GREEN SYNTHESIS OF TITANIUM DIOXIDE NANOPARTICLES USING ETHANOLIC EXTRACT OF GREEN TEA AND THEIR ANTIOXIDANT ACTIVITIES**

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The aim of this work is to prepare and study titanium dioxide nanoparticles (TiO_2 NPs) that were formed from the leaf extract of green tea using the green synthesis method. Plants were dried for seven days at room temperature and finely ground into a fine powder, after which chemical composition, HPLC analysis, characterization of TiO_2 NPs, and biological evaluation processes were performed. In addition, TiO_2 NPs synthesis was confirmed using UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and high-resolution-transmission electron microscope (HR-TEM). A close analysis indicates that the green tea extract contained polyphenols and flavonoids at varied levels, which may be responsible for its biological activities. HPLC examination indicates the presence of Gallic acid, Protocatechuic, p-hydroxybenzoic, Caffeine acid, Catechin, chlorogenic, rutin, coumaric acid, epicatechin gallate, apigenin-7-glucoside, ferulic acid, chrysin, quercetin, and cinnamic acid, might be responsible for their therapeutic potential. In vitro, antioxidant screening revealed that the scavenging effect of green tea extract has 50% inhibition (IC_{50}) at a concentration level of 19.64±0.13 μg/mL, whereas standard Vit. C showed that at 16.81±0.10 μg/mL. However, in the case of the ABTS model, the scavenging effect of green tea extract has 50% inhibition (IC_{50}) at a concentration level of 33.47±0.21 μg/mL, whereas standard Vit. C showed the same at 29.47±0.17 μg/mL. These results demonstrate that green tea is an excellent antioxidant (i.e., an effective anti-DPPH compound).

Keywords: *green tea, TiO_2 NPs, HPLC, total phenolics, antioxidant activity.*

"ЗЕЛЕНЫЙ" СИНТЕЗ НАНОЧАСТИЦ ДИОКСИДА ТИТАНА С ИСПОЛЬЗОВАНИЕМ СПИРТОВОГО ЭКСТРАКТА ЗЕЛЕНОГО ЧАЯ И ИХ АНТИОКСИДАНТНЫЕ СВОЙСТВА

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Наночастицы диоксида титана (НЧ TiO_2) получены методом "зеленого" синтеза из экстракта листьев зеленого чая. Растения высушивали в течение семи дней при комнатной температуре и измельчали в мелкий порошок, после чего выполняли химический анализ методом ВЭЖХ. Синтез НЧ TiO_2 подтвержден с помощью УФ-видимой спектроскопии, ИК-Фурье-спектроскопии (FTIR), рентгеновской дифракции (XRD) и просвечивающей электронной микроскопии высокого разрешения (HR-TEM). Показано, что экстракт зеленого чая содержит полифенолы и флавоноиды, ответственные за его биологическую активность. Исследование ВЭЖХ указывает на наличие галловой кислоты, протокатеховой, п-гидроксибензойной, кофеиновой кислоты, катехина, хлорогеновой кислоты, рутина, кумаровой кислоты, галлата эпикатехина, апигенин-7-глюкозида, феруловой кислоты, хризина, кверцетина и коричной кислоты. Скрининг антиоксидантов in vitro показал, что поглощающий эффект экстракта зеленого чая ингибируется на 50% (IC_{50}) при уровне концентрации 19.64±0.13 мкг/мл, в то время как стандарт — витамин С концентрации 16.81±0.10 мкг/мл. Однако в случае модели ABTS поглощающий эффект экстракта зеленого чая ингибируется на 50% (IC_{50}) при

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концентрации 33.47±0.21 мкг/мл, тогда как стандарт — витамин С концентрации 29.47±0.17 мкг/мл. Результаты подтверждают, что зеленый чай является отличным антиоксидантом.

Ключевые слова: *зеленый чай, наночастицы TiO_2, ВЭЖХ, сумма фенолов, антиоксидантная активность.*

