Journal of Wasit for Science and Medicine 2025: 18, (4), 91-100

Graphene Oxide Nanosheet Synthesis Using a Modified Hammer Process and Toxicity Assessment (LD50)

Shaimaa Hamed Jaber

Department of Chemistry, College of Science, University of Mustansiriyah, Baghdad, Iraq.

Abstract

Two-dimensional (2D) nanomaterials are an extremely complex and sophisticated type of atomically thick nanoparticles composed of one or more layers of molecules. 2D Nanomaterials can have lateral sizes that reach micrometres, and by optimizing the production method, longer ones can be produced. Among the unique characteristics of 2D nanomaterials are high contrast, effective surface area, mechanical strength, plasmonic properties, electron containment, and optical properties. This study used a newly developed sustainable modified Hammers' process to manufacture graphene oxide nanosheets which is considered one of the distinctive two-dimensional nanomaterial substrates. Compared to earlier modified Hammers' process, this process requires less time. The morphological and structural characteristics of GO were investigated using X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), and Raman spectroscopy. The TEM results for the GO nanosheets generated for this investigation showed a single- and double-layer structure. The morphological and structural characteristics were investigated GO exhibited favourable morphological and structural features are present in GO, including a nice graphitic structure and valuable oxygen-containing functional groups such as OH, COOH, and C=O functional groups. There were clear mitigation of waste creation and the possibility of reduced time and cost requirements with this modified hummer approach. The LD50 of GO nanosheets was studied in rats through different concentrations given orally, showing a significant increase in renal function test levels (urea and creatinine), in comparison to the control group at 300 mg/kg. However, only preliminary toxicity data were provided on GO's toxicity in rats in this work; more experimental confirmation and clarification are necessary before GO is extensively employed in biomedical applications.

Keywords: Modified Hammers' process, Graphite oxide nanosheet, Raman spectroscopy.

1. Introduction

Graphene oxide (GO) is a by-product of graphene, one-layer textile with a hexagonal lattice structure made of carbon atoms in one dimension [1, 2]. It is soluble in water and other solvents that can be used to supply graphene due to the oxygen-containing corporations consisting of hydroxyl, carbonyl, carboxyl, and epoxide which can be present on its surface [3, 4].

There have been numerous strategies that scientists may want to use to make graphene oxide nanoparticles. One popular approach becomes the modified Hummer's method, which worried oxidizing graphite via mixing it with potassium permanganate, nitrate, and sulfuric acid. This simple and price-effective approach has the potential to revolutionize the production of graphene-based totally materials and is used extensive range of industries and clinical fields [5, 6].

Graphene oxide nanoparticles have garnered significant hobby within the area of medication because of its homes, such as excessive floor place, remarkable biocompatibility, and capacity to be without problems functionalized with numerous biomolecules [7]. In addition, it is one sort of nanoparticle that has determined a huge variety of packages in fields along with electronics, optics, medication, supercapacitors, food manufacturing, generation, and agriculture specific houses make it a versatile fabric with tremendous capability for advancing healthcare technologies [8-11].

Because of its enormous floor vicinity, electroconductivity, intense flexibility, and thermal stability, graphene oxide most of the numerous nanomaterials generated from carbon has drawn unique interest to be used in biological programs [12]. One of the key medical applications of graphene oxide is in drug shipping systems. Its huge floor location allows for high drug loading potential, at the same time as its biocompatibility guarantees minimum toxicity to cells and tissues [13-15].

Because graphene oxide has many applications, research into its toxicity is crucial for the safe development of nanotechnology based on it and for enabling broad and vital uses [16]. As an efficient initial method for assessing the cytotoxicity of nanomaterials, in vitro experiments are used [17]. The toxicity effect depends on the dose concentration of graphene oxide and the duration of the dose, as in different studies that have shown the toxicity on some cells, including cancer cells [18, 19].

