 to 10.9 ± 0.11 µM/mL . Mn-doped ZnO nano-particles exhibited a higher FRAP than un-doped ZnO nano-particles.[23]

Recent research in the field of nano-science has led to the utilization of nano-particles for cancer therapy through targeted drug delivery, as imaging tools in genetic engineering and in the development of electrochemical biosensors for diagnosis and drug delivery.

CONCLUSION

Based on the in vitro tests to evaluate antioxidant activity, it can be concluded that the novel nano-composite containing ZnO-TiO2and green tea extract has good anti-oxidant properties. The advantages of natural antioxidants have non-toxic effects on biological systems, potency, and cost-effectiveness. Owing to their high catalytic and photochemical capabilities, ZnO nano-particles exhibit antibacterial and antifungal properties even at low concentrations.

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