Introduction. Nanotechnology has recently become a buzzword in several scientific fields, including the area of drug delivery [1]. Within nanotechnology, synthetic materials are in the size range of 1 to 100 nm. Many of these materials are synthesized from many sources such as microorganisms, animals and plants; nature can certainly be synthesized and used in laboratories even on a large scale. These materials are rapidly finding their way into an increasing number of industrial products (e.g., automotive, biomedical, environmental remediation, catalysis) [2, 3] and in an ever-increasing number of consumer products (e.g., clothing, cosmetics, sunscreens) [4, 5]. An eco-friendly or so-called green synthesis is considered to be a very attractive possibility. The use of plants in the synthesis of nanoparticles is quite novel, leading to the truly green chemistry that technologists are looking for. Titanium dioxide (TiO_2) is a stable compound, non-volatile, non-toxic material chemically inert, and extremely insoluble, its low thermal conductivity has a refractory character and it has been used in various industrial applications, such as white pigment, gas sensors, corrosion protective, and optical layers [6], solar cells [7], the purification of the environment [8], high dielectric constant and high electrical resistance [9, 10], the decomposition of carbon dioxide, and because of their catalytic activities, it is used in the generation of hydrogen gas [11]. Besides these, they can also be applied as coatings on glass, forming self-cleaning glass [12].

Material and methods. All reagents used for metal analysis include titanium (IV) tetraisopropoxide, TTIP ≥ 97% purity (Sigma-Aldrich, Co., USA), green tea extract, ammonium hydroxide, NH_4OH (Fluka Analyticals, Sigma-Aldrich, Co., Germany), purchased pure anatase, ≥99% (Sigma-Aldrich, Co., USA), and methylene blue, MB (QREC, Grade AR, (Asia) Sdn. Bhd, Malaysia). The solvents used during the extraction and analysis (ethanol and water) were of spectroscopic grade. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt) and Trolox were bought from Sigma-Aldrich (St. Louis, MO, USA).

The green tea was collected and obtained from a local market in Zagazig city, Sharkia Government, Egypt, and ground to obtain a fine powder. According to the authorization of the Department of Botany in the Faculty of Science, Zagazig University, Egypt, all plant materials have been certified and a voucher (voucher number: 20180905) sample was deposited for further reference.

Plants were dried for seven days at room temperature. Then they were finely ground into a fine powder. Green tea aqueous extract was prepared by the cold maceration method. Green tea powder was soaked in alcohol and kept in a flask at room temperature for two weeks. Then, the extract was filtered and stored at room temperature for further investigation. A part of the green tea extract was used in the Rotary apparatus (evaporation) to get rid of the alcohol also for further analysis.

Preliminary phytochemical screening of the extract was carried out to identify the active constituents, using standard methods. Phytochemical screening for flavonoids, alkaloids, tannins, saponins and terpenoids was done following standard methods (Selim, 2020).

The chemical composition was at a temperature of 22°C, the humidity rate was 23% and it was further dried in the air, whereby the results are shown in Table 1. Ground powder of dried leaves, dried Ethanol extract and dried Methanol extract were used for the analysis of the total composition: total protein %, total fat %, total fiber %, humidity % and total ash %. 0.2 g of dried thyme powder gm HNO_3 were used resulting in a clear pale-yellow solution.

TABLE 1. Phytochemical Screening of Green Tea Extract

Test	Steroids	Terpenoids	Flavonoids	Alkaloids	Saponins	Tannins
Result	++	+++	++++	++	+++	++

N o t e. Highly positive ‘++++’, Moderate ‘++’.

Using a modified methodology, this investigation was carried out to identify and measure specific phenolic chemicals found in the ethanolic extract of green tea (Shuangchan et al., 2013). An Agilent 1260 series was used for the HPLC analysis. 4.6×250 mm i.d., 5 m, C18 column was used for the separation. Water (A) and acetonitrile (B) were the components of the mobile phase, and their flow rate was 1 mL/min. The fol-

lowing linear gradient was used to programme the mobile phase in the following order: 0 min (80% A), 0–5 min (80% A), 5–8 min (40% A), 8–12 min (50% A), 12–14 min (80% A), and 14–16 min (80% A). At 280 nm, the multi-wavelength detector was observed. The injection volume for each was 10 μL for each of the sample solutions. The column temperature was maintained at 35°C. HPLC analysis revealed the presence of a number of polyphenols in both extracts of green tea.

TiO_2 NPs were synthesized in a typical procedure; green tea extract was dissolved in 150 mL of boiling distilled water. Then, 0.01 mol of titanium tetraisopropoxide (TTIP) was added to the green tea extract under stirring for 5 min at 70ºC. Yellow solution with white precipitates was created after adjusting the pH of the solution, whereby the acidic value was at (pH 5.0), the basic was at (pH 9.0) and the neutral was at (pH 7.0), by slowly adding ammonium hydroxide solution ($NH_4(OH)$) with constant stirring for 30 min. The precipitate was centrifuged at 8500 rpm for 10 min, followed by a number of cycles of washing using distilled water, and finally dried at room temperature to obtain TiO_2 NPs.