Nanomaterials demonstrate potential for treatment and diagnosis, though they still need to be further studied to determine the LD50, because even after the nanomaterials have been effectively transported into the cells, they may result in the organisms' mortality [20]. Reactive oxygen species (ROS) production, DNA destruction, and damage to the plasma membrane are the ways in which graphene oxide causes cellular toxicity [21]. This study aims to fabricate sustainable graphene oxide nanosheets using a modified Hammer method and their LD50.

2. Experimental Part

All chemicals, including graphite powder, $KMnO_4$ (99 %), H_2O_2 (97 %), HCl (37 %) H_2SO_4 (99 %), were used as start materials. Graphene oxide (GO) was synthesized using a modified Hummers process. Added 100 mL of H_2SO_4 with 20 mL of HNO_3, then added 2 g of graphite powder, and stirred the mixture for 30 minutes in an ice bath.

Subsequently, 4 g of $KMnO_4$ is added dropwise, and the mixture is stirred for 4 hours. Next, added 500 mL of distilled water, followed by 15 mL of H_2O_2, while continuously mixing. The product was repeatedly cleaned with distilled water and HCl, dried for 24 hours at room temperature to obtain graphene nanosheets.

2.1 Evaluation of LD50 of GO Nanosheets

2.1.1 Experimental Animals

Twenty-four male albino BALB/c rats, weighing 200-300 g, were kept in plastic cages at 20 to 25 degrees Celsius. Four groups of rats were created; each group included six rats. The oral administration period was 5 days as an acute exposure. Group 1 (control): received deionized water orally. Groups 2, 3 and 4: each were daily (100, 200, and 300) mg/kg of GO nanosheets, respectively. Blood samples were collected by cardiac puncture to assay biochemical parameters. Serum samples were stored at -20 ºC until the time of analysis.

2.1.2 Statistical Test

Microsoft Office IBM SPSS statistics version 26 was used to conduct a statistical test that included the student t-test, mean ± standard deviation, and $P<0.05$ was considered statistically significant. Independent-samples t-test or one-way ANOVA was used to assess the data.

3. Results and Discussion

3.1 Diagnosis of GO Nanosheets

The XRD pattern of graphene oxide nanosheets is shown in figures one. graphene oxide typically exhibits a characteristic diffraction peak at $2\theta \approx 10$-

12º corresponding to the (001) plane, 26º (002) and 2θ at 44º (100). The broad diffraction peak indicates reduced crystallinity or small crystallite size and does not directly confirm as nanosheets shown by the broad peak at $2\theta = 26º$, which corresponding to the (002) plane, which may indicate residual graphite or partially reduced graphene oxide [22, 23].

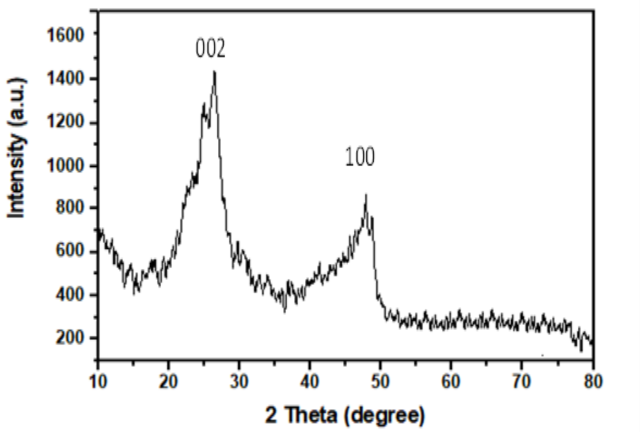

Figure 1: XRD of GO Nano-sheets.

The morphology of GO nanosheets was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Figure 2, and figure 3 show SEM images of samples prepared by the modified Hummers' process, which are in the form of Go nanosheets. This research agrees with M. Muniyalakshmi and co-workers [24].

