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Phytochemical Analysis of *Wrightia tinctoria* Leaves and Synthesis of ZnO Nanoparticles for the Remediation of Water Pollutants

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In this work, zinc oxide nanoparticles (ZnO NPs) were fabricated using aqueous extract of *Wrightia tinctoria* leaves for evaluating its catalytic and antibacterial efficacy against water contaminants. The qualitative phytochemical estimation showed the presence of secondary metabolites alkaloids, tannins, terpenoids, steroids, glycosides, flavonoids and biomolecules carbohydrates and proteins in different solvent extracts. The quantitative estimation for phytochemicals showed total phenols (39.59 mg/g), total flavonoids (44.34 mg/g), total alkaloids (149.56 mg/g) and total tannins (56.47 mg/g). The GC-MS analysis revealed the bioactive compound mome inositol as the major phytochemical present in *W. tinctoria* leaves. The characterization of ZnO nanoparticles with XRD showed Bragg's reflections at 2θ angles of 31.76º, 34.47º, 36.23º, 47.48º, 56.59º, 62.99º and 68.00º which confirmed the hexagonal wurtzite structure and size (86 nm) of the nanoparticles. The SEM analysis showed that the morphology of synthesized was spherical structure with uniform distribution. The ZnO nanoparticles effectively inhibited the growth of water contaminating bacterial pathogens *viz*. *Escherichia coli*, *Staphylococcus aureus*, *Klebshiella pneumoniae* and *Streptococcus pneumoniae*. The photocatalytic activity of ZnO oxide nanoparticles in degrading textile dyes, methylene blue and methyl orange was found to be effective.

Keywords: *Wrightia tinctoria*, Antimicrobial, Photocatalytic activity, Methylene blue, Methyl orange.

INTRODUCTION

Water is a non-renewable resources that occupies 71% of earth's total surface and forms the basic need for all the life forms on earth. However rapid urbanization, industrialization and economic growth have paved the way for several polluting industries that contaminate the environment particularly, water. Because of severe water pollution accumulation of several biological and chemical contaminants resulted in several diseases. The discharge of noxious chemicals affects the symbiotic processes in aquatic ecosystem, resulting in reduced photosynthetic activity [1,2]. Wastewater from textile industries containing dye molecules are the primary source of chemical contaminants. These dyes contain acidic, basic, anthroquinone, azo and metal complexes that are mutagenic and carcinogenic. The structural complexity, solubility, low biodegradability and resistance to heat and light are the challengeable factors in the remediation of dyes [3,4]. Several conventional treatment procedures employed in effluent treatment includes adsorption, chemical oxidation, coagulation, flocculation, filtration and precipitation [5,6] are ineffective and insufficient. The major disadvantage of these methods is that they are highly expensive and requires additional treatment procedures. The anaerobic degradation of dyes results in carcinogenic byproducts [7]. The biological treatment processes results in incomplete degradation of dye and their effectiveness is restricted in controlling the BOD and COD [8].

Similarly, microbial contamination is another important issue in water pollution that causes several infectious and severe diseases. The disinfection of water by various conventional treatments such as chlorination, ultraviolet radiations and ozonation had their own limitations. For instance, chlorination is ineffective against multidrug resistant pathogenic microbes. Nanotechnology is an alternative eco-friendly platform to counter challenge the drawbacks associated with the removal of dyes and in inhibiting the growth of harmful bacterial pathogens. The development of nanoparticles through green route are non-toxic and do not produce any unwanted by

products that are harmful to humans and environment [9] in detoxification and removal of toxic materials from aquatic ecosystem [10,11]. Among the various nanoparticles, ZnO nanoparticles are distinctive electronic and photonic wurtzite n-type semiconductor with a wide direct band gap (3.37 eV) and high binding energy (60 meV) [9,12]. Zinc oxide nanoparticles are ideal and promising candidate in photocatalytic application [13].