Initially, the UV-Vis spectroscopy of the Rigol ultra-3660 was used to investigate the TiO_2 NPs between 200 and 800 nm. The functional groups and different phytochemical components involved in the reduction and stability of the produced nanoparticles were then determined using FTIR. FTIR was performed with a Jasco FTIR 4100 spectrophotometer in the attenuated total reflectance (ATR) mode (Japan). The findings were measured between 400 and 400 cm^{-1}. To validate the existence of ZnO and examine the crystallite structure and size, the powdered sample was exposed to CuK1-X Ray diffractometer radiation (1.5406 Å) running at 40 kV and 30 mA with 2θ ranging from 30–140°. TiO_2 nanopowder was sonicated, deposited onto a copper grid while suspended in ethanol, allowed to dry, and then analyzed with a JEOL-2100. HR-TEM illustrates the particle size of TiO_2 NPs (Fig. 1).

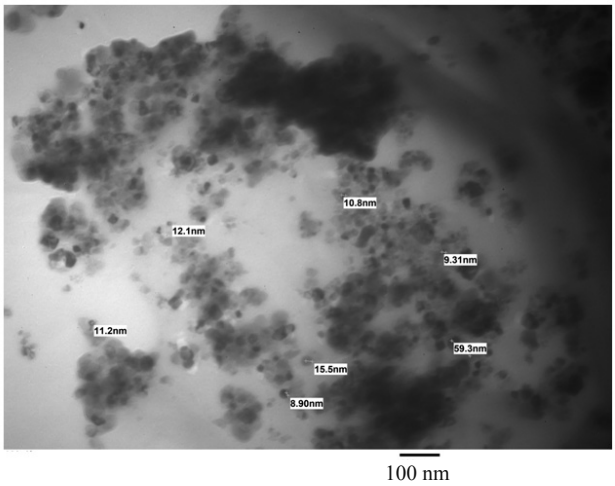

Fig. 1. The size particle of TiO_2 using high resolution-transmission electron microscope (HR-TEM).

In vitro, antioxidant assays used in this present work for the antiradical pharmacological evaluation of the green tea are the ABTS test (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay) and the DPPH test (2,2-diphenyl-1-picrylhydrazyl free radical bleaching assay), which are two of the most common and famous assays used as a base for the further biological evaluation of the antioxidant activities of new organic compounds. Via antioxidant activity assay, the free radical scavenging activity of green tea was measured by the DPPH method with some modification [13]. The mixtures of synthesized compounds at different concentration aliquots were taken and the adjusted volume was up to 3 mL with methanol. 1 mL of 0.1 mM solution of DPPH in methanol was added to the mixture. In addition, the resulting mixture was kept in the dark for 30 min. The free radical scavenging activity of synthesized compounds was compared to standard (Vit. C). One milliliter of 0.1 mM of methanolic solution of DPPH and 3 mL of methanol was considered as the control solution:

$$\text{Inhibition}(\%) = \left[\left(A_{\text{cont}} - A_{\text{test}} \right) / A_{\text{cont}} \right] \times 100,$$

where A_{test} is the absorbance of the test sample, and A_{cont} is the absorbance of the control. The sample concentration accounting for 50% inhibition (IC_{50}) was determined. All the experiments were performed in triplicate and IC_{50} values were expressed as mean ± standard deviation.

To begin with, 0.36 g of ABTS was dissolved in some distilled water. The following stage was 0.0662 g dissolved to which a solution of potassium persulfate ($K_2S_2O_8$) was added, and the mixture was filled with water up to a final volume of 100 mL. To create the ABTS radical cation (ABTS+), the mixture was allowed to sit at room temperature in the dark for 16 hours, after which ethanol was used to dilute the solution 100 times. Water was added to the green tea infusions to dilute them (1+3 v/v); 10 mL of the sample plus 1 mL of (ABTS+) added to the mixture, which was then incubated at room temperature in the dark for 6 min to measure the absorbance at a wavelength of 736 nm. The antioxidant capacity was demonstrated employing Trolox-like substances. Several Trolox concentrations in water were created for this purpose (0–15 mM/L) and handled in the same manner as the samples under investigation.

