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ZnO Nanoparticles bio-synthesized using *Hibiscus subdariffa* Leaf extract for Potential Medicinal Application in hyperbilirubinemia

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ABSTRACT: Zinc oxide nanoparticles (ZnO NP) were synthesized following the green route by whole leaf extract of the plant *Hibiscus subdariffa*. The formation of ZnO NPs was confirmed by X-ray diffraction (XRD) and UV-visible (UV-VIS) spectroscopy. The morphology and size distribution of synthesized particles were analysed by Field emission scanning electron microscopy (FESEM) and High resolution transmission electron microscopy (HRTEM). This study also provides experimental evidence for its ameliorative action against CCl_4-induced hyperbilirubinemia in Swiss albino mice. Liver function enzyme assays like ALT (alanine transaminase), ALP (alkaline phosphatase), AST (aspartate aminotransferase), GGT (Gamma-glutamyl Transferase) and biochemical studies showed that altered biochemical profiles due to jaundice were restored significantly after the administration of *H. subdariffa*-mediated synthesized ZnO nanoconjugate over control and other treatments. Insignificant changes in hematological parameters such as Hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelets and white blood cell counts among different treatment groups suggested the bio-friendly nature of the green NPs. *H. subdariffa*-mediated synthesized ZnO nanoconjugate exhibits a synergistic effect of both chemically synthesized ZnO NPs and *H. subdariffa* herb against hyperbilirubinemia.

Keywords: Zinc oxide nanoparticles, *Hibiscus subdariffa*, hyperbilirubinemia, jaundice, hematological, mice.

INTRODUCTION

Hyperbilirubinemia or Jaundice is a condition in which a person's skin and the cornea are discoloured yellowish due to an increased level of the bile pigment bilirubin in the blood resulting from liver disease (Joseph and Samant 2022). Obstructive jaundice is a medical condition caused by blocking in the common bile duct or its tributaries that may result in difficulties like biliary infection in the bile duct, hepatic parenchymal damage, and multi-organ dysfunctions which in term increases the risk of death (Williams, 2011). It was evident that hyperbilirubinemia is associated with clinical situations like gallstones, tumours inthebile duct, pancreatitis, gastric haemorrhage and cardiac complications (Kingham *et al*., 2015). A healthy liver helps in the maintenance of normal body functions, the homeostatic mechanism of metabolites, and maintains a constant tissue mass in relation to the metabolic load in the body (Fabbrini *et al*., 2010). It is yet unknown what processes and mediators lead to acute biliary obstruction-induced liver injury.

Advances in nanotechnology have shown the potential applications of inorganic nanomaterials in various fields. In the last decade, several biological researchers have been interested in ZnO NPs for their unique and tunable optical and chemical behaviours (Nag *et al*., 2022; Sahoo *et al*., 2021; Pineda-Reyes, 2021; Xiong, 2013; Mohanasundar *et al*., 2021). For biomedical applications, researchers developed many biofriendly approaches for the synthesis of ZnO NPs. These green synthesized NPs showed excellent results on antibacterial, antifungal, acaricidal and larvicidal activities along with their effectiveness on some medical conditions like diabetes, jaundice, and cancer (Applerot *et al*., 2009; Sharma *et al*., 2010; Kirthi *et al*., 2011; Bala *et al*., 2015; Bala *et al*., 2015).

As reported by the World Health Organization (WHO), plant-based medications serve as the primary form of health care for 80% of the world's population (Bodeker *et al*., 2005). Indian, African, and Mexican natives use *Hibiscus subdariffa* L. (Family: Malvaceae) as traditional medicine. The plant's leaf and calyx extract were reported to be diuretics, choleretics, blood pressure suppressive, chemo-protective, anti-oxidative, anti-diabetic, anti-tumor, and anti-cancerous agents. (Jiménez-Ferrer *et al*., 2012; Lin *et al*., 2011; Fullerton *et al*., 2011; Saravanan *et al*., 2011; Salem *et al*., 2022).