Medicinal plants have been used for treating chronic and communicable diseases, since ages [14]. The medicinal plants contain different phytochemicals that possess therapeutic properties in the treatment of diseases. Identification of phytochemical composition of the plants and the advent of modern technologies has paved the way for the development of new modern drugs from plant sources [15]. *W. tinctoria* is a deciduous tree belonging to the family Apocynaceae is widely distributed throughout India [16]. The plant is used in traditional medicine system of India such as Siddha and Ayurveda [17,18]. The leaves of *W. tinctoria* are rich in alkaloids, cardiac glycosides, flavonoids, terpenoids and tannins [19,20]. The advent of nanotechnology further increased the drive in exploring the enhanced biological properties of phytochemicals. The structural complexity of phytochemicals contributes to its therapeutic potential [21-24]. The easy availability, low cost and minimal or absence of adverse effects increased the demand in utilizing these natural resources in the treating various diseases [25]. Therefore, this study attempt to synthesize zinc oxide nanoparticles (ZnO NPs), using the *Wrightia tinctoria* leaves for the synthesis of ZnO NPs for evaluating its photocatalytic efficacy against textile dyes (methylene blue and methyl orange) under solar irradiation and its antibacterial potency against water contaminating bacterial species.

EXPERIMENTAL

Preparation of solvent extracts: Leaf powder of *W. tinctoria* (20 g) was extracted with 250 mL of solvents such as water, benzene, petroleum ether, chloroform, acetone and ethanol using Soxhlet's apparatus. Extraction was continued till the extractor becomes colourless. The collected solvent extracts were condensed under reduced pressure using rotary evaporator. The crude extract obtained was stored at 4 ºC.

Qualitative estimation of phytochemicals: The crude extract obtained with Soxhlet extraction was dissolved in respective solvent for the identification of phytochemicals (secondary metabolites). The secondary metabolites present in *W. tinctoria* leaves were identified (qualitative) with preliminary phytochemical screening following the procedure of Kokate [25] and Harborne [26].

Quantitative estimation of phytochemicals: Phytochemicals such as total phenol and tannin content were estimated by Folin-Ciocalteau method following the method of Rasool *et al.* [27] and Afify *et al.* [28], respectively. The total flavonoid content was estimated using calorimetric method of Atanassova *et al.* [29]. Alkaloid content was determined following the method of Rao *et al.* [30].

Preparation of aqueous extract: Fresh healthy leaves of *W. tinctoria were* collected from Kumbakonam, Thanjavur District, Tamilnadu, India were used. The leaves were rinsed with water to remove any surface contaminations. The washed leaves were shade dried for 10 days and macerated into a fine powder. 5 g of the leaf powder was dissolved in 100 mL of distilled water and boiled to 80 °C for 30 min. The leaf extract was cooled, filtered using Whatmann filter paper (No. 1) and stored at 4 °C until future experiments.

Green synthesis of ZnO nanoparticles: The ZnO NPs were prepared with aqueous *W. tinctoria* leaf extract following the protocol of Karimi *et al.* [31]. 5% aqueous leaf extract was mixed 0.1 M zinc nitrate solution at a ratio of 1:5 and heated to 80 °C for 2 h. The solution cooled to room temperature and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and pellets were re-suspended in distilled water, centrifuged for 10 min at 1000 rpm. The pellets were dried in an oven for 12 h at 70 °C and calcinated at 350 °C for 2 h for the formation of ZnO NPs.

Characterization: The characterization of *W. tinctoria* mediated ZnO nanoparticles were carried out with XRD and SEM microscopic techniques. The crystallinity and size of the nanoparticles were determined with XRD analysis. The distribution and morphology of the synthesized ZnO NPs were observed with SEM analysis.

Particle size measurement: Particles size (L) of the ZnO NPs was calculated using (PAN analytical X pert PRO Model) following Debye-Scherrer's equation:

$$L = \frac{0.9\lambda}{\beta h\theta}$$

where λ is the wavelength of the X-ray, β is full width and half maximum and θ is the Bragg's angle.

