RESEARCH PAPER

Characterization & evaluation of cytoprotective effect of *O. indicum* derived silver nanoparticles

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Abstract
Background: *Oroxylum indicum* is a well-researched plant; however, its endangered status has raised concerns regarding its overexploitation in research. This study focuses on utilizing the abundant *O. indicum* flower buds as a means to support research while minimizing the impact on natural resources.
Methods: *Oroxylum* bud extract was prepared using the decoction method, followed by filtration. *Oroxylum* bud nanoparticles (OBNps) were synthesized using silver, and characterized using UV-Vis spectrophotometry, FT-IR, SEM-EDAX, and XRD. An MTT assay was performed to evaluate the effects of the developed OBNps on human lymphocytes.
Results: The prepared OBNps exhibited a surface plasmon resonance (SPR) peak in the UV-Vis spectra at wavelengths ranging from 426 to 432 nm, which was further supported by FT-IR analysis. The SEM-EDAX images showed highly agglomerated and irregularly shaped OBNps. XRD analysis indicated cubic structures with sizes ranging from 70–75 nm. The extract concentrations of 6.25%, 12.5%, and 25% resulted in cell survival rates of 95.01%, 106.03%, and 107.52%, respectively. OBNps demonstrated a positive ameliorative effect, with a cell survival rate of 95.95%.
Conclusion: The developed OBNps showed promising results for both proliferative and ameliorative effects on human lymphocytes, indicating their potential for further experimental and clinical studies based on the characterization presented in this study.
Keywords: *Oroxylum indicum*; Biosynthesis; Nanoparticles; FT-IR; XRD; Cypermethrin; MTT Assay.
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Introduction

Research on 'Nanoparticles' has been ongoing for decades, with many advancements. It continues to present avenues for advanced research in almost every field, including healthcare [1,2] and engineering [3, 4]. The advent of nanoparticles has significantly affected research leading to improvements in drug development, screening, manufacturing, and drug delivery systems [5,6,7]. These breakthroughs in biological research have led to the use of different metals and sources for nanoparticle formation [8,9]. With innumerable combinations of metals and well-known herbal concoctions, the field of medicine is being enriched with a high number of sustainable drug alternatives [10,11,12]. Nanoparticle biosynthesis with silver has been a proven solution for many problems [13]. AgNPs have been established as potent antimicrobials and anticancer agents, and they have been used as wound healers and to repair burn damage [14,15,16]. The use of silver since ancient times, in the form of utensils and silver-derived products for water disinfection or food preservation [17,18], provides evidentiary value for further research on the biosynthesis of nanoparticles utilizing silver.

The perfect combination of silver with medicinal plants can generate a remedy with the potential to transform *in vitro* results into a sustainable product. The synergy between silver and the bio-source intended for nanoparticle formation highly reflects the use of the developed product in various fields. In this study, we utilized the widely researched tree, *Oroxylum indicum*. One indicum is a highly sought-after medicinal tree that is used to treat many diseases, including obesity [19], arthritis [20], cancer [21], ulcers [22], diabetes [23], diarrhoea [24], and inflammatory [25] diseases. It is also useful as a hepatoprotective [26], analgesic [27], antioxidant [28], and antimicrobial [29, 30]. Such wide applicative measures have made research avenues rich in terms of data isolation and application.

With so many opportunities for research and given the status of the tree as endangered, we thought of utilizing the source differently. The observations revealed that *Oroxylum indicum* flowers during the early summer and rainy seasons. Flower bud

initiation was observed in the last week of May, and peak flowering was observed in the third week of July. The average production of flowers was 524.15±120.11; however, fruit production was only 11.61±4.99 [31]. Such statistics showcase the higher frequencies of the developing bud, higher numbers of flowers, and their decreased transformation into fruit. Most flowers wilt and fall off after the flowering season. Thus, flower buds can be used in large quantities. This idea led to the study of the synergy between the flower bud extract and silver nitrate, which further led to *Oroxylum* bud nanoparticles (OBNps).

