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Green synthesis of reduced graphene oxide using *Indigofera tinctoria* leaf extract and its antibacterial activity against *Escherichia coli*

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ABSTRACT

This article describes a green and sustainable approach for the synthesis of reduced graphene oxide (rGO) using *Indigofera tinctoria* leaf extract as a natural reducing agent. The biogenic IT-rGO was characterized using UV-Vis spectroscopy, FTIR, XRD, and SEM to confirm its successful reduction and to determine its structural properties. The phytochemicals present in the extract, including flavonoids, polyphenols and alkaloids, played a crucial role in reducing GO to rGO. The synthesized rGO exhibited significant antibacterial activity against *Escherichia coli*, with the highest antibacterial activity observed at a 100% IT-rGO concentration, resulting in an inhibition zone value of 10.25 mm against the *E. coli* bacteria. The antibacterial mechanism is proposed to involve membrane disruption and the induction of oxidative stress. The use of *Indigofera tinctoria* not only offers an eco-friendly and cost-effective route for rGO production but also imparts enhanced bioactivity, making the material a promising candidate for biomedical and environmental antibacterial applications.

Keywords: reduced graphene oxide (rGO), green synthesis, *Indigofera tinctoria*, antibacterial, *Escherichia coli*.

INTRODUCTION

The aquatic environment is something that must be preserved. Pollution of water bodies can cause several problems, including the onset of diseases caused by pathogenic microorganisms. *Escherichia coli* (*E. coli*) is a Gram-negative bacterium whose presence in water can cause health problems, such as diarrhoea or digestive tract infections (Abavisani et al., 2023). Along with the increasing resistance of bacteria to antibiotics, the development of effective antibacterial alternatives has become increasingly urgent. Carbon-based nanomaterials, notably reduced graphene oxide (rGO), have attracted attention as potential antibacterial agents (Mann et al., 2021).

Reduced graphene oxide (rGO) is a carbon-based material derived from graphene, exhibiting graphene-like characteristics in terms of its mechanical, optical, and electrical properties. rGO is produced by removing some oxygen groups from graphene oxide (GO) through the production process and retaining some COOH functional groups at the edges. Some of the advantages of rGO are its good electrical conductivity properties and its large surface area, which is attributed to the presence of defects and oxidised functional groups. rGO has been widely used in various fields, such as sensors (Karim et al., 2024), supercapacitors (Panicker and Sahu, 2021), adsorbents (Wijaya et al., 2020), photocatalysis (Parthipan et al., 2021), and antibacterial (Rakkimuthu et al., 2022). Oxygen functional groups on rGO can interact with bacterial cell membranes, as well as their high conductivity properties, which can induce oxidative stress in microorganisms (Agarwal and Zetterlund, 2021).

A widely used approach in the synthesis of rGO is chemical reduction. The problem is that all the chemicals used, including hydrazine, sodium

borohydride, sodium citrate and hydroquinone, are potentially toxic to human health and pollute the environment (Hu and Gao, 2023). It is essential to develop an environmentally safe method for producing reduced graphene oxide using natural materials, such as microbes and plants.

Green synthesis offers an eco-friendly alternative by utilising natural products as reducing agents. Plant extracts, in particular, have proven to be effective in reducing GO due to the presence of bioactive compounds. These compounds not only facilitate the reduction of GO but also stabilise the resulting rGO through surface functionalisation (Singh et al., 2023). This approach aligns with the principles of green chemistry, emphasising the use of renewable resources and minimising environmental impacts. Biomolecules from the polyphenol group and other secondary metabolites in plants serve as reducing agents in the synthesis of rGO (Kurian, 2021).

Among the various plants explored for green synthesis, *Indigofera tinctoria*, a member of the Fabaceae family, stands out due to its rich phytochemical composition. Traditionally used as a source of natural indigo dye, *Indigofera tinctoria*. Includes several bioactive molecules, namely alkaloids, flavonoids, and tannins, which possess substantial reducing and capping capabilities (J.O, 2021; Wahyuningsih et al., 2017). Hese properties make it an ideal candidate for reducing GO under environmentally benign conditions. The identified Indigofera tinctoria leaf extract contains phenolic compounds (Mishra et al., 2020), such as 5-Hydroxy-L-trypthopan (Rajan et al., 2015; Rajab et al., 2025). The reducing ability of Indigofera tinctoria leaf extract has been proven by its ability to reduce gold and silver to nanoparticle size (Vijayan et al., 2018).

The importance of applying green solutions to solve environmental problems, such as environmental pollution caused by bacteria, highlights the need to utilise rGO synthesised from *Indigofera tinctoria* leaf extract as a solution for environmentally friendly antibacterial purposes. This research aims to investigate the effectiveness of *Indigofera tinctoria* extract in reducing GO, characterise the resulting rGO, and evaluate its potential as an antibacterial agent against *E. coli*. By adopting a green synthesis approach in the preparation of rGO, this study contributes to the development of sustainable nanotechnology while reducing the environmental risks associated with conventional chemical reduction methods. Additionally, it provides a solution for treating diseases caused by *E. coli* bacteria originating from aquatic environments.

