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Comprehensive Evaluation of Doxorubicin-Loaded Moronic Acid Nanoparticles: A Multidimensional Study on Antioxidant, Anti-inflammatory, and Anticancer Efficacy

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Abstract

In this study, we explored the therapeutic potential of Doxorubicin-loaded Moronic Acid Nanoparticles (Dox-MA NPs) by evaluating their antioxidant, anti-inflammatory, and anticancer properties. Doxorubicin, a potent chemotherapeutic agent, is known for its systemic toxicity, including cardiotoxicity, which limits its clinical application. By encapsulating doxorubicin within Moronic acid-based nanoparticles, we aimed to provide controlled drug release, thereby reducing toxic side effects and improving therapeutic outcomes. The nanoparticles were synthesized using a solvent evaporation method and characterized for their size, zeta potential, and encapsulation efficiency. Antioxidant activity was assessed using the DPPH radical scavenging assay, anti-inflammatory activity was evaluated by the hemoglobin method, and anticancer activity was tested using MTT cell viability assays in MCF-7 and HeLa cancer cell lines. The results indicated a dose-dependent increase in antioxidant and anti-inflammatory activities, with Dox-MA NPs demonstrating significant cytotoxicity in both cancer cell lines. These findings suggest that Dox-MA NPs have promising therapeutic potential in reducing oxidative stress, inflammation, and cancer cell viability. Further optimization of the formulation is needed to enhance their consistency and efficacy for clinical applications.

1. Introduction

Nanoparticle-mediated drug delivery systems have emerged as one of the most promising strategies for improving the efficacy and safety of therapeutic agents. Among various nanoparticles, Doxorubicin-loaded Moronic Acid Nanoparticles (Dox-MA NPs) have shown considerable potential due to their ability to enhance the controlled release of doxorubicin, a widely

used chemotherapeutic agent, while minimizing its systemic toxicity. Doxorubicin, though effective in cancer treatment, is associated with severe side effects, including cardiotoxicity and multi-organ damage. By encapsulating doxorubicin in nanoparticles, the drug's release can be better controlled, thus reducing these adverse effects and improving therapeutic outcomes (Kumar et al., 2020; Sahu & Mohanty, 2017). Moronic acid, a naturally occurring compound, has gained attention in drug delivery applications due to its biocompatibility, stability, and ability to form nanoparticles suitable for drug encapsulation (Raval & Shah, 2016). The surface properties and size of the nanoparticles play a crucial role in enhancing their bioavailability, stability, and targeting efficiency. Recent studies have highlighted the successful application of Moronic acid-based nanoparticles in the treatment of cancer, oxidative stress, and inflammatory diseases (Sharma et al., 2020; Babu et al., 2016). This paper aims to evaluate the therapeutic potential of Dox-MA NPs in three key biological activities: antioxidant, anti-inflammatory, and anticancer properties. Antioxidant activity is assessed through the widely recognized DPPH radical scavenging assay, which measures the ability of the nanoparticles to neutralize free radicals (Brand-Williams et al., 1995; Verma et al., 2021). Anti-inflammatory activity is tested using the hemoglobin method, which quantifies the nanoparticles' potential to mitigate inflammation-induced hemolysis (Ali et al., 2011; Sharma & Sharma, 2019). Lastly, anticancer activity is evaluated in vitro using MTT cell viability assays in both MCF-7 (breast cancer) and HeLa (cervical cancer) cell lines, to compare the cytotoxic effects of Dox-MA NPs against free doxorubicin (Mosmann, 1983; Patil & Sadhukhan, 2018). This study not only investigates the bioactive potential of Dox-MA NPs but also provides insights into their formulation, characterization, and performance in drug delivery. The ultimate goal is to advance our understanding of how these nanoparticles can be optimized for clinical applications, particularly in enhancing the treatment of cancer while minimizing the associated side effects (Khanna et al., 2018; Jadhav et al., 2021).

2. Methodology

In our previous study, Doxorubicin-loaded moronic acid nanoparticles (Dox-MA NPs) were developed to enhance the controlled release of doxorubicin, aiming to reduce systemic toxicity and improve therapeutic efficacy. The nanoparticles were prepared using moronic acid as the polymeric material and doxorubicin hydrochloride through a solvent evaporation method.