Photocatalytic activity: The catalytic activity of the biosynthesized ZnO NPs was studied using methyl orange (MO) and methylene blue (MB) as model system dyes under direct sunlight. A solution of dye (100 mL) was added to 10 mg/L of *W. tinctoria* mediated ZnO NPs. A control was also maintained containing only dye. The mixture was stirred magnetically for 30 min in dark condition prior to exposure to direct sunlight irradiation. At specific intervals (every 30 min), an aliquot of the suspension was centrifuged for 10 min at 10,000 rpm to obtain a clear supernatant and the optical density was measured using a UV-visible spectrophotometer in the wavelength range from 300 to 800 nm [32]. The λ_{max} of MB and MO was at read at 660 nm and 460 nm, respectively. The concentration of dye after photocatalytic experiments was evaluated by measuring the absorbance of dye. The photocatalytic degradation efficiency (R) of ZnO NPs was calculated using the following equation [33].

$$R = \frac{A_o - A_t}{A_o} \times 100$$

where A_o = absorbance of dye at time 0; A_t = absorbance of dye at time t.

Bactericidal efficacy of *W. tinctoria* mediated ZnO NPs: Gram-negative bacteria (*Klebsiella pneumonia* and *Escherichia coli)* and Gram-positive bacteria (*Staphylococcus aureus* and *Strepcoccus pneumoniae*) were obtained from IMTECH, India.

TABLE-1
PRELIMINARY SCREENING OF PHYTOCHEMICALS IN *W. tinctoria* LEAVES

Phytochemicals	Solvent extracts					
	Water	Ethanol	Acetone	Chloroform	Benzene	Petroleum ether
Carbohydrates	+	+	+	+	+	+
Proteins	–	–	–	+	+	+
Saponins	+	+	+	–	–	–
Alkaloids	+	+	+	+	–	–
Glycosides	–	–	+	+	–	–
Flavonoids	+	+	+	+	+	+
Terpenoids	+	+	–	+	–	–
Sterols	+	+	+	–	+	+
Tannins	+	+	+	+	–	–

The bactericidal activity of *W. tinctoria* synthesized ZnO NPs was evaluated using agar well diffusion assay following the method of Kumar & Rao [34]. These bacterial strains were inoculated in the nutrient broth medium and cultures were adjusted to 0.5 McFarland standards (1×10^8 CFU mL^{-1}) and spread on to sterilized nutrient agar plates. The plates were dried for 15 min before being used for sensitivity test. Standard antibiotic amoxicillin (positive control) and different concentrations of *W. tinctoria* mediated ZnO NPs (40, 50 and 60 µg/mL) were added to wells made on nutrient agar surface. The plates were incubated at 37 ºC for 24 h and the zone of inhibition was measured.

RESULTS AND DISCUSSION

The qualitative determination of phytochemicals in *Wrightia tinctoria* leaves of was examined using different solvent extracts based on polarity. The ethanol extract of *W. tinctoria* reported flavonoids, saponins, alkaloids, tannins, terpenoids and sterols. Carbohydrates, proteins, alkaloids, glycosides, flavonoids, tannins and terpenoids were identified in the chloroform extract (Table-1). However, Vedhanarayanan *et al.* [35] identified alkaloids and saponins in the ethanolic extract. Similarly, saponins and steroids were also identified in chloroform extract. The aqueous extract of *W. tinctoria* had carbohydrates, saponins, alkaloids, flavonoids, terpenoids, sterols and tannins which was in agreement with findings of Maddila & Hemalatha [36]. The difference in phytochemicals might be attributed to the difference in geographic distribution, environmental parameters and the capacity of solvent in dissolving the phytochemicals [35].

The quantitative estimation of phytochemicals (Table-2) revealed a total phenolic content 39.59 ± 0.18 mg/g equivalent of gallic acid. Iqbal *et al.* [37] reported that *W. tinctoria* leaves collected from Amity University campus, Noida, India had a total phenol content of 18 mg/g equivalent of gallic acid, which was much lower than the phenolic content recorded in this work. The total phenolic content of *W. tinctoria* leaves obtained from different localities in Gujarat province of India ranged between 15.52 to 29.83 mg/g equivalent of gallic acid [38]. Similarly, high phenolic content of 67 mg/g equivalent of gallic acid was reported with *W. tinctoria* roots [39]. The difference in phenolic content might be attributed to the influence of soil constituents.