MATERIALS AND METHODS

Materials

GO as a precursor in synthesizing rGO was obtained from Nanomaterials for Renewable Energy Research Center (NRE LAB) Indonesia. Fresh leaves of *Indigofera tinctoria* were obtained from Bulukumba Regency, South Sulawesi Province.

Extraction of *Indigofera tinctoria*

The leaves of *Indigofera tinctoria* were initially cleaned, dried, and then ground into a powder. A total of 10 grams of this leaf powder was extracted using 100 mL of distilled water as the solvent, which was then heated to boiling for 30 minutes. Following this, the mixture was filtered to separate the precipitate from the filtrate. The filtrate was collected in a bottle for use as a bioreductor in the synthesis of rGO (Yasser et al., 2021; Yasser et al., 2021; Yasser et al., 2020). The extract was concentrated using a rotary vacuum evaporator. The concentrated extract was then characterized using phytochemical testing, IR spectroscopy, and Gas Chromatography-Mass Spectrometry (Alamsjah et al., 2024 ; Yasser et al., 2020).

Green synthesis of IT-rGO

In this project, we green-synthesised rGO using the aqueous extract of *Indigofera tinctoria* leaves as a reductant. The green synthesis was achieved by reacting GO with an aqueous extract from *Indigofera tinctoria* leaves in a 1:1 (v/v) ratio at 85 °C. A 50 mL GO solution (1 mg/mL) was reacted with 50 mL of an aqueous extract of Indigofera tinctoria leaves. The mixture was reacted on a hot plate at a heating temperature of 85 °C and stirred using a magnetic stirrer for 12 hours. After the heating process was complete, the mixture was filtered to separate the precipitate and filtrate. The precipitate was then washed with water and ethanol until the pH of the precipitate reached 7. The precipitate was then oven-dried at 60 °C for 12 hours (Wijaya et al., 2020).

UV-Vis spectroscopy characterization

The IT-rGO solid formed from the green synthesis process was dissolved in aqueous solvent. UV-Vis spectroscopy was employed to characterize the IT-rGO solution at wavelengths between 200 to 700 nm.

Fourier transform infrared spectroscopy (FTIR) characterization

The IT-rGO material was mixed with KBr crystals until a homogeneous mixture was obtained. The homogeneous mixture was characterized with IR Prestige-21 Shimadzu at a measurement range of 4000–500 cm^{-1}.

X-ray diffraction (XRD) characterization

The crystal confirmation was determined using a Bruker D8 Advance X-Ray diffractometer (XRD) at a measurement range between 5° – 89,990°, a scan speed of 0.10 deg/min and at room temperature (25 °C).

Scanning electron microscope-energy dispersive X-ray characterization

The morphology of IT-rGO was examined utilizing SEM, Hitachi SU3500, accelerating voltage 10kV and maximum magnification 100 k. The elemental composition was examined using EDAX TEAM.

Escherichia coli antibacterial activity

Sensitivity testing is done to assess the effectiveness of antibiotic use. This antibiogram uses the Kirby-Bauer method by taking colonies of each test bacterium *Escehrichia coli* ATCC 25923 which were grown for 24 hours previously and suspended in 1 ml of 0.9% NaCl to reach turbidity (Sabee et al., 2020). Then using a sterile swab, the bacterial suspension was applied (swab) evenly on Muller Hinton Agar (MHA) media and placed paper disks that previously contained IT-rGO with various concentrations, concentrations of 100% (100 mg/100 µL), 50% (50 mg/100 µL), 25% (25 mg/100 µL), 12.5% (12.5 mg/100 µL), 6.25% (6.25 mg/100 µL), 3.125% (3.125 mg/100 µL), on the media and incubated for 24 hours (10). Positive control used chloramphenicol antibiotic 30 µg/disk and negative control used distilled water. Observations were made on the disc paper to measure the diameter of the inhibition zone, the value of the inhibition zone produced indicates the level of the IT-rGO ability as an antibacterial against the test bacteria *Escehrichia coli* ATCC 25923.

RESULTS AND DISCUSSION

Characterization of extract

The secondary metabolite compounds of each plant depend on several factors, including differences in the growth environment and the polarity of the solvents. The qualitative identification of secondary metabolites is characterised by physical changes, such as colour changes and the formation of specific precipitates when the sample is reacted with a specialised reagent (Mulia et al., 2023). Phytochemical tests on the aqueous extract of Indigofera tinctoria yielded positive results for the presence of secondary metabolite compounds, including alkaloids, flavonoids, and phenolics (Table 1). These results demonstrate differences in the outcomes of phytochemical testing using various solvents and regions, specifically in the case of Inigofera tinctoria plants growing. Venkatachalam (2018) demonstrated that the aqueous extract of Indigofera tinctoria leaves contained secondary metabolite compounds, including flavonoids, phenolics, and terpenoids. The difference in the results obtained for alkaloids and terpenoids shows that differences in plant growing locations affect the content of secondary metabolites (Venkatachalam, 2018).