The study aims to synthesize ZnO NPs in the green route using *H. subdariffa* leaf extract, giving special emphasis on the significant treatment of hyperbilirubinemia. UV-VIS, XRD, FESEM, and HRTEM spectra were used to analyse the produced ZnO NPs. The anti-hyperbilirubinemia activity was conducted on Swiss albino mice. In response to NPs

treatments, many biochemical parameters correlated with jaundice were studied.

MATERIAL AND METHODS

Materials: The plant material *Hibiscus subdariffa* belonging to the family Malvaceae, has been collected from the Jadavpur University campus. Carbon tetrachloride (CCl_4), Zinc acetate dihydrate and all other chemicals utilized in this experiment are research grade and were procured from Merck (India).

Green synthesis of zinc oxide nanoparticles: *H. sabdariffa* leaves extract-mediated ZnO NPs were prepared using a standard protocol (Bala *et al*., 2015). Chemically synthesized NPs were prepared following the protocol of Hasnidawani *et al*. (2016).

Anti-hyperbilirubinemia study: Randomly Swiss albino mice (32-35gm) were divided into five groups (n=5) which were acquired from a CPCSEA (Control and Supervision of Experiments on Animals, Chennai, India) approved animal house (50/CPCSEA/1999). A standard laboratory diet, Hindustan Lever, Kolkata and water *ad libitum* was served throughout the period of the experiment. All through the study period, the animals were kept in spacious, clean polypropylene cages in a controlled environment (Temp.: 25±2ºC, RH: 45-60%) with a12-hour day/night cycles. Acclimatization was performed for a week before the trial. The animals were maintained according to the guidelines recommended by CPCSEA and permitted by Institutional Animal Ethics Committee (AEC/PHARM/1503/03/2015 dated 30th November 2015). Jaundice was induced by treating mice following standard methods (Bala *et al*., 2016). Table 1 summarises the experimental approach. Upon the end of the experiment (4 weeks) the animals were sacrificed by cervical dislocation on the 29th day.

Biochemical and Hematological Estimation: Blood samples were taken from the retro-orbital vein in sterile tubes soon before sacrifice for biochemical testing using standard methods (Bhattacherjee *et al*., 2021; Bala *et al*., 2016). Liver function was evaluated by the estimation of liver enzymes like ALT (alanine transaminase), ALP (alkaline phosphatase), AST (aspartate aminotransferase), GGT (Gamma-glutamyl Transferase) and Total protein rom hemolysis free sterile serum. For hematological studies, blood was collected in heparin-zed tubes. Different hematological parameters like Hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelets and white blood cell have been studied.

Statistical Analysis: A mean ±standard deviation is employed to express all quantitative data. The one-way analysis of variance was carried out to compare various parameters between groups, followed by Tukey's multiple comparison tests by GraphPad Prism (v. 5.0), USA. $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Characterization of synthesized ZnO NPs: Synthesized nanoparticles have been optically analysed by UV-VIS spectra. The formation of ZnO NPs is confirmed by a strong peak at 378 nm (Fig. 1A). That was further confirmed by the XRD spectrum.2θ values at 31.77°, 34.40°, 36.22°, 47.61°, 56.58°, 62.85°, 66.41°, 67.93°, 69.08°, 72.54°, and 76.85°, which correspond to planes (100), (002), (101), (102), (110), (103), (112) and (202) respectively, these peaks were duly assigned by JCPDS file no. 361451. The characteristic peaks for NPs that were present in the XRD showed well crystallinity. These data were further confirmed by the previously published work (Bala *et al*., 2015; Nag *et al*., 2022).

Table 1: Treatment protocol for different groups of mice.