Characterization of the developed OBNps was another step towards developing a sustainable alternative. One of the preliminary techniques for the characterization of nanoparticles is the identification of characteristic peaks of associated metals via UV-Visible spectroscopy [32]. This technique is one of the most widely used methods because of its simplicity, precision, inexpensiveness, non-destructiveness, and analytical approach [33]. Another spectroscopic study was performed using FT-IR spectroscopy, which provides the structural and functional characterization of the sample [34]. Thus, FT-IR analysis is an efficient tool for the characterization of nanoparticles. Structural analysis of nanoparticles is another technique for the structural characterization of powdered nanoparticles. The derived nanoparticles were characterized for their chemical properties using FT-IR. The characterization of nanoparticles has been improved using Scanning Electron Microscopy. High-resolution imaging of nanoparticles establishes a stronger foundation, stating the acceptable size and presence of associated metals for further studies [35]. We utilized SEM-EDAX for characterization. The XRD pattern of the biosynthesized nanoparticles provides insight into the metal peak and proper structural design [36]. XRD was used to better characterize the developed nanoparticles after SEM characterization. To characterize the biological activity, we performed the MTT assay. This study mainly focused on the ameliorative effects of nanoparticles on human lymphocytes.

Clinical trials are one of the most expensive fields of research, and their excruciating levels of norms make them a remote area for many researchers [37]. In such situations, the MTT Assay is a solution for *in vitro* testing. This makes the experiment feasible and adaptable, and its accurate analysis becomes a scaffolding platform for further studies [38]. The assay provides critical information about cell proliferation and/or cytotoxicity. Cytotoxicity screening is one of the most important tests for evaluating new drugs [39, 40]. The biosynthesis of nanoparticles makes this test important, as it leads to the development of an alternative that could be industrially significant [41]. In this study, we compared the ameliorative effects of both OB extract and OBNps on human lymphocytes. Toxicity studies of cypermethrin [42,43,44] provided a standard positive control for comparing the effects of OB extract and OBNps.

1. Material and Methods

2.1 Collection of plant material- O. *indicum* flower buds were collected from the reserve forest area of Jabalpur, Madhya Pradesh (Fig. 1, Fig. 2). The collected buds were dried in a hot air oven at a temperature of 100℃, with monitoring at regular intervals for two days. The dried buds were ground in a clean pestle mortar, and the collected powder was sieved to obtain a fine and uniform powder (Fig. 3).

Fig. 1
Fig. 2
Fig. 3

2.2 Preparation of extract- The obtained powder was used to prepare a 70% ethanolic extract of 1 mg/ml concentration using the hot extraction method. The powder was boiled for 20 min at 100°C until a color change was observed and solvent reduction was observed. The boiled solution was later filtered using Whatman filter paper No.1 to obtain the extract. (Fig.4, Fig.5)

Fig. 4

Fig. 5

2.3 Biosynthesis of Silver Nanoparticles- The *O. indicum* bud nanoparticle synthesis was accomplished after testing different temperature treatments given to 1mM AgNO3 Solution & 70% ethanolic *O. indicum* bud extract mixed in 9:1 ratio respectively (Fig. 6, Fig. 7, Fig.8) The characteristic colour change & Surface Plasmon Resonance peak of silver nanoparticles was tested for each treatment (Fig. 9). The nanosilver solution was centrifuged, and the pellets were collected and air-dried to obtain nanoparticles (Fig. 10).

Fig. 6
Fig. 7

Fig. 8

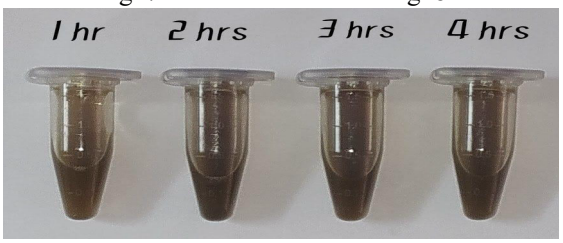

Fig. 9

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2.4 2.4 Characterization of nanoparticles

2.4.1 UV-Visible spectroscopy- The samples were scanned in UV-vis spectrophotometer 1800 (Shimadzu A116352) at 300–800 nm. The samples were analyzed for the characteristic Surface Plasmon Resonance Peak of silver nanoparticles in the region.

2.4.2 FT-IR Spectroscopy- The samples were scanned using an IRAffinity-1 S instrument (Shimadzu A221353). A comparative analysis of the peaks was performed for the bud powder and derived nanoparticles.