The phytochemical analysis of *Indigofera tinctoria* leaf extract was strengthened by the results of measuring the extract using IR spectroscopy to determine the functional groups in the extract (Figure 1 and Table 2). The extract was identified as containing an aromatic ring

Table 1. Phytochemical test results of *Indigofera tinctoria* leaf extract

Metabolite compounds	Test result
Alkaloid	+
Flavonoid	+
Polyphenols	+
Triterpenoid	-
Steroid	-

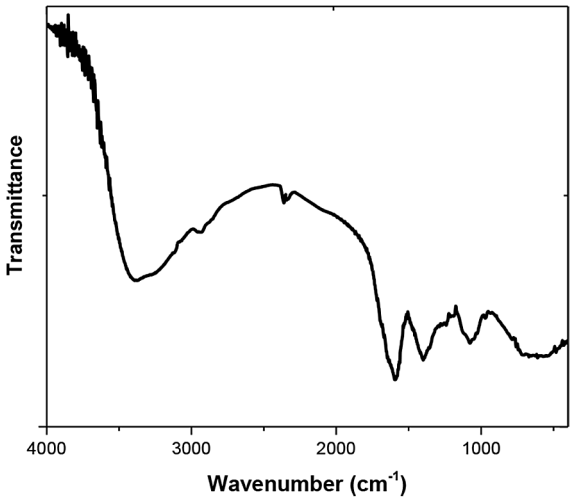

Figure 1. FTIR spectrum of *Indigofera tinctoria* leaf extract

Table 2. Possible functional group of *Indigofera tinctoria* leaf extract

Wavenumber (cm^{-1})	Possible functional group
3394.83	Hydroxyl (-OH)
1591.33	Aromatic ring (C=C-C)
1400.37	Phenol or tertiary alcohol
1076.32	C-O bonds or primary amines (-CN stretch)
711.76	C-H bond (ortho aromatic)

(C=C-C) characterized by the presence of absorption at wave numbers around around 1591.33cm^{-1} (Coates, 2000). The presence of this aromatic ring is confirmed by the absorption at wave number 711.76 cm^{-1}, which is a C-H bond (ortho aromatic). Hydroxyl (-OH) groups were identified at 3394.83 cm^{-1} and 1400.37 cm^{-1} as phenol or tertiary alcohol. The IR spectrum also found an absorption at 1076.32 cm^{-1} which allows the presence of C-O bonds (alcohols, ethers or glycoside groups) or the presence of primary amines (-CN stretch) (Nandiyanto et al., 2019). The results of characterization by IR spectroscopy of *Indigofera tinctoria* leaf extracts are in consistence with the results of its phytochemical test. The presence of flavonoid and phenolic secondary metabolite compounds is characterized by the identification of aromatic rings, hydroxyl groups (-OH) and phenol groups. While the presence of alkaloids is confirmed by the presence of primary amine (-CN) contained in the extract. The presence of nitrogen atoms (N) is a factor that indicates the presence of Alkaloid group compounds.

The chromatogram (Figure 2) of *Indigofera tinctoria* leaf extract was identified as containing 59 compounds (Table 3), some of which consisted of polyphenol, flavonoid (Phenanthrenol) and alkaloid (Quinoline) compounds. The presence of the three types of compounds confirmed the results of phytochemical tests and IR spectroscopy, which also found the presence of these compounds in the extract.

Characterization of rGO

The UV-Vis spectrum (Figure 3) of GO shows maximum absorption at a wavelength of 230.64 nm, which means π-π* transition of aromatic C=C bond interaction (Tahir et al., 2023; Mombeshora and Muchuweni, 2023) and a shoulder peak at ~300 nm due to n-π* transition of carbonyl group (Jin et al., 2018 ; Lingaraju et al., 2019). The UV-Vis spectrum of IT-rGO synthesized using Indigofera tinctoria leaf extract has a maximum absorption at a wavelength of 270 nm. This shows that there has been a red shift from a wavelength of 230 nm to a wavelength of 270 nm, which means that there has been a π-π * transition from the C = C aromatic bond. The removal of oxygen groups has occurred through reduction with Indigofera tinctoria leaf extract (Perumal et al., 2022; Ghosh et al., 2021).