TABLE-2
QUANTITATIVE ESTIMATION OF
PHYTOCHEMICALS IN *W. tinctoria* LEAVES

Phytochemicals	Content in leaf (mg/g)
Total phenol	39.59 ± 0.18
Total flavonoids	44.34 ± 0.28
Total alkaloids	149.56 ± 0.26
Total tannins	56.47 ± 0.41

The *W. tinctoria* leaves alkaloid content of was 44.34 ± 34 mg/g equivalent of atropine. Nath *et al.* [38] reported total alkaloid content of 48.40 mg/g dry weight of *W. tinctoria* leaves collected from Shamlaji, India was in agreement with the present study. The tannin content was 149.56 ± 0.26 mg/g equivalent of gallic acid. Though the quantitative estimation of tannin content in *W. tinctoria* leaves was not reported earlier, tannin content of *W. tinctoria* seeds (240.67 ± 5.21) reported by Subhashini Devi *et al.* [39] was high compared to leaves. Similarly, the flavonoid content (56.47 mg/g) equivalent of quercetin was comparatively high to the flavonoid content (16.16, 14.46, 22.89, 13.95 mg/g) equivalent of quercetin documented in the leaves of *W. tinctoria* collected from different stations in Gujurat [38]. Total flavonoid content of 65.21 mg/g equivalent of gallic acid from *W. tinctoria* leaves was in agreement with the findings of this study [37].

The GC-MS analysis revealed the presence mome inositol (74.75%) as the major compound in the ethanol extract of *W. tinctoria* leaves (Table-3 and Fig. 1). The compounds identified in the ethanol extract of leaves were alcohol, carboxylic acids, fatty acids and terpenoids. Mome inositol was reported with anti-cirrhotic, anti-alopecic, anti-neuropathic, lipotropic, anti-cholesterolytic activities. It is generally used as a sweetening agent [40]. Similarly, hexadecanoic acid (CAS) palmitic acid is a 5-α-reductase inhibitor and a hemolytic agent [41]. 9-Octadecenoic acid (or) oleic acid is a saturated fatty acid that possess anti-inflammatory and anti-tumor properties [42]. 2-Methoxy-4-vinylphenol is an antioxidant, antimicrobial, anti-analgesic, anti-inflammatory and anti-germination agent. Heptadecene-(8)-carbonic acid was reported with antibacterial activity [43]. From the results of GC-MS analysis, it is evident that bioactive compounds such as mome inositol, 9-octadecenoic acid, dihydrohenzofuran, hexadecanoic acid, 2-methoxy-4-vinyl phenol and methyl linoleate contribute to the antibacterial efficacy of *W. tinctoria*.

TABLE-3
IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM ETHANOL EXTRACT OF *W. tinctoria*

S. No.	Area (%)	Retention time	m.f.	Compound name
1	0.44	5.254	C_8H_8O	2.3-Dihydrobenzofuran
2	0.54	5.587	$C_8H_{14}O_4$	1,4-Diacetoxybutane
3	3.57	6.500	$C_{11}H_{14}O_4$	2-Methoxy, 4-vinylphenol
4	0.26	7.108	$C_5H_7O_2$	4-Deuterio-*trans*-3,4-dihydroxy-cyclopentene
5	0.35	9.707	$C_8H_{10}O_2$	O-Anisyl alcohol
6	0.39	9.933	$C_{12}H_{18}$	1-(3,3-Dimethyl-but-1-ynyl)-1,2-dimethyl-3-methylene-cyclopropane
7	0.25	10.005	$C_{12}H_{14}O_4$	Diethyl phthalate
8	2.61	10.753	$C_7H_{12}O_6$	Quinic acid
9	74.75	11.155	$C_7H_{14}O_6$	Mome inositol
10	1.07	11.806	$C_{14}H_{28}O_2$	Mystric acid
11	1.52	11.932	$C_{10}H_{12}O_3$	Coniferyl alcohol
12	0.25	12.531	$C_{11}H_{18}O_2$	2,6,8–Trimethylbicyclo(4.20) oct-2-ene-1,8-diol
13	0.23	12.759	$C_{10}H_{18}O$	Eucalyptol
14	0.48	13.574	$C_{19}H_{36}D_2O_2$	Methyl 17, 18 – dideutero octadeconate
15	0.23	13.692	$C_{14}H_{22}N_2O$	Xycaine
16	3.93	13.988	$C_{16}H_{32}O_2$	Palmitic acid
17	1.06	15.571	$C_{20}H_{40}O$	Phytol
18	2.48	15.863	$C_{18}H_{34}O_2$	Heptadecane–(8)–carbonic acid
19	4.25	15.928	$C_{19}H_{32}O_2$	Methyl linolenate
20	1.38	16.085	$C_{18}H_{34}O_2$	Oleic acid