2.4.3 XRD- The nanoparticle solution was centrifuged, and the pellets were collected and air-dried. The instrument (PANalytical Empyrean XRD in the instrumentation facility at IISER, Bhopal, was used for the analysis. The sample was analyzed at a voltage of 30 kV, and a current of 100 mA. The scan speed was 4.0000 °/min, and the step size was set to 0.0200°/min. The scherrer equation was used to estimate the average crystal size

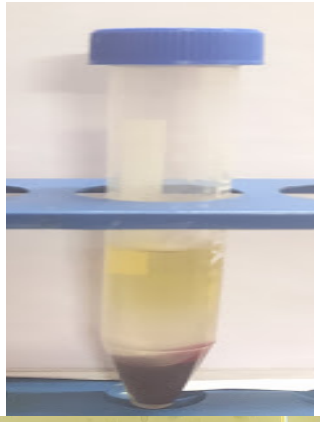
$D = k\lambda / \beta * \cos\theta$

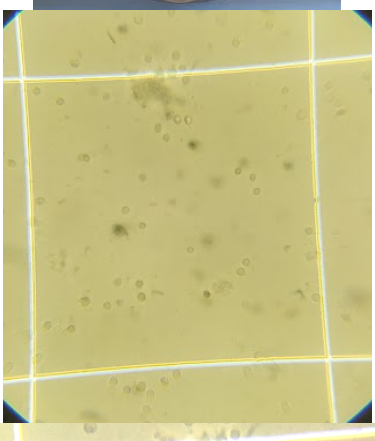
2.4.4 SEM-EDAX - An Energy Dispersive X-ray spectrometer (EDAX), manufactured by Oxford Instruments Nanoanalysis, was used at the instrumentation facility at IISER, Bhopal. The sample was coated with copper using a sputter coater for better spatial resolution, and the processing was performed with three iterations.

2.5 Ameliorative effect of OB Extract & OB Nps on human lymphocytes- The protocol described by Gokhale et al. 2015 [45], was followed to study the ameliorative effect of both the extract and derived nanoparticles.

Isolation of lymphocytes from whole blood

Blood (3 ml) was collected in sterile heparinized vials. This was diluted with an equal volume of PBS (1×). Three millilitres of HiSep™ Lymphocyte Separation Medium (LSM) 1077 (Hi Media) was aseptically transferred into a centrifuge tube. This was carefully overlaid with 6 ml of diluted blood. The mixture was centrifuged at 400 × g at room temperature (RT) for 20 min. The erythrocytes sedimented, and the lymphocytes formed a layer above the HiSep layer. (Fig. 11) Most of the supernatant was aspirated, and the lymphocyte layer along with half of the HiSep layer was carefully aspirated into a separate centrifuge tube. The cells were then washed twice with isotonic PBS. Cells were counted using a hemocytometer. Approximately 0.5 ml of the suspension was appropriately diluted in TC 199 medium (Hi Media) supplemented with fetal bovine serum to give a final concentration of 5×10^4 cells/ml. (Fig. 12, Fig. 13)





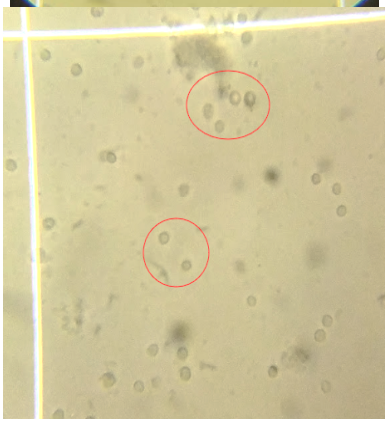


Fig. 11

Fig. 12

Fig. 13

MTT Assay

MTT assay was performed according to Mosmann, 1983 [46] with some modifications. Aliquots (180 µl) of the prepared lymphocyte suspension (5×10^4 cells/ml) were seeded into a 96-well polystyrene tissue culture plate in ten replicates. One row containing only medium and cells served as a control.

Aliquots of 40 µl of the 0.5% v/v concentration of cypermethrin were added to the wells. Each concentration was tested in ten

replicates. The plate was incubated for 2 hrs. at 37°C in a moist CO_2 incubator (at 5% CO_2 concentration).