The functional group of GO and IT-rGO was examined within 4000–500 cm^{-1} (Figure 4 and Table 4). FTIR spectrum of GO shows the presence of functional groups such as hydroxyl groups (-OH) with a broad peak at 3444.02 cm^{-1}, the presence of a peak at 1703.29 cm^{-1} indicating

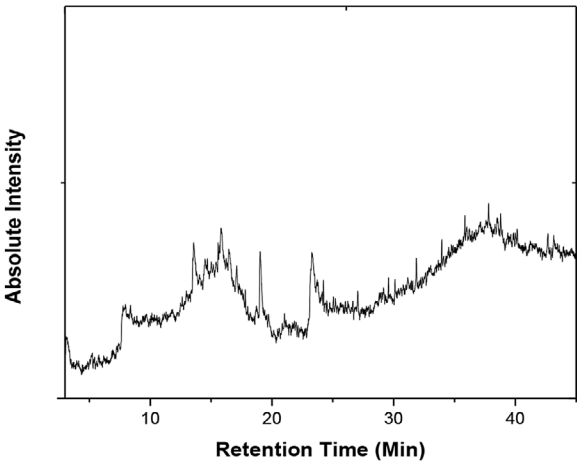

Figure 2. Chromatogram of *Indigofera tinctoria* L. leaf extract

Table 3. Chemical composition of *I. tinctoria* L. leaf extract

No.	RT (min)	Compounds name	No.	RT (min)	Compounds name
1	7.258	2,4-Octadienoic acid, 7-(t-butyldimethylsilyloxy)-, methyl ester (E,E)-	31	24.387	Heptadecyl acetate
2	7.375	7-(N,N-Diallylamino)-2-methyl-5-heptyn-3-ol	32	24.510	1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide
3	7.683	1-BUTANOL, 3-METHYL-, ACETATE	33	25.050	18-Nonadecenoic acid
4	8.382	2-Penten-1-ol, (Z)-, TMS derivative	34	26.038	Cyclotetradecane
5	13.333	Quinoline-5,8-dione-6-ol, 7-[[(4-cyclohexylbutyl)amino]methyl]-	35	26.637	Octyl palmitoleate
6	13.500	3,6,9,12-Tetraoxatetradecan-1-ol	36	26.783	3.ALPHA.,20.ALPHA.-BIS-TERT-BUTYLDIMETHYLSILYL-ETHER
7	13.567	5-Aminosalicylic acid, N,O,O'-tris(trimethylsilyl)-	37	27.045	Amodiaquine, 2TMS derivative
8	13.733	1-Bromo-1-methyl-1-silacyclohexane	38	27.766	1H-phosphindole, 2,3-dihydro-1,3,3,4,6-pentamethyl-, 1-oxide
9	13.875	p-Mentha-6,8-dien-2-one, semicarbazone	39	28.808	1,5-Naphthalenedione, octahydro-4a-methyl-
10	14.042	ETHANE, 1,1'-OXYBIS[2-ETHOXY-	40	29.169	Bicyclopropyl derivative
11	14.483	Oleyl alcohol, trifluoroacetate	41	29.580	1H-Purin-6-amine, [(2-fluorophenyl) methyl]-
12	14.695	Octadecane (CAS)	42	30.109	trans-2-Hexadecenoic acid
13	15.015	3,7,11-Trimethyldodecylacetate	43	31.565	2,3 Dinor thromboxane b2 tetra-TMS
14	15.392	1-Heptatriacotanol	44	31.861	Silicone oil
15	15.576	5-Bromo-3-ethyl-3-hydroxyindolin-2-one, 2TBDMS derivative	45	33.956	Cyclodecasiloxane, eicosamethyl-
16	15.650	MOME INOSITOL	46	35.533	Dihydroindolizine derivative
17	15.806	Tetradecanoic acid (CAS)	47	35.700	Trimethylbenzimidazole derivative
18	16.217	1,3,6-Trioxa-2-silacyclooctane, 2,2,-dimethylsilyl-	48	35.848	Biphenylenecarboxylic acid derivative
19	16.461	PLUCHIDIOL	49	35.975	Spiro dioxaspiro derivative
20	17.108	3,6,9,12-Tetraoxatetradecan-1-ol	50	36.21	Silicone oil
21	17.817	EICOSAMETHYLCYCLODECASILOXANE	51	36.449	Isopropyl .gamma.-(p-nitrophenyl)-.alpha.-methylene-.beta.-hydropropanoate
22	19.029	n-Hexadecanoic acid	52	37.806	Silicone Oil
23	21.031	Benzoic acid, 4-amino-2-hydroxy-, tris(trimethylsilyl) deriv.	53	38.490	9.beta.-Acetoxy-3,5.alpha.,8-trimethyltricyclo[6.3.1.0(1,5)]dodec-3-ene
24	22.130	1,2-Epoxynonane	54	38.782	3-PHENANTHRENOL, 4B,5,6,7,8,8A,9,10-OCTAHYDRO-4B,8,8-TRIMETHYL-, (4
25	22.883	(E)-4-Chloro-2,3-dimethyl-1,3-hexadiene	55	40.003	Acetamide, N-[[2-(1,1-dimethylethyl)-6-oxo-1,3-dioxan-4-yl]methyl]-, (2R-cis)-
26	23.183	Oleic Acid	56	40.145	Silikonfett
27	23.275	9-Octadecenoic acid, (E)-	57	42.114	Heptasiloxane, hexadecamethyl- (CAS)
28	23.508	2,3,6-Trimethylhept-6-en-1-ol	58	42.677	2-[3-(AMINOMETHYL)-5,7-DIMETHYL-1-ADAMANTYL]ETHANAMINE
29	23.718	Octadecanoic acid	59	43.964	Tetrasiloxane, decamethyl- (CAS)
30	24.226	5-Bromo-3-ethyl-3-hydroxyindolin-2-one, 2TBDMS derivative			