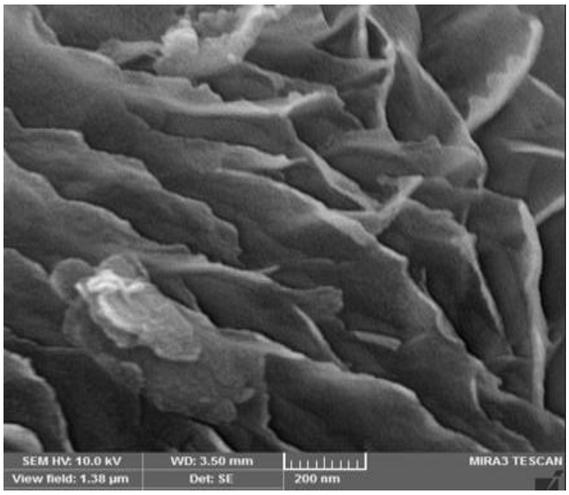

Figure 2: SEM images of GO nanosheets.

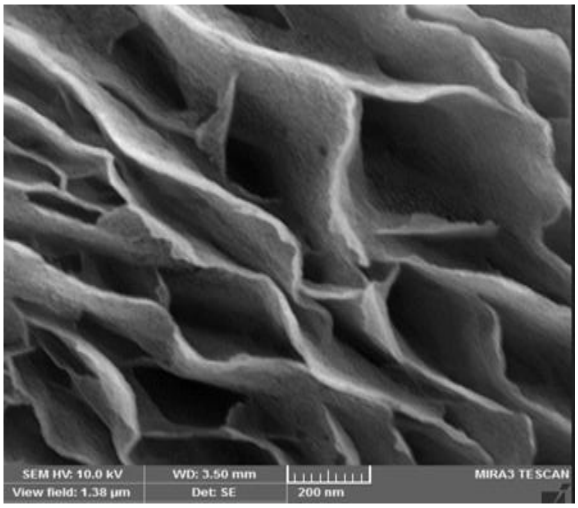

Figure 3: SEM images of GO nanosheets.

The surface morphology of GO was observed using a FESEM. The GO surface is ridged and composed of overlapping structural layers. As another researcher observed, the folded and continuous layers have a high thin-film surface area. This feature of graphene oxide (GO) is highly relevant in a different of applications including biosensors, where the large surface area of the GO enhances the interaction between the functional groups and chemical molecules, such as ethanol and benzene [25].

Images from the transmission electron microscope (TEM) show a sheet of the area under investigation with different morphologies, each of them exhibits a unique thickness figure 4, and figure 5. Dark areas are densely stacked nanostructures made of many layers of GO with certain oxygen characteristic groupings. The TEM results for the GO nano-sheets generated for this investigation showed a single- and double-layer structure.

The increased levels of clarity demonstrate that stacking exfoliation resulted in noticeably a few layers of graphene oxide. Graphene oxide notably greater surface area and extremely clear delaminated graph layers. The reason for the increased thickness is that oxygenated functional groups have been introduced, which has changed the surface of the GO sheet and increased its thickness. This agrees with Ruba and co-workers [26].

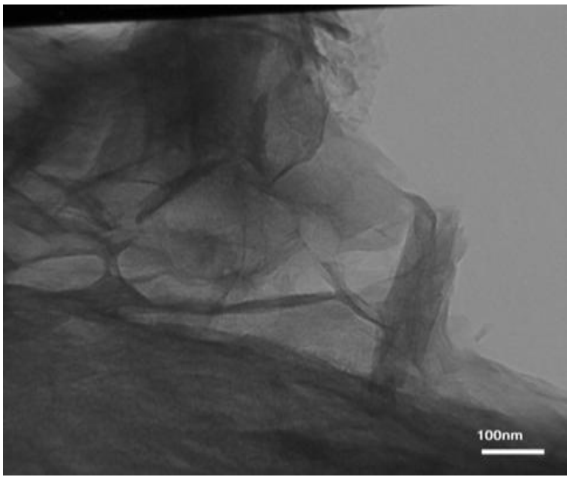

Figure 4: TEM images of GO nanosheets.