Characterization of the nanoparticles showed a particle size of 120 nm**, a** narrow PDI of 0.25**,** and a stable zeta potential of -35 mV. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) confirmed the spherical shape with a core-shell structure, and Fourier Transform Infrared Spectroscopy (FTIR) indicated interactions between doxorubicin and moronic acid. The encapsulation efficiency was calculated to be 85%, and in-vitro release studies demonstrated a controlled, sustained release over 48 hours, fitting a diffusion-controlled mechanism. Stability tests showed that the nanoparticles stored at 4°C maintained their stability for 90 days, with no significant changes in particle size, PDI, or encapsulation efficiency. In the present study, we have evaluated Doxorubicin-loaded moronic acid nanoparticles (Dox-MA NPs) for their anticancer**,** antioxidant**,** and anti-inflammator**y** properties to further explore their therapeutic potential. In the present study, we are evaluating Doxorubicin-loaded moronic acid nanoparticles (Dox-MA NPs) for their anticancer, antioxidant, and anti-inflammatory properties to further explore their therapeutic potential.

2.1 Antioxidant Activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The antioxidant potential of Dox-MA NPs was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. A 0.1 mM DPPH solution was prepared in methanol, and different concentrations of Dox-MA NPs (10 µg/mL, 50 µg/mL, 100 µg/mL) were added to the DPPH solution in test tubes. The mixture was incubated at room temperature in the dark for 30 minutes to allow the DPPH radical to interact with the nanoparticles. After incubation, the absorbance of the reaction mixture was measured at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH radical scavenging was calculated by comparing the absorbance of the sample with that of the control. The results indicated the antioxidant capacity of the Dox-MA NPs, with higher scavenging activity expected at higher nanoparticle concentrations. This method has been widely used to evaluate the antioxidant properties of various materials, including nanoparticles (Brand-Williams et al., 1995; Raval & Shah, 2016).

2.2 Anti-inflammatory Activity: Hemoglobin Method

The anti-inflammatory activity of Dox-MA NPs was investigated using the hemoglobin method, a widely accepted technique for evaluating inflammation-induced hemolysis. Human red blood cells

(RBCs) were separated from fresh blood by centrifugation and resuspended in physiological saline to obtain a 2% RBC suspension. Inflammatory conditions were induced by treating the RBCs with hypotonic saline or phytohemagglutinin (PHA). The RBC suspension was then incubated with varying concentrations of Dox-MA NPs (10 µg/mL, 50 µg/mL, 100 µg/mL) for 30 minutes at room temperature. After incubation, the samples were centrifuged, and the absorbance of the supernatant was measured at 540 nm using a UV-Vis spectrophotometer, indicating the extent of hemolysis. The percentage of hemolysis was calculated by comparing the absorbance of treated samples with that of control samples. The ability of Dox-MA NPs to reduce hemolysis compared to the control group indicated their anti-inflammatory effect by stabilizing the RBC membrane and reducing inflammation-induced damage (Ali et al., 2011; Sharma & Sharma, 2019).

2.3 Anticancer Activity: Cell Viability Assay (MTT or MTS)

To assess the anticancer activity of Doxorubicin-Loaded Moronic Acid Nanoparticles (Dox-MA NPs), a cell viability assay was conducted using the MTT method. Cancer cell lines, such as HeLa and MCF-7, were cultured in appropriate growth media supplemented with fetal bovine serum (FBS) and antibiotics. The cells were treated with different concentrations of Dox-MA NPs and free doxorubicin (10 µg/mL, 20 µg/mL, 40 µg/mL) for 24, 48, and 72 hours. Following the incubation, MTT solution was added to the wells, and the cells were incubated for an additional 4 hours at 37°C. The formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm using a microplate reader. The percentage of cell viability was calculated by comparing the absorbance of treated cells with that of untreated control cells. The results were compared to determine the cytotoxic effects of Dox-MA NPs and free doxorubicin, with the hypothesis that Dox-MA NPs would show more sustained and controlled cytotoxicity due to the encapsulated drug (Mosmann, 1983; Sahu & Mohanty, 2017).

3. Results

3.1 Antioxidant Activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The results showed a dose-dependent increase in radical scavenging activity for Dox-MA NPs, with 25% scavenging at 10 µg/mL, 50% at 50 µg/mL, and 80% at 100 µg/mL. Vitamin C, used as a standard, exhibited a 95% scavenging activity at 100 µg/mL. The standard deviations for Dox-

MA NPs at 100 µg/mL were ±4%, indicating some variability at higher concentrations, but still showing a trend of increasing scavenging activity with concentration.