the presence of C=O bonds, the presence of epoxy groups at a peak of 1221.44 cm^{-1}, the C-O stretch of C-OH at a peak of 1037.44 cm cm^{-1}; and the bond between C=C at a peak of 1642.39 cm^{-1} indicating the basic framework of graphene in the form of aromatic carbon (Parthipan et al., 2021). FTIR spectrum of IT-rGO, it appears that the typical peak of the C=O functional group (1703.29 cm^{-1}) has not been detected in the FTIR spectrum, the intensity of the typical peak of the epoxy group and the C-O stretch of C-OH decreased in intensity as the synthesis time is lengthened (Shahane and Sidhaye, 2018). This shows that the reduction process has been successfully carried out with the

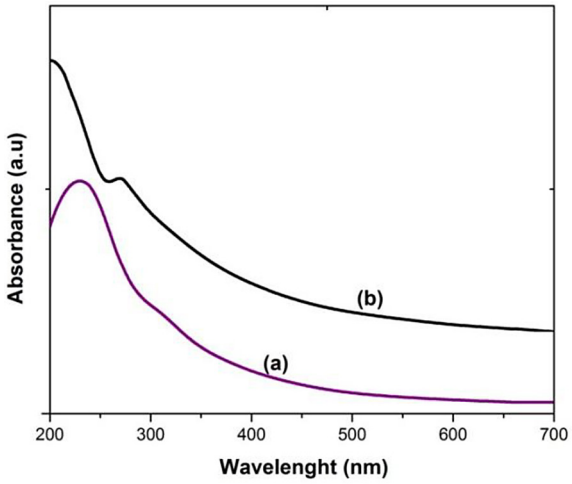

Figure 3. UV-Vis spectrum of GO (a) and IT-rGO (b)

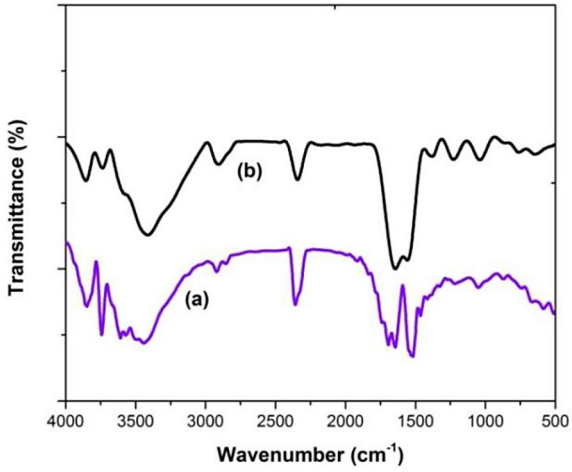

Figure 4. FTIR spectrum of GO (a) and IT-rGO (b)

removal of several oxygen groups (Parthipan, Cheng, et al., 2021).

The XRD patterns produced on GO and IT-rGO show their distinctive peaks (Figure 5). The GO diffraction peak appears at 9.362°, while the IT-rGO synthesized with *Indigofera tinctoria* leaf extract has a broad diffraction peak of about 23.885°. The peak shift from 9.362° to 23.885° indicates that a reduction process has occurred by reducing the number of oxygen functional groups present in GO (C. Agarwal et al., 2019 ; Faiz et al., 2020). It also indicates that the conjugated graphene network of sp^2 hybrid carbon has been restored during the reduction process (Shubha et al., 2017 ; Nawaz et al., 2017; Rai et al., 2020).

The morphological characteristics of GO and IT-rGO samples were examined using SEM imaging (Figure 6). The GO samples exhibit smooth, wrinkled, and folded surfaces. The presence of wrinkles and folds on the GO surface is attributed to the abundance of functional groups, such as epoxy, hydroxyl, and carboxyl, attached to it (Mahendran et al., 2020 ; Meka Chufa et al., 2021). In contrast, the surface morphology of IT-rGO reveals a reduction in wrinkles, resulting in a structure characterized by folded and overlapping sheets (Kumar, et al., 2022 ; Shubha et al., 2017). This observation indicates the successful reduction of GO using *Indigofera tinctoria* leaf extract.

The Energy Dispersive Spectroscopy (EDS) analysis depicted in Figure 7 demonstrates the composition ratio of carbon (C) and oxygen (O) elements (C/O) before and after the reduction of GO using *Indigofera tinctoria* leaf extract. The presence of numerous epoxy, hydroxyl, and carboxyl groups remains attached to the GO structure, resulting in a C/O ratio of 1.3 (56.51 : 43.49). In contrast, the C/O ratio for the rGO is 3.8 (79.17 : 20.83), indicating the successful reduction of GO through the application of *Indigofera tinctoria* leaf extract. The shift in the C/O atomic ratio from 1.3 to 3.8, as determined by EDS analysis, quantitatively confirms the reduction efficiency. This finding aligns with the changes observed in UV-Vis, FTIR, and XRD analyses, collectively corroborating the restoration of the sp^2 carbon network and the partial removal of oxygen-containing groups.