By adding 40 µl of OB extract & OBNps separately to the cells in the same microtitre plate and incubating for an overnight period (Fig. 14). Cells incubated in culture medium alone served as control for cell viability which was taken as 100%. After overnight incubation, (Fig. 15), 20 µl aliquots of MTT solution (5 mg/ml in PBS) were added to each well and reincubated for 2 h at 37°C. 100 µl Dimethyl sulfoxide (DMSO) was then added to each well to dissolve the formazan crystals followed by incubation at 37°C for 30 min.

The culture plates were then placed in an ELISA microplate reader and absorbance was read at 600 nm. The amount of color produced was directly proportional to the number of viable cells. OD of various concentrations of bud extract and a 1% dilution of DMSO dissolved OBNps was noted and the final OD was calculated after making the due adjustment for these factors. Data were statistically analyzed applying the student's t test. Cell viability rate was calculated as the % of MTT absorption as follows:

% survival = (Mean experimental absorbance/Mean control absorbance) × 100.

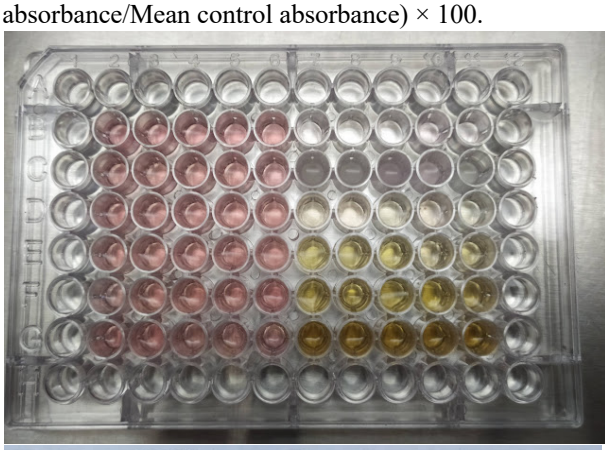


Fig. 14

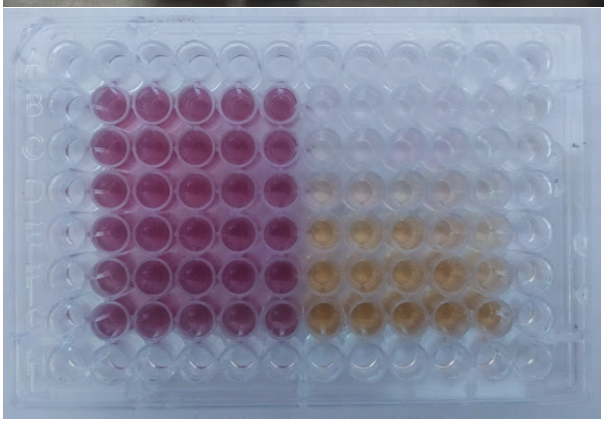


Fig. 15

2. Results

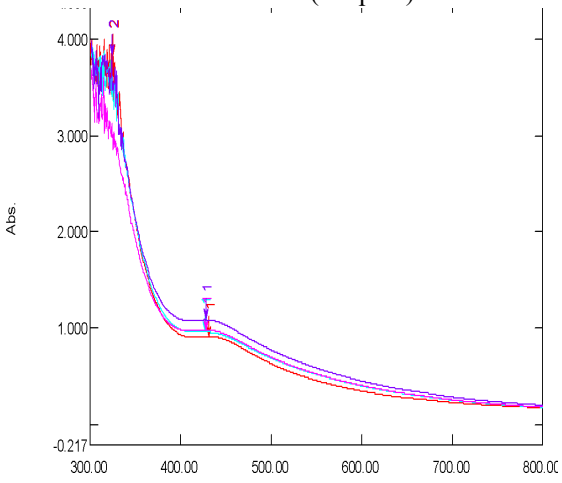
3.1 Characterization of nanoparticles

3.1.1 UV-Visible spectroscopy- The Characteristic peak of UV- Vis spectrum of silver nanoparticles was also used to confirm the biosynthesis of silver nanoparticles. All the nanosilver solutions, irrespective of the treatment duration, demonstrated the characteristic SPR peak for silver at 426- 432 nm. The absorbance recorded for solutions treated for different time durations was similar (in the range of 0.909- 1.085) (Table 1).