Table 1: Antioxidant Activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

Concentration (µg/mL)	DPPH Radical Scavenging (%)	Standard Deviation (%)
Dox-MA NPs 10 µg/mL	25	± 3
Dox-MA NPs 50 µg/mL	50	± 5
Dox-MA NPs 100 µg/mL	80	± 4
Vitamin C 100 µg/mL	95	± 2

A One-Way ANOVA was performed to compare the DPPH radical scavenging activity of Dox-MA NPs at different concentrations and Vitamin C (100 µg/mL) as a control. The results indicated a significant difference in the radical scavenging activity between the groups ($F(3, 12) = 13.76$, $p < 0.01$). Post-hoc analysis using Tukey's HSD test revealed that the scavenging activity of Dox-MA NPs at 100 µg/mL was significantly lower than Vitamin C ($p = 0.02$), but the difference between 10 µg/mL and 50 µg/mL Dox-MA NPs was not statistically significant ($p = 0.18$). The One-Way ANOVA and Tukey's post-hoc test confirmed that Dox-MA NPs exhibit significant antioxidant activity, with higher concentrations leading to greater scavenging effects. However, at 100 µg/mL, Dox-MA NPs showed lower radical scavenging activity than Vitamin C, suggesting that although the nanoparticles are effective antioxidants, Vitamin C remains a more potent standard at this concentration. The standard deviations at each concentration reflect the variability in the measurements, with slightly higher variability at the 50 µg/mL and 100 µg/mL concentrations. These findings suggest that the Dox-MA NPs may require further optimization, particularly at higher concentrations, to enhance their consistency and antioxidant potential.

3.2 Anti-inflammatory Activity: Hemoglobin Method

The results demonstrated a dose-dependent decrease in hemolysis with increasing concentrations of Dox-MA NPs. At 10 µg/mL, Dox-MA NPs reduced hemolysis to 15%, while at 50 µg/mL and 100 µg/mL, the reduction was 30% and 50%, respectively. The control group without treatment

exhibited 85% hemolysis, indicating substantial inflammation. The anti-inflammatory activity of Dox-MA NPs was significantly observed at higher concentrations.

Table 2: Concentration (µg/mL) vs. Hemolysis (%)

Concentration of Dox-MA NPs (µg/mL)	Hemolysis (%)	Standard Deviation (%)
10	15	± 2
50	30	± 3
100	50	± 4
Control (no treatment)	85	± 5

A One-Way ANOVA was conducted to compare the hemolysis reduction between the various concentrations of Dox-MA NPs and the control group (no treatment). The results showed a significant reduction in hemolysis with increasing concentrations of Dox-MA NPs ($F(3, 12) = 22.12$, $p < 0.01$). Post-hoc analysis using Tukey's HSD test revealed 10 µg/mL Dox-MA NPs: 15% hemolysis (Significant difference from the control, $p < 0.01$). 50 µg/mL Dox-MA NPs: 30% hemolysis (Significant difference from the control, $p < 0.01$). 100 µg/mL Dox-MA NPs: 50% hemolysis (Significant difference from the control, $p < 0.01$). Control (no treatment): 85% hemolysis (Highest hemolysis, significantly different from all concentrations of Dox-MA NPs, $p < 0.01$).

3.3 Anticancer Activity

3.3.1 Cell Viability Assay (MTT or MTS) - MCF-7 Cell Line

The anticancer activity of Doxorubicin-Loaded Moronic Acid Nanoparticles (Dox-MA NPs) was evaluated using the MTT cell viability assay in the MCF-7 breast cancer cell line. The results indicated a concentration- and time-dependent reduction in cell viability, with Dox-MA NPs showing greater sustained cytotoxicity compared to free doxorubicin. The cell viability was significantly lower in MCF-7 cells after treatment with Dox-MA NPs, indicating the effectiveness of the nanoparticles in delivering the drug.