Groups	Treatment	Drug administration
I: Control	Nil	Nil
II: Auto recovery	CCl_4 in olive oil (1 ml/kg body weight)	Nil
III: Herb control	CCl_4 in olive oil (1 ml/ kg body weight)	3 ml/ kg body weight of the plant extract
IV: Chemically synthesized ZnO NPs control	CCl_4 in olive oil (1 ml/ kg body weight)	ZnO NPs (5 mg/ kg body weight)
V: Green synthesized ZnO NPs	CCl_4 in olive oil (1 ml/ kg body weight)	Green ZnO NPs (5 mg/ kg body weight)

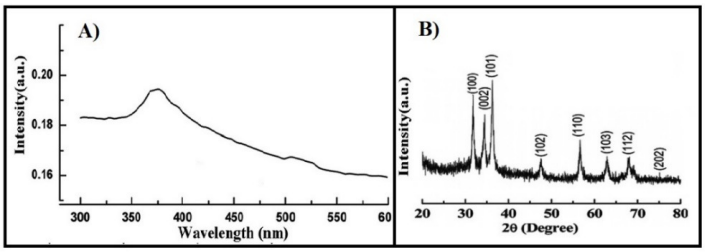

Fig. 1. (A) UV-VIS spectrum of ZnO NPs synthesized by *H. subdariffa* B) XRD pattern of the same NPs.

The surface morphology of synthesized NP was studied by FESEM micrographs (Fig. 2A). It was observed that synthesized NPs were spherical in shape. On higher magnification, this structure showed aggregation of a group of smaller spherical particles (16–60 nm) organized in a larger construction of about 300–400 nm in diameter. Well crystallinity of the particles observed in the micrograph was previously confirmed by XRD (Fig. 1B). The result obtained from FESEM was further confirmed by the HRTEM micrograph (Fig. 2B). This analysis validated the shape and size of the bio-synthesized NPs obtained by FESEM. HRTEM of NPs

in the aqueous solution showed isolated small-sized particles with a diameter ranging from 12–46 nm in diameter. When the FESEM and HRTEM results are compared, it is feasible to conclude that particle aggregated construction lost its organization patterns in the solution phase, resulting in a smaller size and a larger surface-to-volume ratio.

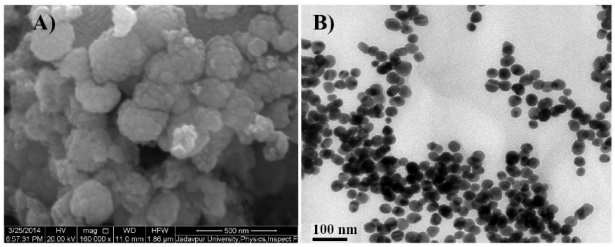

Fig. 2. (A) FESEM micrograph of synthesized NPs at 500nm scale bar and (B) HRTEM micrograph shows isolated spherical particles ranging from 12–46 nm in diameter.

Anti-hyperbilirubinemia activity of synthesized NPs: As compared to the control group (Group-I 0.3mg/dl), Group II-V showed significantly increased serum bilirubin level, which was a sign of hyperbilirubinemia (Table 2). Group II was not treated for one week in order to study the auto-recovery process. Group III and IV had a marginal decrease in bilirubin levels compared to the control set after treatment. The group V which was treated with *H. subdariffa*-mediated synthesized ZnO nanoconjugate showed more significant remediation. The hyperbilirubinemia triggered by CCl_4 resulted in a considerable increase in liver function enzyme levels such as AST., ALP, ALT, GGT, and Total Protein compared to the control set (Table 3). Groups III and IV significantly lower elevated enzyme levels compared to group II. Group V recovers changed enzyme levels to near normal levels, indicating regeneration of injured liver cells or preservation of hepatocytic cell membrane (Bala *et al*., 2016; Drotman and Lawhorn 1978). The evaluation of enzyme levels such as AST and ALT is widely utilised in the assessment of liver damage caused by hyperbilirubinemia. High AST levels suggest liver damage from viral hepatitis, as well as heart attack and muscular injuries (Giannini *et al*., 2005; Baars *et al*., 2016). AST catalyses the conversion of alanine, pyruvate, and glutamate. As a result, ALT was more specific to the liver and a superior measure for diagnosing cellular leakage and diminished functional stability of the cell membrane in the liver (Rajkapoor *et al*., 2008). ALP rises due to increased production &membrane shedding of ALP from hepatocytes subjected to cholestasis's increased biliary pressure, whereas bilirubin rises due to bilirubin excretion failure (Polley *et al*., 2015). The enzymatic profile obtained from the experiment clearly demonstrates *H. subdariffa*-mediated synthesized ZnO nanoconjugate (group V) shows a more effective medication comparison to that of mice treated with chemically synthesized ZnO NPs (group IV) and Herb extracts (group III).