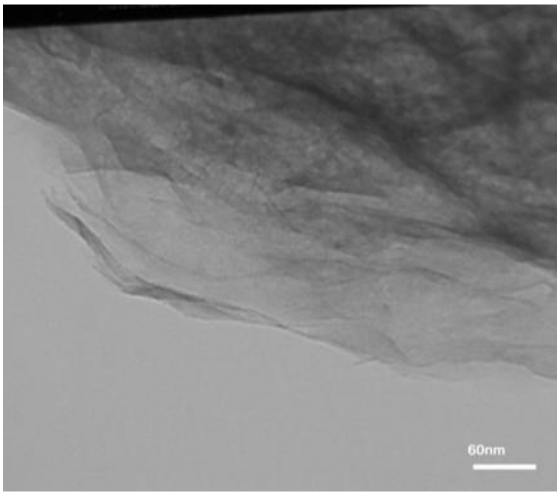

Figure 5: TEM images of GO nanosheets.

Raman spectroscopy is a versatile and well-established technique for recognizing and characterizing the physical and chemical characteristics of carbon compounds. Due to the double bonds between carbon atoms, conjugation exhibits the characteristic D and G and 2D, as shown in figure 6.

The band D is approximately 1350 cm^{-1}, which is attributed to sp2 carbon rings activated, while at approximately 1600 cm^{-1} is the G band, corresponding to the vibrational mode of sp2 bond carbon atoms. The 2D band appears as two peaks at 2700 cm^{-1} and 2980 cm^{-1}, which constitute the graphene sheet. The ID/IG intensity ratio provides an additional indicator of structural disorder. The high ID/IG density ratio confirms increased irregularity and defects, which are consistent with the expected structure of graphene oxide. This research and diagnosis by Raman agree with Muhammad et. al [27].

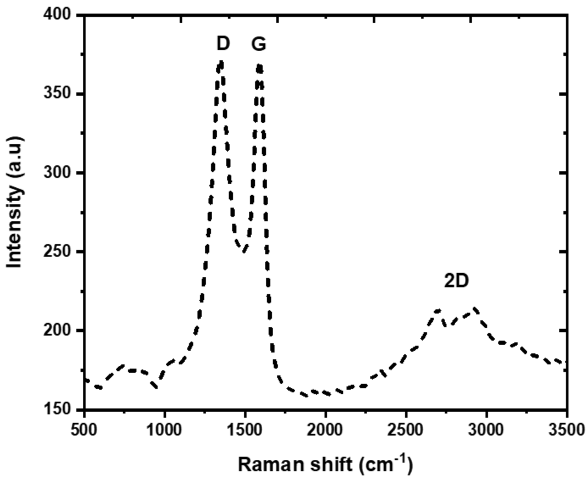

Figure 6: Raman spectra of GO nano-sheets.

3.2 Evaluation of oral LD50 of GO Nanosheets

The oral acute toxicity of GO nanosheets in rats was evaluated using the oral acute LD50 was examined, based on the Karber equation [28]. As indicated by equation 1 and table 1. The LD50 was 166.6 mg/kg after five days of monitoring period the number of dead rats in various groups. At 200 mg/kg of oral GO nanosheet, one rat from the third group died. On the other hand, two of the six rats in the fourth group had died at an oral GO nanosheet concentration of 300 mg/kg.

Table1: Shows the results of the GO nanosheet's toxicity for calculating the LD50 following five days of oral administration to male rats.

Groups	Rats number	GO nanosheet dose mg/kg	Total number of killed rats	(A)	(B)	(A×B)
1	6	0	0	0	0	0
2	6	100	0	100	0	0
3	6	200	1	100	0.5	50
4	6	300	2	100	1.5	150

LD50 =LD - sum (A ×B)/N ....1 [28, 29].