Results and discussion. All results of the phytochemical analysis are shown in Table 1. In the present study, the green tea extract showed positive results for triterpenes and/or steroids as measured by the Liebermann-Burchard reaction. It was found that green tea extract contained polyphenols and flavonoids, which may be responsible for the biological activities found. The chemical composition of the investigated green tea was presented in Table 2. Moisture, ash, crude protein, crude fat, crude fiber, and carbohydrate contents were tested. HPLC analysis indicates the presence of gallic acid, protocatechuic, *p*-hydroxybenzoic, caffeine, acid, catechin, chlorogenic, rutin, coumaric acid, *Epicatechin gallate*, apigenin-7-glucoside, ferulic acid, chrysin, quercetin, and cinnamic acid (Fig. 2 and Table 3), which might have been responsible for their therapeutic potential.

TABLE 2. Chemical Composition of Green Tea

Measuring	Standart Deviation		Method used in measuring
Protein	21.5	±0.82	ES:5465-1/2006
Fat	2.3	±0.05	EN(EC)NO.152/2009
Fiber	12.4	±0.93	
Humidity	8.17	±0.15	ES:5464/2006
Ash	6.1	±0.06	

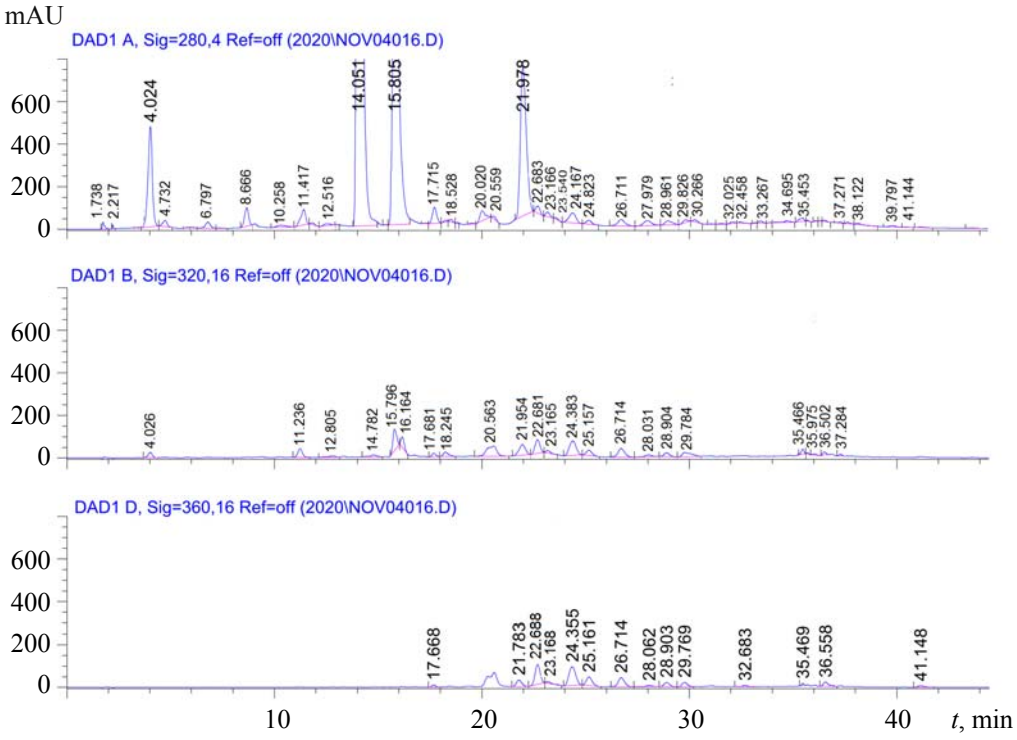

Fig. 2. HPLC chromatogram of green tea.

During synthesis, the change in color of the solution and formation of a yellow solution-white precipitate was an indication that titanium oxide had been reduced. The formation of TiO_2 NPs was initially confirmed by UV-Vis spectroscopy within the range of 200 to 600 nm. The absorption spectrum of green synthesized TiO_2 NPs showed a characteristic peak at 315 nm (Fig. 3). The direct energy gap (E_g) calculated was 3.32 eV.