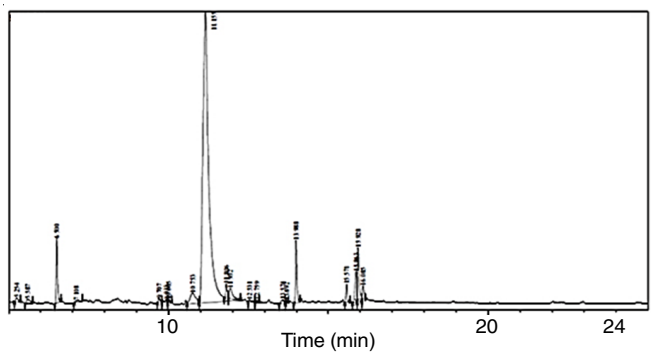

Fig. 1. GC-MS chromatogram of ethanol extract of *W. tinctoria* leaves

Preparation of ZnO NPs: Aqueous *W. tinctoria* leaf extract (10%) solution was used to reduce 0.1 M $Zn(NO_3)_2$. The stoichiometric concentration was fixed at 1:5 ratio. The reacting solution was heated for 2 h at 70 ºC and the pH was adjusted to 10 with NaOH solution. The change in the colour of the reaction mixture from light brown to pale yellow indicated the formation of zinc-phyto complex [31]. The pellets collected after centrifugation were calcinated at 350 ºC for the formation of ZnO NPs. The ZnO NPs mediated through *W. tinctoria* leaf extract exhibited an absorption peak at a wavelength measuring 340 nm [44]. XRD analysis showed that ZnO NPs were crystalline. The nanoparticles were confirmed to possess hexagonal wurtzite structure. The Bragg's reflections at 2θ angles of 31.76º, 34.47º, 36.23º, 47.48º, 56.59º, 62.99º and 68.00º corresponding to miller index (100), (002), (101), (102), (103) and (112), respectively (Fig. 2). A similar XRD spectrum with peaks at 31.72º, 34.38º, 36.20º, 47.48º, 56.52º, 62.81º, 67.81º and 67.89º was obtained with *Leucaena leucocephala*. Corresponding to miller indices of (100), (002), (101), (102), (110), (103), (112) and (201) planes, respectively [45] was consistent with XRD spectrum obtained with ZnO NPs mediated through *W. tinctoria* leaf extract. The