Time (in hrs.)	Wavelength & Absorbance at different temperature treatments				
	4 °C	10 °C	45 °C	80 °C	Water Bath (100-125 °C)
1	-	-	-	-	431/0.909
2	-	-	-	-	**427.50/1.085**
3	-	-	-	-	428/0.975
4	-	-	-	-	426.50/0.955

Table 1 Table summarizing different treatments performed for nanoparticle preparation & presence of SPR Peak observed for water bath treatment at different time intervals

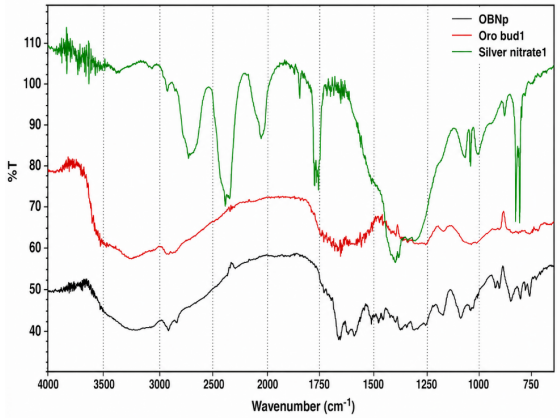
The single peak in the 'Surface Plasmon Resonance Region' (370 nm -450 nm) suggests the presence of nanosilver in the solution. Peak in similar regions was observed in all the treated solutions for different times. (Graph 1)



Graph 1 Coinciding UV-Vis Spectra of water bath treated solutions for different time intervals (1 hr, 2 hrs, 3 hrs & 4 hrs in Pink, blue, sky blue and red colour respectively)

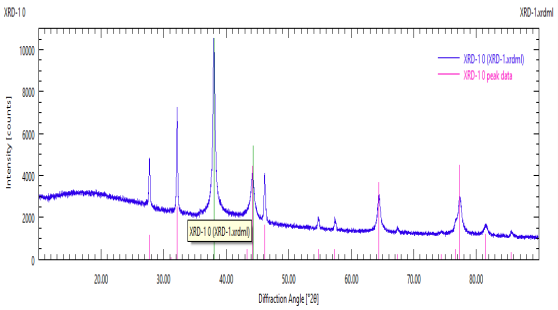
3.1.2 FT-IR Spectroscopy- Dark colored powder of OBNps was obtained after centrifugation of nanosilver solution & air drying of the sample. The comparative FT-IR Spectra of Bud, Derived Nps & $AgNo_3$ shows the stronger vibrations of $AgNO_3$ reflecting in the Nps in the 700 - 100 cm^{-}

[1] region, which were completely weak in Bud. (Graph 2)



Graph 2 Comparative FT-IR Spectra of Bud (red), Derived NPs (black) & $AgNO_3$ (green)

3.1.3 XRD- The highest peak was found at the 38.06° (2**θ** position) (Graph 3). The crystal system was found to be cubic and the average size was calculated to be approximately 70-75 nm. The obtained data for peaks at different 2**θ** position and the corresponding h,k,l values are mentioned in the table 2. The h,k,l values for 100% intensity were found to be (1,1,1).



Graph 3 XRD Diffractometer of developed OBNps

Table2 Peak list for the XRD of OBNps.

S. No.	h	k	l	d (A)	2Theta (deg)	I (%)
1.	1	1	1	2.35905	2.429	100.0
2.	0	0	2	2.04300	2.805	47.5
3.	0	2	2	1.44462	3.967	27.8
4.	1	1	3	1.23198	4.652	30.9
5.	2	2	2	1.17953	4.859	8.9

3.1.4 SEM-EDAX- The images received from the facility showed high agglomeration of the particles. Other than high agglomeration the rough texture and irregular boundaries of the nanoparticles can also be noted distinctly at both 10 µm & 40 µm scale. (Fig. 16, Fig. 17). The EDAX data for the first iteration shows 100% presence of silver and the increase in oxygen content over iterations might suggest progressive oxidation, surface contamination, or a silver oxide (Ag_2O) formation. The weight % of Ag was found to be 74.04% with the presence of 25.96% weight of Oxygen after the third iteration. The spectrum of both the iterations can be viewed in Fig. 18 & Fig. 19.