TABLE 3. Polyphenolic Compounds of Green Tea Extract

Compound	Conc., µg/g	Compound	Conc., µg/g
Gallic	2351.62	Sinapic	ND
Protocatechuic	266.13	*Epicatachin gallate*	216.85
p-hydroxybenzoic	157.67	*p*-coumaric	244.61
Gentisic	ND	Rutin	67.86
Cateachin	1638.30	Rosmarinic	ND
Chlorogenic	68.91	Apigenin-7-glucoside	132.68
Caffeic	ND	Cinnamic	57.21
Caffeine	37740.99	Quercetin	44.68
Syringic	ND	Apigenin	ND
Vanillic	ND	Kaempferol	ND
Ferulic	301.97	Chrysin	7.44

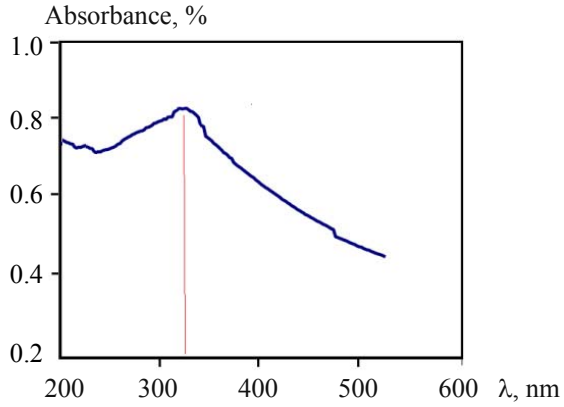

Fig. 3. UV spectrum of TiO_2 NPs synthesized from green tea.

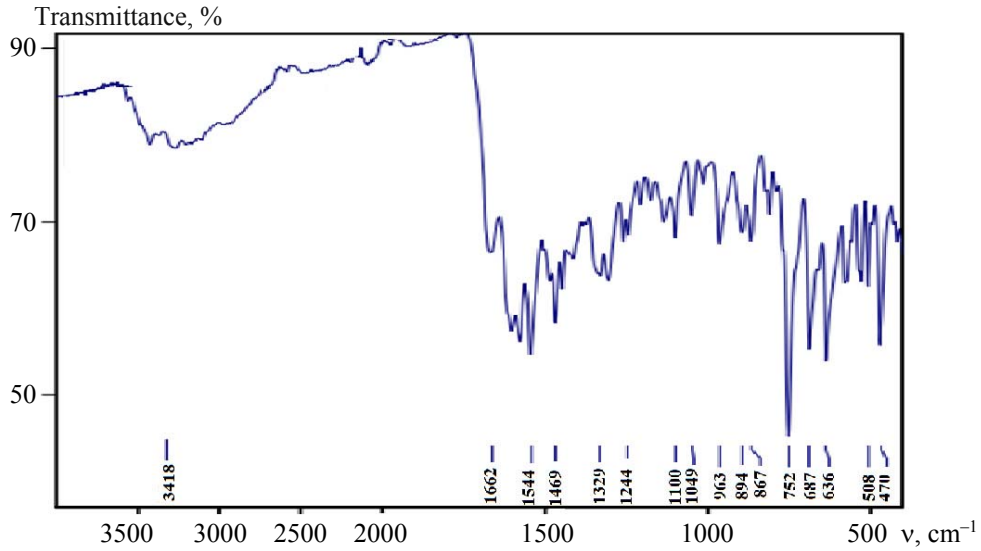

Fig. 4. FTIR spectrum of TiO_2 NPs synthesized from green tea.

FTIR is used as a confirmatory technique for nanoparticle formation and offers an impression of the vibrational and rotational modes of the existing molecules; hence, it helps to identify the functional and possible phytochemical molecules involved in the reduction and stabilization of TiO_2 NPs. Figure 4 represents the FTIR spectrum of TiO_2 NPs synthesized by the green approach in the range of 400–4000 cm^{-1}; peaks at 470 and 752 cm^{-1} are for O–Ti–O bonding in anatase morphology [14, 15]. Bands centered at 1544 and 3418 cm^{-1} are characteristic of surface-adsorbed water and hydroxyl groups [15]. Peaks at 479 and 652 cm^{-1} are contributions of the anatase TNPs [14]. Peaks centered at 1662 cm^{-1} indicated the characteristic of δδ-H_2O bending and the vibration of hydroxyl groups [15]. There is no peak at 2900 cm^{-1} for the curve of TNPs regarding the C–H stretching band, which means all organic compounds were removed from the samples.

XRD peaks in Fig. 5 indicating a wide-angle range of 2θ (20° < 2θ < 90°) were ascertained, whereby the peaks in 25.287, 36.940, 37.844, 38.511, 48.110, 53.892, 55.152, 62.779, 68.718, 70.402, 75.062, and 76.013° can be attributed to the 101, 103, 004, 112, 200, 105, 211, 204, 116, 220, 215, and 224 crystalline structures of anatase synthesized TNPs, respectively.