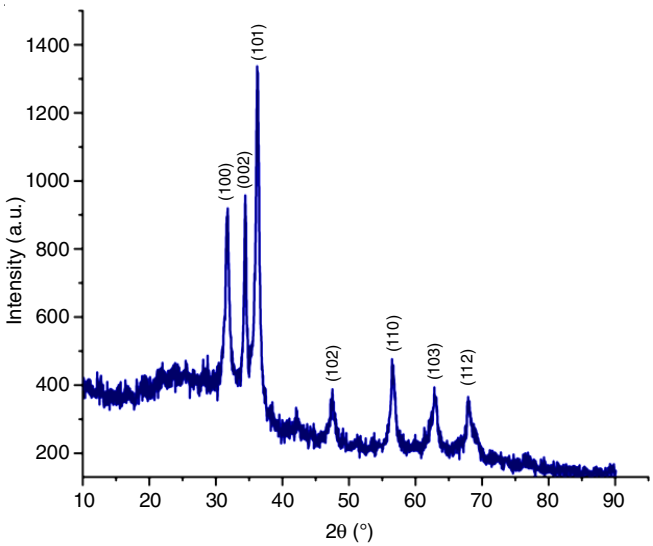

Fig. 2. XRD spectrum of ZnO NPs mediated through *W. tinctoria* leaves

size of the synthesized nanoparticles was found to be 86 nm using Debye-Scherrer's equation. The SEM analysis showed that the synthesized nanoparticles were agglomerated spherical and hexagonal structures (Fig. 3).

Antibacterial activity: Zinc oxide nanoparticles are particularly less toxic compared to silver nanoparticles [46-48] and are involved in the generation of reactive oxygen species (ROS) such as hydroxy radicals, superoxides and hydrogen peroxides. The interaction of ROS with cellular components such as proteins, lipids and DNA cause cell death by initiating apoptosis. Literature suggests that Zn^{2+} ions interact with biomolecules of bacterial cell through electrostatic forces. The coordination of Zn^{2+} ion with proteins is brought about by the lone pair of electrons present in the nitrogen atom of proteins. ZnO NPs are effective towards both aquatic and terrestrial microbes [46,49-51]. The surface area of ZnO NPs is increased upon

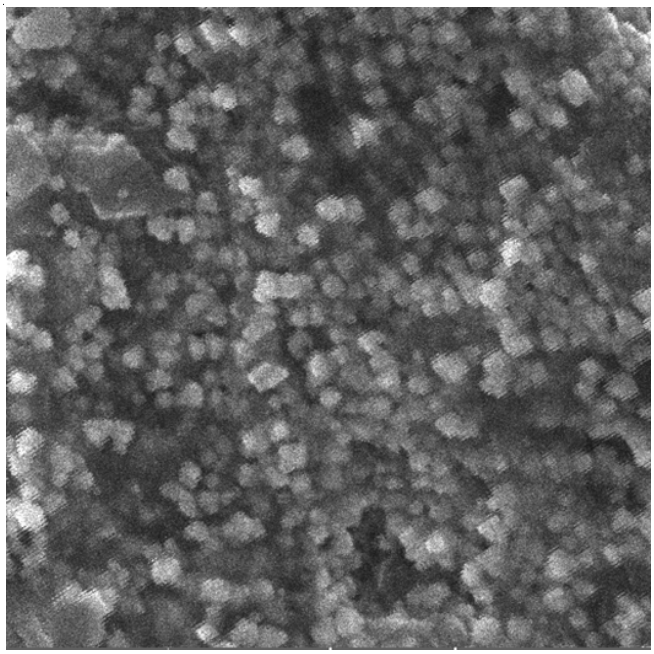

Fig. 3. SEM images of ZnO NPs synthesized using *W. tinctoria* leaves

absorption and interaction with pathogens and enzymes [52]. In present study, ZnO NPs mediated through aqueous *W. tinctoria* leaf extract of were effective towards both Gram-positive and Gram-negative bacteria. Further, the antibacterial activity was dose dependent. The study observed no significant difference in the bactericidal effect of ZnO NPs against *S. aureus* and *E. coli* (16 mm) at 60 µL concentration. *K. pneumoniae* was susceptible at higher concentration (60 µL) compared to *S. pneumoniae* (Table-4).