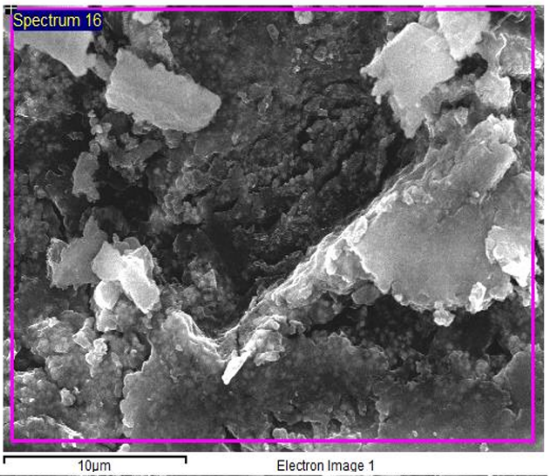


Fig. 16

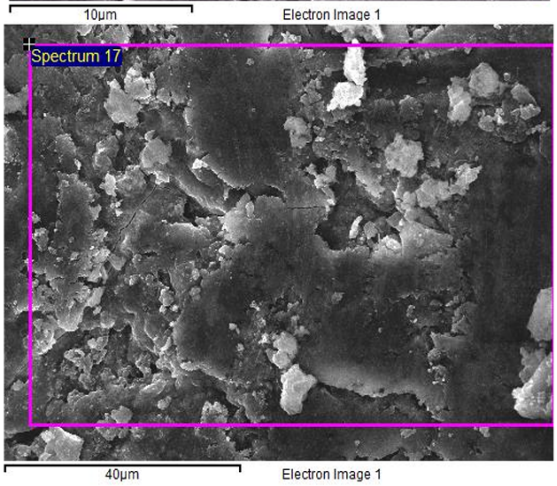
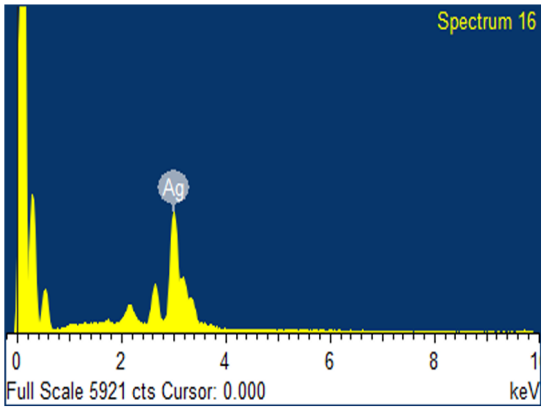


Fig. 17



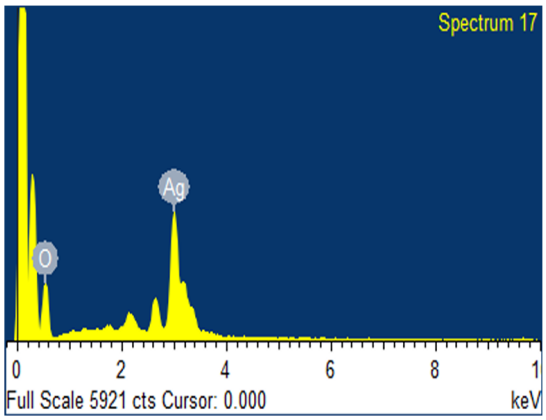


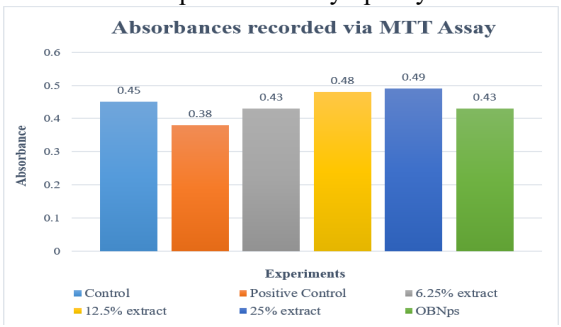
Fig. 18

Fig. 19

3.2 Ameliorative effect on human lymphocytes- The microplate readings after overnight incubation of the plate were noted and cell survival was calculated with respect to controls. The mean absorbance calculated for the negative control was 0.45 ± 0.03. The calculated mean absorbances are given in Table 3 and Graph 4. The addition of OB extract showed a significant and gradual increase in survival. The concentration of extracts, 6.25%, 12.5 % and 25% reflected a cell survival rate of 95.01%, 106.03% & 107.52% respectively. The OBNps reflected a similar observation as noted for 6.25% extract with a cell survival % of 95.95%. The increasing cell survival rate presented high proliferation for higher concentrations of OB extract.