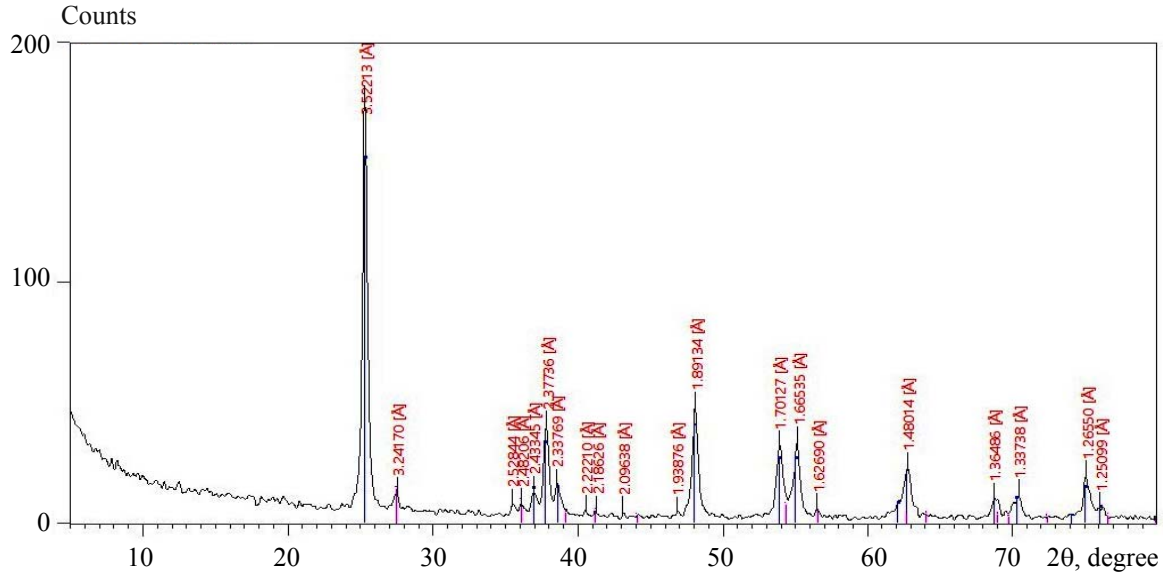

Fig. 5. XRD of TiO_2 NPs synthesized from green tea.

TABLE 4. Results of the Antioxidant Capacities (Expressed as IC_{50} Values) of the Green Tea in the DPPH and ABTS Test (Using Vit. C as Antioxidant References)

Comp.	Concentration, μg/mL						IC_{50}
	10	20	40	60	80	100	
	% Inhibition						
DPPH							
Vit. C	38.7	52.1	69.6	81.8	87.4	94.5	16.81±0.10
Green tea	32.6	50.0	64.8	78.3	91.2	97.8	19.64±0.13
ABTS							
Vit. C	24.2	37.4	54.9	66.7	78.5	94.3	29.47±0.17
Green tea	21.5	36.5	50.1	64.5	71.0	89.5	33.47±0.21

The structural elucidation of the titled synthesized compounds showed the presence of phenolic groups. In general, nitrogen-bearing heterocyclic rings and phenolic compounds have free radical scavenging activity. DPPH radicals accept the hydrogen atom or electron from the organic molecules and can form stable diamagnetic molecules. The scavenging effect of green tea extract showed 50% of inhibition (IC_{50}) at a concentration level of 19.64±0.13 μg/mL, whereas standard Vit. C showed that at 16.81±0.10 μg/mL (Table 4). However, in the case of the ABTS model, the scavenging effect of green tea extract showed 50% of inhibition (IC_{50}) at a concentration level of 33.47±0.21 μg/mL, whereas standard Vit. C showed the same at

29.47±0.17 μg/mL (Table 4). These results prove that green tea is obviously a good-excellent antioxidant (i.e., effective anti-DPPH compounds). Being very close and relatively different, the differences in values in this assay can be explained by and attributed to the same effects of structural modifications (i.e., differences) that were previously mentioned under the ABTS test.

Conclusions. The TiO_2 nanoparticles were successfully synthesized using a green synthesis method of green tea extract. The formation of the nanoparticles was confirmed by UV-spectrophotometer, XRD, and HR-TEM. The particle size and crystalline phases of the prepared biomaterial have been determined. The present work proves that biosynthesis of TiO_2 nanoparticles using green tea extract contains polyphenols and flavonoids, which may be responsible for the biological activities found in living beings. Finally, the results investigate that the formation of particles in a nano-size range can be further used in various biomedical applications.

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