Photocatalytic activity: The photocatalytic activity of *W. tinctoria* mediated ZnO NPs was monitored for the degradation of methylene blue (Fig. 4a) and methyl orange (Fig. 4b) at wavelength 660 and 460 nm, respectively. From the UV-Visible spectroscopic analysis, it was evident that the degradation of dyes was proportional to the time of exposure to ZnO NPs. The reaction was carried out for a period of 240 min. The degradation of dye was achieved completely as there

TABLE-4
ANTIBACTERIAL ACTIVITY OF ZnO NPs
MEDIATED BY *W. tinctoria* LEAVES

Bacterial species	Antibiotic amoxicillin	Zone of inhibition (mm)		
		40 µg/mL	50 µg/mL	60 µg/mL
Staphylococcus aureus	11.0	14.5	15.5	16.0
Streptococcus pneumoniae	18.5	13.5	14.0	14.5
Escherichia coli	20.5	14.5	14.0	16.0
Klebsiella pneumoniae	16.0	13.0	15.0	15.5

were no additional peaks in UV spectrum indicating the absence of intermediate products. Siripireddy & Mandal [53] reported on the biogenic preparation of ZnO NPs using *Eucalyptus globus* leaf extract which was effective in degrading methylene blue (98.2%) and methyl orange (84.46%) was in agreement with present findings.

Conclusion

The qualitative and quantitative estimation of phytochemicals revealed that the leaves of *W. tinctoria* are rich in flavonoids, phenol, alkaloids and tannins. The zinc oxide nanoparticles (ZnO NPs) synthesized using *W. tinctoria* effectively inhibited the growth of Gram-positive and Gram-negative bacterial pathogens and was also observed to be an effective photocatalyst in the preferential degradation of textile dyes methylene blue and methyl orange. Hence, the study suggested that ZnO NPs mediated throught *W. tinctoria* would be effectively utilized in the degradation of textile dyes as well as for inhibiting the water contaminating bacterial pathogens.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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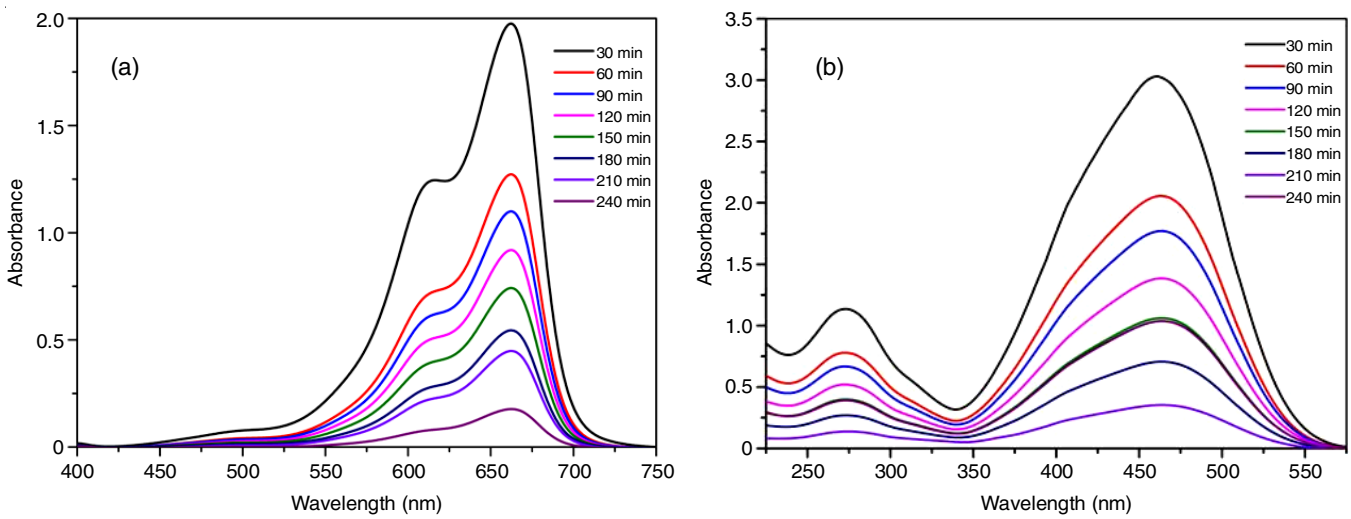

Fig. 4. Photocatalytic degradation of (a) methylene blue dye and (b) methyl orange dye by *W. tinctoria* mediated ZnO NPs

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