The group's lowest lethal dose is (LD), (N) is each group's total number of rats. The dose difference is (A). The average mortality is (B).

LD50 = 200-200/6 = 166.6 mg/kg

Table 2: Biochemistry test of rats exposed to GO nanosheet for 5 days compared with control
300 mg/kg of GO nanosheet.

Parameters	Control Mean ± SD (n = 6)	100 mg/kg of GO nanosheet Mean ± SD (n = 6)	200 mg/kg of GO nanosheet Mean ± SD (n = 6)	300 mg/kg of GO nanosheet Mean ± SD (n = 6)
Serum urea (mg/dL)	25.13 ± 4.24	26.55 ± 3.23 [a]	28.43 ± 7.54 [b]	45.98 ± 5.65 [c*]
Serum creatinine (mg/dL)	0.419 ± 0.08	0.865 ± 0.18 [a]	0.835 ± 0.27 [b]	1.176 ± 0.21 [c*]
ALT (U/L)	10.25 ± 2.54	11.95 ± 1.77 [a]	12.99 ± 1.8 6 [b]	12.89 ± 2.49 [c]
AST (U/L)	12.55 ± 3.88	12.98 ± 4.11 [a]	13.45 ± 3.65 [b]	13.95 ± 3.94 [c]

Where a, b, c = non-significant ($P \geq 0.05$) of oral administration of GO nanosheet group (100, 200, and 300) mg/kg respectively, compared with control group. c* = significant ($P \leq 0.05$) of oral administration of GO nanosheet group 300 mg/kg, compared with control group.

Results, as shown in table 2, represented a non-significant difference in the levels of serum urea, creatinine, AST and ALT at 100, 200 and 300 mg/kg of the GO nanosheet group. Except for significantly elevated serum levels of urea, serum creatinine at 300 mg/kg compared to the control group. Mohammad A.S. [30], observed a non-significant increase in serum (urea, creatinine, ALT, and AST).

Additionally, histological findings in the brain were tiny foci of necrosis and neuronal degeneration at 500 mg/kg of GO nanosheet. The liver, kidney, spleen, and brain were shown to be poisoned by graphene oxide nanosheets, but not the heart. GONs accumulated inside the body and then caused inflammation in a dose-dependent way.

4. Conclusion

In this study, the modified Hammers' process is considered a suitable method because it provides rapid and continuous production of graphene oxide nanosheets compared to the traditional method. The method encourages a more environmentally friendly synthesis by using less H_2SO_4 and Unwanted chemicals, which decreases waste production. To study toxicity, the LD50 test was applied to rats. There was an LD50 of 166.6 mg/kg of the GO nanosheets, and a non-significant change in serum level of liver enzymes (ALT and AST), and a significant increase in the levels of renal function test (urea and creatinine) in comparison to the control group, at 300 mg/kg. Therefore, graphene oxide nanosheets have the potential to have wide-ranging medical and therapeutic applications due to their low toxicity.

5. References

1. Haishan S., Byungkwon J., Jiyoung P., Jiyoung P., Hyung-jin M., Hong-Baek C., and Yong-Ho C., (2022). In Situ Synthesis of a Bi_2Te_3-Nanosheet/Reduced-Graphene-Oxide Nanocomposite for Non-Enzymatic Electrochemical Dopamine Sensing. Nanomaterials Basel. 12, 12, 2009.
2. Biswajit P., Niharika D., Ruturaj S., Dipak K.S, Biswajit D., Chhatrapati P., and Sarat K. D., (2023). Synthesis, Characteristics and Applications of Graphene Composites: A Survey. Patra B et al. Journal of The Turkish Chemical Society. 10, 3, 757-772.
3. Alam S. N., Sharma N., and Kumar L., (2017). Synthesis of graphene oxide (GO) by modified hummers method and its thermal reduction to obtain reduced

graphene oxide (rGO). Graphene. 6, 1, 1-18.