The GO reduction process that occurs cannot be separated from the presence of polyphenol

Table 4. FTIR spectrum analysis of GO and rGO

Wavenumber (cm^{-1})		Functional group	References
GO	IT-rGO		
3444.02	3421.30	Hydroxyl (-OH)	(Nandiyanto et al., 2019 ; Mahendran et al., 2020)
1703.29	-	Carbonyl/Carboxyl (C=O)	(Mahiuddin and Ochiai, 2021 ; Sabayan et al., 2020)
1642.39	1645.33	C=C	(Bhattacharya et al., 2017 ; Shubha et al., 2017)
1221.44	1226.76	Epoxy (C-O)	(Yang et al., 2021 ; Punniyakotti et al., 2021)
1053.32	1039.66	C-O stretch of C-OH	(Phukan et al., 2019; Sabayan et al., 2020)

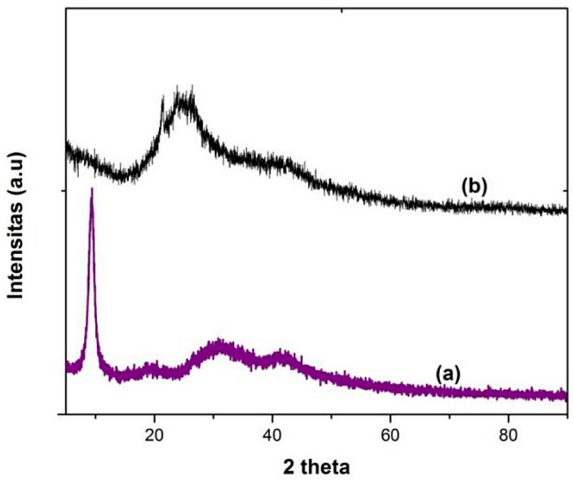

Figure 5. Diffractogram of GO (a) and IT-rGO (b)

groups in the *Indigofera tintoria* leaf extract. Oxidized polyphenols in the extract can interact with rGO sheets through π-π stack interactions, causing electrostatic repulsion between the rGO layers that triggers stabilization (Qi et al., 2021). aqueous extracts from *Indigofera tinctoria* leaves were found to contain various classes of chemical compounds, including polyphenol, flavonoids and alkaloid (Venkatachalam, 2018). An illustration of the rGO synthesis mechanism can be seen in Figure 8.

Antibacterial activity of IT-rGO

The antibacterial efficacy of IT-rGO against *E. coli*, a gram-negative bacterium, was assessed. The results, as depicted in Figure 9, demonstrate the extract's inhibitory effect on bacterial growth, evidenced by the inhibition zones observed at various IT-rGO concentrations. Specifically, the inhibition zones for *E. coli* at IT-rGO concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125% were measured at 10.25 mm, 8.60 mm, 6.70 mm, 4.50 mm, 2.00 mm, and 1.30 mm, respectively (Table 5).

The antibacterial efficacy of IT-rGO against *E. coli* exhibits varying levels of inhibition, classified as strong, moderate, and weak. This variability in bacterial inhibition is attributed to the concentration of materials employed in the antibacterial activity assays. This observation aligns with the findings of Thiyagarajulu et al. (2020), who reported positive inhibition against pathogenic bacteria at

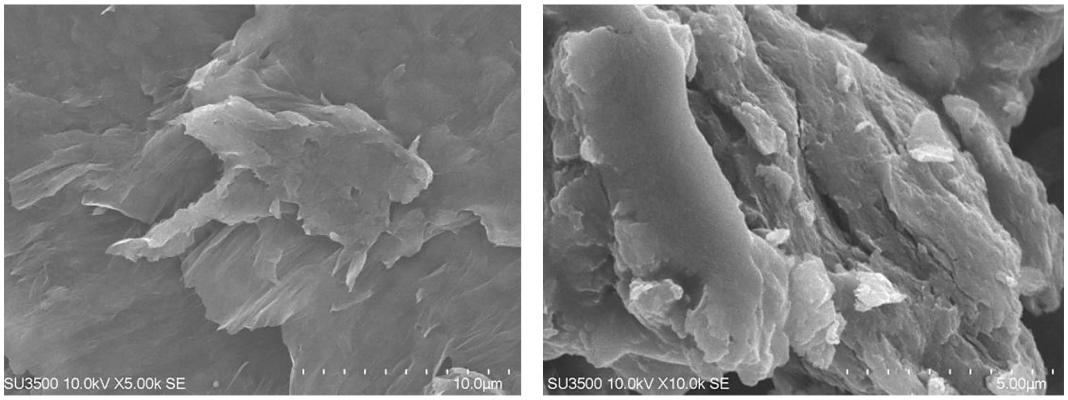

Figure 6. SEM image of GO (a) and IT-rGO (b)