S. No.	Experiment	Absorbance
1	Negative Control	0.45 ± 0.03
2	Positive Control	0.38 ± 0.03
3	6.25% extract	0.43 ± 0.01
4	12.5% extract	0.48 ± 0.02
5	25% extract	0.49 ± 0.07
6	OBNps	0.43 ± 0.05

Table 3 Absorbances recorded via MTT Assay for estimating effect of different concentrations of OB extract and OBNps on human lymphocytes.

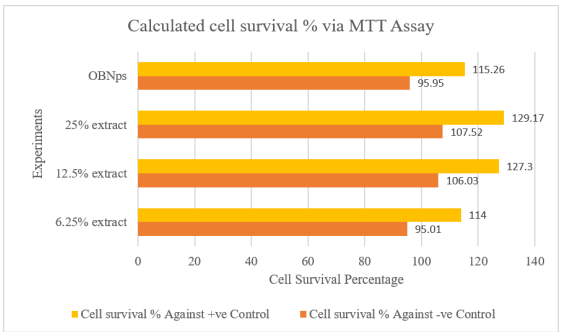


Graph 4 Recorded absorbances of different concentrations of acts and OBNps to study their respective effects on human lymphocytes data is calculated as mean ±SD)

In comparison with positive control, the ameliorative effect of the OB extract and OBNps can be deduced as the cell survival percentage is noted to be significantly increased. The effect of cypermethrin showed a cell survival with absorbance of 0.38 ± 0.03. The calculated cell survival percentages were 114, 127.3, 129.17 & 115.26 for 6.25%, 12.5%, 25% OBExtract and OBNps respectively. The statistical analysis, using ANOVA, shows a significant difference at 0.1% α level of significance (n= 5, 0.05<p>0.1). A summarized table and graph for the same data have been presented (Table-4, Graph- 5). The t-test showed significance of difference only between positive and negative control, whereas was found to be insignificant for negative control versus all experiments. This insignificance of difference for all the experiments reflects a successful ameliorative effect by OB Extract and OBNps (n=5, p>0.05).

S. No.	Experiment	Cell survival percentage (%)	
		Against -ve Control	Against +ve Control
1	6.25% extract	95.01	114
2	12.5% extract	106.03	127.3
3	25% extract	107.52	129.17
4	OBNps	95.95	115.26

Table 4 Calculated cell survival percentages for human lymphocytes against both positive and negative controls.



Graph 5 Calculated cell survival percentage showing the proliferative and ameliorative effect of different concentrations of OB extract and OBNps.

3. Discussion & Conclusion

Research conducted on *Oroxylum indicum* has always been positive for drug development, and thus, this resource needs to be studied more in accordance with its endangered status. The present work therefore is a step towards creating an equilibrium between the resource and utilization. Abundant data is establishing the importance of *O. indicum* phytochemicals [47,48,49]. This makes *O. indicum* an exquisite raw material for research. The nanoparticle development from such an applicative material seems just another step towards research in this era, where nanoparticle research is one of the most important fields of applied sciences [50, 51]. The development of nanoparticles, in this paper, the OBNps, further led to characterization. Nanoparticle characterization for the physical, chemical, and biological properties is essential to investigate in order to find an optimized application [52,53]. The UV spectroscopic analysis was a significant characterization of the peak of surface plasmon resonance. The peak in the 430 nm region concluded the presence of interaction of the same with the extract, resulting in the synthesis of silver nanoparticles [54]. Similarly, the characterization through FT-IR supported the results obtained through UV-Visible spectroscopy. Along with this data, sophisticated techniques like SEM-EDAX and XRD have been employed for better characterization. The surface characterization was made easier through EDAX analysis. The 3-D image and surface characterization provide an insight into further application strategies [55]. With the structural characterization and 3-D images from SEM, the size estimation was highly essential to conclude the material as a nanoparticle. The analysis provides a reliable estimate of the nanoparticle size [56]. The nanosize of the synthesized particles sets in motion limitless applications. These employed analytical techniques have been an efficient way of identifying the potential application of synthesized nanoparticles [57].