4. Raghad M., Qusay F., Sama A, Issam K. S., Adnan A. A, Mohammed A. S., Zoltán N., and Klara H., (2022). Classification of nanomaterials and the effect of graphene oxide (GO) and recently developed nanoparticles on the ultrafiltration membrane and their applications: A review. Membranes. 12, 11, 1043.
5. Kaviyarasan G., Sharmila R., Shanmugasundaram R., Palaniselvam V., and Saranya N., (2023). Biosynthesis of Graphene Oxide Nanoparticles from Coconut Fronds. International Journal of Environment and Climate Change. 13, 10, 3007-3014.
6. Zainab B., Nasser A., and Abbas K., (2022). A Physico-chemical Contribution to the Conventional Technique of Preparation Graphene Oxide. Journal of Applied Sciences and Nanotechnology. 2, 2, 70-78.
7. Tsai M. H., Chao H. R., Jiang J. J., Su Y. H., Cortez M.P., Tayo L. L., Lu I.C., Hsieh H., Lin C.C., Lin S.L., Wan Mansor W. N., Su C. K., Huang S. T., and Hsu W. L., (2021). Toxicity of Low-dose Graphene Oxide Nanoparticles in an in-vivo Wild Type of Caenorhabditis elegans Model. Aerosol Air Qual. Res. 21, 200559.
8. Jino R. A., (2024). Large-scale Production and Application of Graphene Oxide Nanoparticles to Meet Agriculture Needs. Current Applied Science and Technology. 24, 1, e0254564.
9. Vaishnavi S., Shreeya R., Debal G., Nityananda A., Palash R. C., Arnab M., and Dibakar R. C., (2023). Terahertz sensing of reduced graphene oxide nanosheets using subwavelength dipole cavities. Scientific Reports. 13, 1, 12374.
10. Al Hawash M., Rajeev R., and Mohamed A. B., (2022). Fabrication of Polyaniline/Graphene Oxide Nanosheet Tea Waste Granules Adsorbent for Groundwater Purification. Nanomaterials. 12, 21, 3840.
11. Pallellappa C., Madhan M. R., Giridharet U. K., and Rao C. N. R., (2020). Simple synthesis of nanosheets of rGO and nitrogenated rGO. Beilstein J. Nanotechnol. 11,1, 68-75.
12. Domi B., Rumbo C., García-Tojal J., Elena Sima L., Negroiu G., and Tamayo-Ramos J. A., (2019). Interaction Analysis of Commercial Graphene Oxide Nanoparticles with Unicellular Systems and Biomolecules. International journal of molecular sciences. 21, 1, 205.
13. Mai S., and Inkielewicz-Stepniak I., (2024). Graphene Oxide Nanoparticles and Organoids: A Prospective Advanced Model for Pancreatic Cancer Research.

International journal of molecular sciences. 25, 2, 1066.