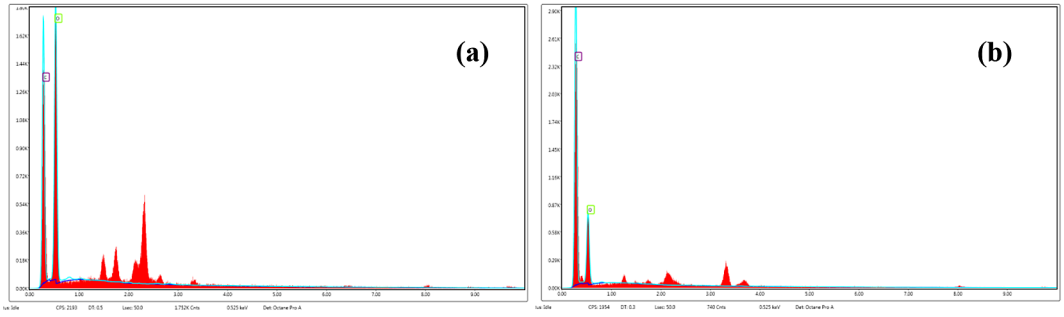

Figure 7. EDS spectra of GO (a) and IT-rGO (b)

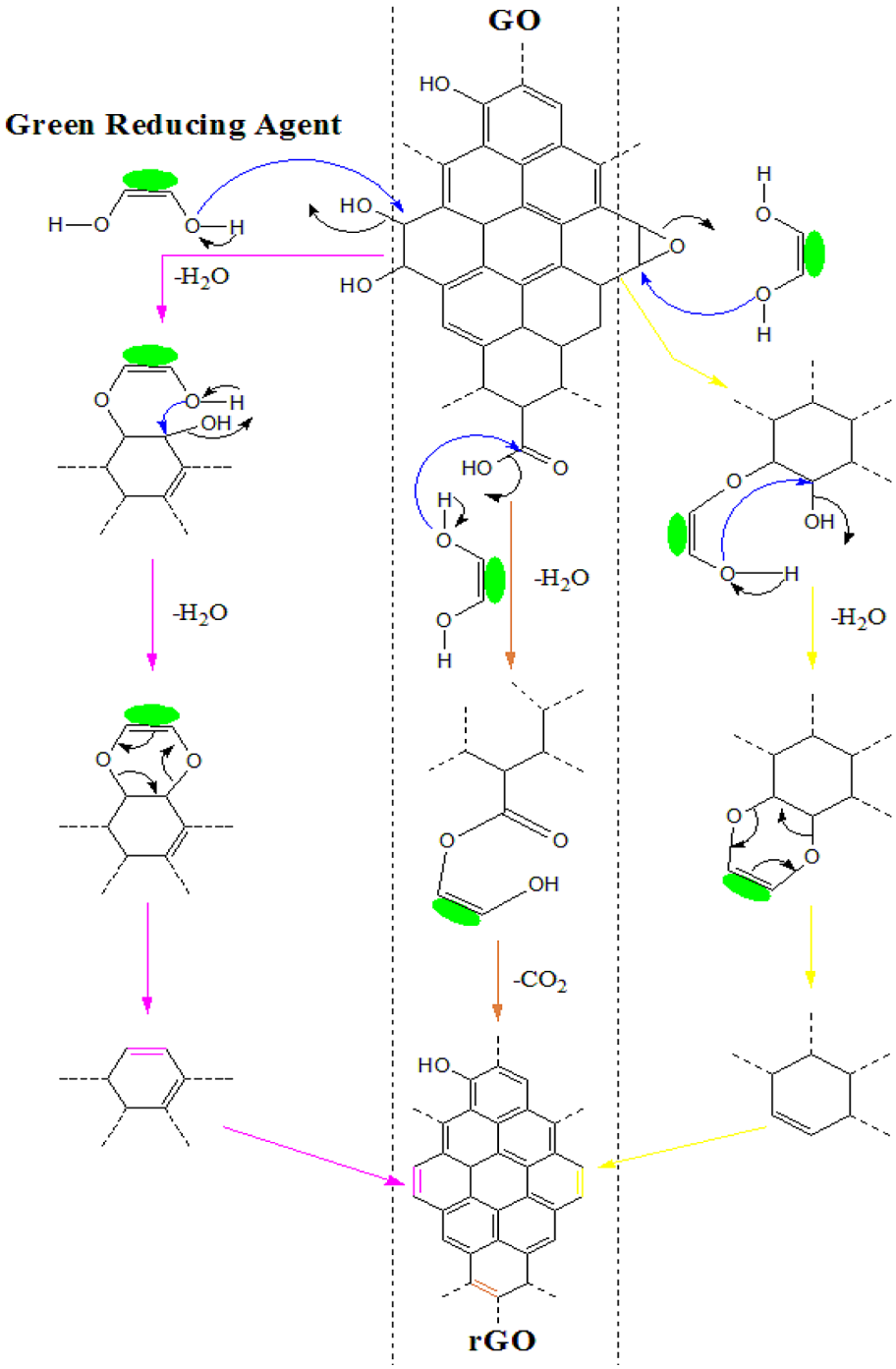

Figure 8. Green synthesis mechanism illustration of rGO

IT-rGO concentrations of 25%, 50%, 75%, and 100%, with inhibition zone values increasing proportionally with higher IT-rGO concentrations (Thiyagarajulu and Arumugam, 2021). The antibacterial activity of IT-rGO demonstrated the highest efficacy against *E. coli* at a 100% IT-rGO concentration. At a 100% concentration, IT-rGO produced an inhibition zone of 10.25 mm, while the standard antibiotic (positive control) exhibited an inhibition zone of 19.50 mm. According to established criteria for antibacterial efficacy, inhibition zones greater than 10 mm are classified as indicative of strong antibacterial activity. Thus, although IT-rGO exhibited a slightly lower efficacy than conventional antibiotics, its activity remains within a strong category. Beyond this, the practical applicability of IT-rGO is noteworthy. The green synthesis approach eliminates toxic reducing agents, offering an environmentally friendly and cost-effective route to produce rGO.

Table 5. Inhibition zone diameter antimicrobial test of IT-rGO

Concentrations	Inhibitory zone diameter (mm)	Inhibition category
Chloramphenicol (+) 30 µg/disk	19.50	Strong
100%	10.25	Strong
50%	8.60	Medium
25%	6.70	Medium
12.5%	4.50	Weak
6.25%	2.00	Weak
3.125%	1.30	Weak

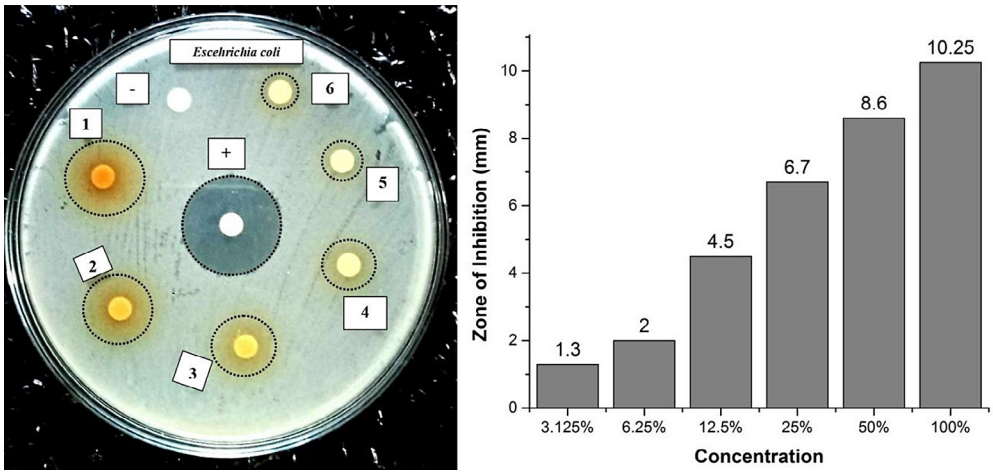

Figure 9. Zone of inhibition against *E. Coli* bacteria by rGO