Although the study was centered on the medicinal benefits of the plant source used, the study of the ameliorative effect of the biogenic nanoparticles was a key takeaway of the study. The *in vitro* method for testing the effects of both the sample, OB extract and OBNps, through MTT Assay provides a better insight for it to actually proceed for more studies. The studies of various biogenic nanoparticles as anticancer drugs have been well established [58,59,60]. Similarly, the toxic effects of cypermethrin on human peripheral blood lymphocytes have been studied [61,62]. Both the study approaches were utilized, and the effect of OB Extract and OBNps were studied on the human lymphocytes incubated with cypermethrin. The obtained results supported the ameliorative effect of OB extract as reported earlier in the works of Soni et al. 2016 [63]. The significant effect of developed OBNps reflects the efficiency similar to 6.25% concentration of OB extract. The experiment clearly indicated the cytoprotective effects of the extracts and OBNps. The reported presence of oroxylin A and chrysin in the stem, leaf, root, and bark of the tree [64,65] supports the hypothesis of the presence of similar bioactive compounds in OB extract exhibiting cytoprotective activity. These bioactive compounds, also present in the OBNps, need to be studied for developing the process of nanoparticle preparation. The high agglomeration of the nanoparticles observed in SEM, suggests studying the capping agents in depth to attain stability of nanoparticles. Although the bioactivity of developed OBNps was favorable for the lymphocytes, the activity can be optimized with more studies. The analysis of the MTT assay results also piqued our attention towards the fact that the OB extract, used for the experiment was freshly prepared; on the other hand, the nanoparticles had been prepared earlier and passed the whole process of chemical characterization (UV-Vis, FT-IR spectroscopy), thus indicating prolonged bioactivity of OBNps over OB extract. The development of OBNps exhibited easier storage and much longer shelf life as compared to the crude extract, which is a characteristic of nanoparticles, also reported by Habibullah et al. 2022 [66]. This particular experiment showcases the benefits of biosynthesis of nanoparticles from important medicinal plants in order to obtain a sustainable solution to the problem of shelf life and storage of fresh extracts [67,68].

The development of OBNps has been done by following the review on the medicinal properties of *O. indicum* and the use of its various parts. This paper describes the characterization of the developed nanoparticles, and the application part provides many avenues for further research. The prospects of the present work include characterization of the bud extract and assessing the cytoprotective effect of OBNps' different concentrations, development and characterization of OBNps by collecting samples from different geographical regions, etc. Furthermore, evaluation of cytoprotective effects can help in alleviating pesticide induced stress in lymphocytes. This

particular study is a step ahead, wherein we tried experimenting with a resource that is in itself wasteful to the plant, but can be more useful for society.

4. Acknowledgement

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5. References

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6. Figure Captions

Fig. 1 *Oroxylum indicum* flower buds
Fig. 2 Separated *O. indicum* flower buds
Fig. 3 Ground *O. indicum* buds
Fig. 4 Filtration of the hot plate extract of *O. indicum* buds
Fig. 5 Prepared ethanol extract of *O. indicum* buds
Fig. 6 Solution of OB extract and $AgNO_3$
Fig. 7 Water bath treatment setup for the OB extract added $AgNO_3$ solution
Fig. 8 Nanosilver solution obtained after 1 Hr treatment

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Contributions

M.G. conceived and designed the study. I.D. performed the experiments and collected the data. I.D. & M.G. contributed to data analysis and interpretation. I.D. assisted in the synthesis and characterization of the nanoparticles. M.S. supervised the project. I.D. contributed to writing and revising the manuscript.

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9. Ethics declarations

Ethical Approval

Not applicable.

Informed Consent

None.

Research Involving Humans and Animals

Not applicable.

Competing Interests

The authors declare no competing interests.