14. Ghulam A. N., Dos Santos O. A. L., Hazeem L., Pizzorno Backx B., Bououdina M., and Bellucci S., (2022). Graphene Oxide (GO) Materials-Applications and Toxicity on Living Organisms and Environment. Journal of functional biomaterials. 13, 2, 77.
15. Tamer M. S., Mohammed F. E., Hend F., Mona O. S., Yasmin M. S., Manal M. A., Mohammed A. M., and Wafaa A. Z., (2024). Multifunctionalization of reduced graphene oxide nanosheets for tumor theragnosis: Synthesis, characterization, enzyme assay, insilico study, radiolabeling and in vivo targeting evaluation. Journal of Pharmaceutical Sciences. 32, 1, 77-95.
16. Balasubramanian K., Karuppiah C., Alagarsamy S., Mohandoss S., Arunachalam P., Govindasamy C., and Ramaraj S. K., (2024). Highly sensitive detection of environmental toxic fenitrothion in fruits and water using a porous graphene oxide nanosheets based disposable sensor. Environmental Research. 259, 119500.
17. Rahaman M. H., Islam M. R., Islam R., Alam S. N., Rahman M. S., Rahman M. A., and Begum B. A., (2024). Preparation, characterization, and adsorption kinetics of graphene oxide/chitosan/carboxymethyl cellulose composites for the removal of environmentally relevant toxic metals. International Journal of Biological Macromolecules. 257, 2, 128357.
18. Su H. M., Vasu D., Chan S. Y., Liu Y. C., Jiang J., You Y. F., and Chen S. C., (2024). Two-dimensional heterojunction layered graphene oxide/graphitic carbon nitride photocatalyst for removal of toxic environmental dye methylene blue. Environmental Pollution. 345, 123556.
19. Yiming S. U. N., Huang A., Zhi Z. H., Chen S. O., and Guihua L. A., (2004). Immunogenic and toxic effects of graphene oxide nanoparticles in mouse skeletal muscles and human red blood cells. Journal of Southern Medical University. 44, 4, 617.
20. Prashant H.G., and Gopal A., (2025). Metal grafted graphene-based nanomaterials towards cancer theranostic efficacy. Explor Target Antitumor Ther. 6,11,1-28.
21. de la Parra S., Fernández-Pampín N., Garroni S., Poddighe M., de la Fuente-Vivas D., Barros R., and Tamayo-Ramos J. A., (2024). Comparative toxicological analysis of two pristine carbon nanomaterials (graphene oxide and aminated graphene oxide) and their corresponding degraded forms using human in vitro models. Toxicology. 504, 153783.

22. Kamil A. F., Abdullah H. I., and Rheima A. M., (2021). Modification of hummers presses for synthesis graphene oxide nano-sheets and graphen, e oxide/Ag nanocomposites. Journal of Ovonic Research. 17, 3, 253-259.
23. Marwa I. I., Elsayed A. A., Salah M. D., and Wafaa M., (2023). Graphene Oxide Synthesis and Characterizations as a Carbonaceous Nanoparticle by Using Modified Hummers' Method. Egyptian Journal of Soil Science. 63, 4, 537-552.
24. Muniyalakshmi M., Sethuraman K., and Silambarasan D., (2020). Synthesis and characterization of graphene oxide nanosheets. Materials Today: Proceedings. 21, 1 408-410.
25. Surani A. H., Rashid A. R., Arshad N., and Hakim A. A. N., (2019). High-Yield and Stepwise Synthesis of Graphene Oxide by Modified Hummers' Method. International Journal of Nanoelectronics and Materials. 12, 4, 413-422.
26. Ruba F. A., Mohammed J. M., and Ahmed M. R., (2024). A Sustainable Modified Hummers Method for Synthesizing Graphene Oxide Nanosheets. Iraqi Journal of Applied Physics. 20, 1, 317-320.
27. Muhammad A., Mohamed K., Khaled G. M., Abdel-Ghany A. M., Ebnalwaled A. A., and Ibrahim E. M. M., (2024). Synthesis, Characterization, and Electrochemical Analysis of Graphene Oxide Prepared by Modified Hummer's Method. Sohag Journal of Sciences. 9, 2, 140-144.
28. Dodehe Y., Rita B., Bernard N.D., Chonta T., and Jean D.N., (2012). Acute and subacute toxic study of aqueous leaf extract of Combretum molle. Tropical Journal of Pharmaceutical Research. 11, 2, 217-223.
29. Mosaad R. M., Samir A., and Ibrahim H. M, (2017). Median lethal dose (LD50) and cytotoxicity of Adriamycin in female albino mice. Journal of Applied Pharmaceutical Science. 7, 3, 77–80.
30. Mohammad A., Mohammad K. K., Ehsan Z., Mohammad H., Hablolvarid., Jalal H., and Alexander M. S., (2018). In vivo toxicological evaluation of graphene oxide nanoplatelets for clinical application. International Journal of Nanomedicine. 13, 4757-4769.