The antibacterial efficacy of IT-rGO is attributed to the interaction between graphene and microbial cells, which leads to adhesion on the bacterial cell surface, resulting in cell membrane disruption and oxidative stress (Lim et al., 2012). Additionally, the presence of secondary metabolite compounds in Indigofera tinctoria leaf extracts, such as alkaloids, phenols, and flavonoids, on the surface of IT-rGO nanosheets further enhances the bactericidal effect (Thiyagarajulu and Arumugam, 2021; Khanam and Hasan, 2019).

CONCLUSIONS

This study successfully demonstrated a green and sustainable route for the synthesis of rGO using *Indigofera tinctoria* leaf extract as a natural reducing agent. The presence of phytochemicals, such as flavonoids, polyphenols and alkaloids, played a significant role in the effective reduction of graphene oxide to rGO. Comprehensive characterization via UV-Vis, FTIR, XRD, and SEM-EDS confirmed the structural and morphological changes associated with the reduction process. The biosynthesized IT-rGO exhibited antibacterial activity against *Escherichia coli*, as evaluated using the Kirby-Bauer disk diffusion method. The highest antibacterial performance was observed at 100% IT-rGO concentration, with an inhibition zone diameter of 10.25 mm, indicating a strong antibacterial effect. These findings suggest that *Indigofera tinctoria*-mediated rGO holds promising potential as an eco-friendly and effective antibacterial nanomaterial for biomedical and environmental applications. This study highlights the potency of rGO as an environmental-friendly antibacterial agent and functional material for applications in sensors, photocatalysis, and energy.

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