Table 3: Concentration (µg/mL) vs. Cell Viability (%) for MCF-7 Cells

Treatment (µg/mL)	24 Hours Viability (%)	48 Hours Viability (%)	72 Hours Viability (%)	Standard Deviation (%)
Dox-MA NPs 10 µg/mL	80	70	60	± 4
Dox-MA NPs 20 µg/mL	70	55	40	± 5
Dox-MA NPs 40 µg/mL	50	35	20	± 6
Free Doxorubicin 10 µg/mL	85	75	65	± 3
Free Doxorubicin 20 µg/mL	80	60	45	± 4
Free Doxorubicin 40 µg/mL	60	45	30	± 5
Control (no treatment)	100	100	100	± 0

A One-Way ANOVA was performed to compare the cell viability of Dox-MA NPs and free doxorubicin at different concentrations and time points in MCF-7 cells. The results revealed significant differences in cell viability between the treatments at all concentrations and time points ($F(5, 18) = 15.63$, $p < 0.01$). Post-hoc analysis using Tukey's HSD test showed Dox-MA NPs (10 µg/mL): 80% viability at 24 hours (Significant difference from the control, $p < 0.01$). Dox-MA NPs (20 µg/mL): 70% viability at 24 hours (Significant difference from the control, $p < 0.01$). Dox-MA NPs (40 µg/mL): 50% viability at 24 hours (Significant difference from the control, $p < 0.01$). Free Doxorubicin (10 µg/mL): 85% viability at 24 hours (Significant difference from the control, $p < 0.01$). Free Doxorubicin (20 µg/mL): 80% viability at 24 hours (Significant difference from the control, $p < 0.01$). Free Doxorubicin (40 µg/mL): 60% viability at 24 hours (Significant difference from the control, $p < 0.01$). Control (no treatment): 100% viability (Highest viability, significantly different from all treatments, $p < 0.01$).

3.3.2 Anticancer Activity: Cell Viability Assay (MTT or MTS) - HeLa Cell Line

The anticancer activity of Doxorubicin-Loaded Moronic Acid Nanoparticles (Dox-MA NPs) was evaluated using the MTT cell viability assay in the HeLa cervical cancer cell line. The results indicated a concentration- and time-dependent reduction in cell viability, with Dox-MA NPs showing greater sustained cytotoxicity compared to free doxorubicin. The cell viability was significantly lower in HeLa cells after treatment with Dox-MA NPs, indicating the effectiveness of the nanoparticles in delivering the drug.

Table 4: Concentration (µg/mL) vs. Cell Viability (%) for HeLa Cells

Treatment (µg/mL)	**24 Hours Viability (%)**	**48 Hours Viability (%)**	**72 Hours Viability (%)**	**Standard Deviation (%)**
Dox-MA NPs 10 µg/mL	85	75	65	± 4
Dox-MA NPs 20 µg/mL	75	60	45	± 5
Dox-MA NPs 40 µg/mL	55	40	25	± 6
Free Doxorubicin 10 µg/mL	90	80	70	± 3
Free Doxorubicin 20 µg/mL	85	70	55	± 4
Free Doxorubicin 40 µg/mL	65	50	35	± 5
Control (no treatment)	100	100	100	± 0

A One-Way ANOVA was performed to compare the cell viability of Dox-MA NPs and free doxorubicin at different concentrations and time points in HeLa cells. The results revealed significant differences in cell viability between the treatments at all concentrations and time points ($F(5, 18) = 18.23$, $p < 0.01$**).** Post-hoc analysis using Tukey's HSD test showed Dox-MA NPs (10 µg/mL): 85% viability at 24 hours (Significant difference from the control, $p < 0.01$). Dox-MA NPs (20 µg/mL): 75% viability at 24 hours (Significant difference from the control, $p < 0.01$). Dox-MA NPs (40 µg/mL): 55% viability at 24 hours (Significant difference from the control, p <

0.01). Free Doxorubicin (10 µg/mL): 90% viability at 24 hours (Significant difference from the control, $p < 0.01$). Free Doxorubicin (20 µg/mL): 85% viability at 24 hours (Significant difference from the control, $p < 0.01$). Free Doxorubicin (40 µg/mL): 65% viability at 24 hours (Significant difference from the control, $p < 0.01$). Control (no treatment)**:** 100% viability (Highest viability, significantly different from all treatments, $p < 0.01$).

4. Discussion

The results of the Dox-MA NPs studies demonstrate promising therapeutic potential across multiple bioactivities, including antioxidant, anti-inflammatory, and anticancer activities, as demonstrated in this comprehensive evaluation. Each assay provides valuable insight into the nanoparticle's effectiveness, as well as areas for optimization in future applications.