Table 2: Effect of nanoparticles on Bilirubin levels in CCl_4 intoxicated mice.

GROUP	TOTAL BILIRUBIN		DIRECT BILIRUBIN	
	Induced	Treated	Induced	Treated
I	0.35±0.3	0.34±0.3	0.19±0.1	0.20±0.2
II	0.92±0.4	0.89±0.7	0.39±0.3	0.33±0.3
III	0.89 ±0.7	0.52±0.4	0.38±0.3	0.25±0.2
IV	0.88±0.6	0.65±0.4	0.38±0.3	0.29±0.2
V	0.88±0.6	0.36±0.3	0.45±0.4	0.21±0.1

Table 3: Consequence of treatments on ALT, AST, ALP, GGT and Total Protein levels in hyperbilirubinemic mice.

GROUP	AST(IU/L)	ALT(IU/L)	ALP(IU/L)	GGT(IU/L)	TOTAL PROTEIN (G/DL)
I	82.43±8.31	20.01±1.41	26.30±2.63	2.60±0.42	6.38±0.55
II	207.45±22.32	59.64±3.72	55.38±5.48	5.49±0.66	5.78±0.40
III	67.98±17.19	41.72±2.28	45.30±4.88	4.78±0.48	5.88±0.44
IV	103.43±9.31	59.58±3.81	49.30±5.88	5.10±0.45	5.98±0.54
V	79.45±8.32	29.38±0.81	35.30±2.88	3.72±0.37	5.68±0.34

Table 4: Effect of treatments on hematological parameters in CCl_4 intoxicated mice.

Hematological parameter	GROUPI	GROUPII	GROUPIII	GROUP IV	GROUP V
Hb ($gmdl^{-1}$)	11	13.05	12.44	12.08	12.88
RBC (10^6 ml^{-1})	6.47	6.22	6.86	6.44	6.50
HCT (%)	57.15	58.20	58.42	60.85	61.22
MCV (fl)	62.50	62.21	62.01	62.23	63.10
MCH (pg)	19.20	21.05	20.40	20.85	20.56
WBC (10^3 ml^{-1})	6.80	7.45	7.95	7.85	7.50
Platelets (10^5 ml^{-1})	634	666	650	687	667

Hb: Hemoglobin, RBC: Red blood corpuscle, HCT: Hematocrit test, MCV: Mean Corpuscular volume, MCH: Mean corpuscular haemoglobin.

Phenols and flavonoids present in nanoconjugate showed better efficacy to restore biochemical and liver function enzyme levels than that of Group III and IV (Salgado *et al*., 2019). Anti-oxidant activity, free radicals scavenging ability and inhibition of lipid per oxidation capability of the herb prevent hepatocellular injury (Diantini *et al*., 2021). In the case of Group V, NPs shows a synergistic effect of both chemically synthesized ZnO NPs and *H. subdariffa* herb.

The hematological condition of mice from Groups I-V was determined (Table 4). It was evident that minute changes in hematological parameters among different groups are insignificant. From the hematological studies, it can be analysed that, the introduction of nanoparticles has no influence on the haematological parameters. i.e. *H. subdariffa*-mediated synthesized ZnO nanoconjugate is bio-friendly in nature.

CONCLUSIONS

In summary, the result of this study demonstrates that*H. subdariffa*-mediated ZnO NPs have a significant hepatoprotective effect in mice against CCl4-induced liver injury. These green synthesized NPs exhibit significant anti-hyperbilirubinemia activities in Swiss albino mice by restoring altered levels of serum bilirubin and liver function enzymes, suggesting the stabilization of hepatocytic cell membrane or recovery of injured liver cells.

FUTURE SCOPE

Green synthesized ZnO NPs by leaf extract of H. subdariffa shows its potential anti-jaundice activity in Swiss albino mice. The mechanism of its ameliorative activity is yet to be studied. Further extensive studies may use to establish its potential medical application.

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Conflict of Interest. None.

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