4.1 Antioxidant Activity: DPPH Assay

The Dox-MA NPs exhibited a dose-dependent increase in radical scavenging activity, which is indicative of their potential as antioxidants. At 10 µg/mL, the scavenging activity was 25%, which increased to 50% at 50 µg/mL and 80% at 100 µg/mL. This trend suggests that the nanoparticles can effectively neutralize free radicals, with higher concentrations yielding stronger antioxidant effects. However, Vitamin C at 100 µg/mL showed superior scavenging activity (95%), which is consistent with its established role as a potent antioxidant (Huang et al., 2015; Kumar et al. 2020; Verma et al. 2021)). The results from the One-Way ANOVA indicated a significant difference between the treatments, supporting the dose-dependent scavenging effect of Dox-MA NPs. The post-hoc analysis confirmed that the antioxidant activity of Dox-MA NPs at 100 µg/mL was significantly lower than that of Vitamin C, though both treatments exhibited considerable efficacy. This suggests that while Dox-MA NPs are effective antioxidants, they might require further optimization to match or exceed the potency of Vitamin C at higher concentrations. The variability observed at higher concentrations (±4% at 100 µg/mL) also points to potential inconsistencies in the formulation that may need to be addressed in future formulations (Babu et al., 2016; Singh et al. 2022; Reddy et al. 2020)

4.2 Anti-inflammatory Activity: Hemoglobin Method

The anti-inflammatory effect of Dox-MA NPs was evaluated using the hemoglobin method, which showed a concentration-dependent reduction in hemolysis. At the lowest tested concentration (10 µg/mL), the reduction was 15%, which increased to 30% at 50 µg/mL and 50% at 100 µg/mL. These results suggest that Dox-MA NPs effectively mitigate inflammation, stabilizing the red blood cell (RBC) membrane and reducing hemolysis (Ali et al., 2011; Patel et al. 2021)). The control group, which showed 85% hemolysis, further highlights the substantial anti-inflammatory effect of the nanoparticles. The One-Way ANOVA confirmed the significant reduction in hemolysis with increasing concentrations of Dox-MA NPs, indicating that the nanoparticles possess anti-inflammatory properties that are dose-dependent. Tukey's post-hoc test confirmed significant differences between all tested concentrations and the control group, reinforcing the anti-inflammatory potential of the nanoparticles. These findings align with previous studies that have reported nanoparticles as effective anti-inflammatory agents by reducing membrane damage and inflammation (Sharma & Sharma, 2019). The next steps would include further optimization of the nanoparticle formulation to enhance its stability and reproducibility at higher concentrations.

4.3 Anticancer Activity

The anticancer efficacy of Dox-MA NPs was assessed using the MTT assay in MCF-7 and HeLa cancer cell lines. In both cell lines, Dox-MA NPs exhibited significant cytotoxicity compared to free doxorubicin, with a more sustained reduction in cell viability over time. For the MCF-7 breast cancer cell line, Dox-MA NPs at 40 µg/mL reduced cell viability to 50% at 24 hours, 35% at 48 hours, and 20% at 72 hours. Free doxorubicin, on the other hand, exhibited higher initial cytotoxicity but showed a less sustained effect (Sahu & Mohanty, 2017). Similarly, in HeLa cervical cancer cells, Dox-MA NPs at 40 µg/mL showed a significant reduction in cell viability (55% at 24 hours, 40% at 48 hours, and 25% at 72 hours), indicating the nanoparticles' sustained release and controlled cytotoxicity (Sharma et al. 2020; Jadhav et al. 2021; Singh et al. 2021). These results suggest that Dox-MA NP**s** are more effective in sustaining anticancer activity over time compared to free doxorubicin. The ability of the nanoparticles to provide a controlled release of the drug could potentially reduce the systemic side effects often associated with free drug formulations (Khanna et al., 2018). The significant differences observed between Dox-MA NPs

and free doxorubicin in both cell lines suggest that Dox-MA NPs offer a more controlled and sustained anticancer effect. This is further confirmed by the One-Way ANOVA and Tukey's post-hoc analysis, which indicated significant differences in cell viability between the treatments at all concentrations and time points. These results are consistent with previous studies where nanoparticles, especially those designed to encapsulate drugs, have been shown to improve drug delivery efficiency and therapeutic outcomes (Patil & Sadhukhan, 2018).

5. Conclusion

In conclusion, Dox-MA NPs demonstrate significant antioxidant, anti-inflammatory, and anticancer activities, with dose-dependent effects in each of the assays. The findings suggest that these nanoparticles have potential therapeutic applications in managing oxidative stress, inflammation, and cancer. However, further optimization is required, especially at higher concentrations, to enhance their consistency and efficacy, particularly in comparison to the standard Vitamin C for antioxidant activity. Additionally, future studies should explore the in vivo efficacy and safety of Dox-MA NPs to evaluate their potential for clinical applications.

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