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Gum-based nanocapsules comprising naphthoquinones enhance the apoptotic and trypanocidal activity against *Trypanosoma evansi*

Ruma Rani [a], Balasubramanian Narsiman [b], Rajender S. Varma [c,*], Rajender Kumar [a,*]

[a] ICAR-National Research Centre on Equines, Hisar-125001, Haryana, India
[b] Faculty of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, 124001, India
[c] Regional Center of Advanced Technologies and Materials, Czech Advanced Technology and Research Institute, Palacky University, Šlechtitelů 27, 78371 Olomouc, Czech Republic

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ABSTRACT

Nanoencapsulation is a promising approach to enhance the therapeutic potential of a drug. Herein, three selected naphthoquinone (NTQ) derivatives, based on the IC_{50} value against *Trypanosoma evansi*, were encapsulated using gum damar as biocompatible and biodegradable natural gum via nanoprecipitation method. Nanoformulation of NTQs (NNTQs) was less than 150 nm in size, was found to be stable and released the drug in a sustained manner. All the three NNTQs exhibited significant antitrypanosomal effect and morphological changes at approximately two to three times lesser drug concentrations. The nanoformulations exhibited enhanced production of reactive oxygen species (ROS) in the axenic culture of *T. evansi* and less cytotoxic effect on horse peripheral blood mononuclear cells relative to pure NTQs. As evidenced by flow cytometry, the NNTQs showed dose-dependent and time-dependent increased transition of live cells (AV^-PI^-) to early apoptotic cells (AV^+PI^-), late apoptotic cells (AV^-PI^+), and necrotic cells (AV^+PI^+) using annexin V/propidium iodide probe analysis. The results concluded that NNTQs induced more ROS, apoptosis and necrotic effects that exhibited more inhibitory effect on the growth of *T. evansi* with respect to respective NTQ by themselves.

1. Introduction

Naphthoquinones (NTQs) are wide spread phenolic compounds present in different families of plants and their molecular structures bestow the property of oxido-reduction. In traditional medicine, especially in some parts of Asia (India, China) and South America, such phytobioactive naphthoquinones are extensively used for the treatment of various cancers and parasitic diseases. Juglone, lawsone, and plumbagin are the most widespread bioactive naphthoquinones owing to their various biological and pharmacological activities such as antibacterial (Nair et al., 2016), anticancer (Shen et al., 2020), antifungal (Futuro et al., 2018), anti-inflammatory (Vanco et al., 2020), antileishmanial (Mendonça et al., 2018), antimalarial (Brandao et al., 2018) and antitrypanosomal (Pinto and de Castro, 2009; Salas et al., 2011; da Silva et al.,2013). Various studies in the recent past have documented that the NTQ compounds promote significant increase in free radicals or reactive oxygen species (ROS) at mitochondrial level responsible for cell death in parasite (Silva Junior et al., 2014; Bombaça et al., 2019; Rani et al., 2021).

Trypanosoma evansi is one of the causative agents of animal trypanosomosis, also known as 'Surra' and represents a major limitation to livestock productivity that bring about severe economic losses especially in Asia, Africa, and South America (Desquesnes et al., 2013; Kumar et al., 2017; Aregawi et al., 2019). This parasite multiplies by binary fission in the blood of mammals and trypomastigotes are further transmitted to other domestic and wild animals by biting flies, mainly *Tabanus, Stomoxys, Hematopoda*, and *Chrysops* species. It has been mainly restricted to animals but rarely parasitized human as some reports have been documented that the parasite can cross the species boundaries and can be zoonotic (Joshi et al., 2006; Van Vinh Chau et al., 2016). Quinapyramine methyl sulfate/chloride, diminazene aceturate and isometamedium chloride have been dispensed for the treatment of animal trypanosomosis although they have high toxicity besides the emergence of drug resistance (Kumar et al., 2016). Therefore, efforts have been made for the development of an economic, antitrypanosomal chemotherapeutic molecules with less cytotoxic effect as compared to the currently used drugs. Nanomedicine approach have been studied for the treatment of parasitic infections as this strategy can effectively

* Corresponding authors.
E-mail addresses: rajender.kumar@icar.gov.in (R.S. Varma), rkg.nrce@gmail.com (R. Kumar).

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deliver the drug molecules to the target site and enhanced the biological effect with sustained release of drug from the nanocarrier (Branquinho et al., 2014; Baldissera et al., 2016; Rani et al., 2020).

In the present investigation, we applied the nano-based strategy for the nanoformulations of selected NTQ derivatives from our previous study and comparatively examined their enhancement of trypanocidal effect via different analysis with native NTQ compounds. The three selected compounds exhibited good parasite inhibition effect (Table 1) and were further encapsulated in gum-based nanocapsules, as the nanoparticulate drug delivery systems are known to enhance the solubility, absorption, bioavailability, and therapeutic potential of drugs (Dahiya et al., 2018; Rani et al., 2019; 2020). For drug delivery applications, several natural gums, *viz.*, guar gum, tragacanth gum, gellen gum, xanthan gum etc. have been extensively reported. However, only a few studies have been conducted for gum damar, a biocompatible and biodegradable natural gum produced by tapping trees of *Shorea wiesneri* (Family: *Dipterocarpaceae*) and have been used as a micro- and nano-encapsulating material for sustained release of drugs (Choudhary and Pawar, 2014; Sethi et al., 2019; Amiri et al., 2021). To date, no study has been conducted on enhancement of antitrypanosomal potency of naphthoquinone derivatives using nanoencapsulation approach that further improve via increasing ROS and apoptotic-like effect against *T. evansi*. Thus, in the present study, gum damar was deployed for nanocapsules preparation via nanoprecipitation method of selected NTQ derivatives (NNTQs) and were comparatively evaluated for their antitrypanosomal potential and cytotoxic effects relative to respective NTQ derivatives. The NNTQs were assessed for the generation of apoptotic cells and intracellular ROS in axenic culture of *T. evansi*.

2. Experimentation

Six selected naphthoquinone derivatives (NTQ1 to NTQ6; Table 1) have been evaluated for their antitrypanosomal effect on *T. evansi* (Rani et al., 2021). Among these six, three compounds exhibited good parasite inhibition effect against *T. evansi*. Therefore, the NTQ derivatives (NTQ1, NTQ3, and NTQ6) were selected and carried forward from our initial study (Table 1) and used as a drug for preparation of NNTQ1, NNTQ3, and NNTQ6 respectively.

2.1. Preparation and characterization of gum damar-loaded naphthoquinone nanocapsules (NNTQ)

The gum damar-loaded naphthoquinone nanocapsules were prepared by nanoprecipitation method in which two phases, organic phase and aqueous phase are required as reported previously (Rani et al., 2018); concentration of excipients used in were optimized by Box-Behnken statistical analysis of Design Expert Software (Version 8.0.4, Stat-Ease Inc., Minneapolis, MN). The aqueous phase (distilled water) contained the surfactant or stabilizing agents- polyvinyl alcohol (0.26% w/v) and polysorbate 80 (1 ml), while the organic phase (acetone) contained the gum damar (0.08% w/v), active substance (NTQ derivative– 20 mg), oleic acid (1 ml), and lecithin (a water in oil surfactant, 0.40% w/v in chloroform). The selected NTQs were used for the preparation of NNTQ by drop wise addition of the organic phase to the aqueous phase with constant vigorous stirring at room temperature. The ensued NNTQs were analysed for particle size and zeta potential using Zetasizer instrument (Nano ZS-90, Malvern Instruments, UK) to check the size and stability.

Table 1
Detail of naphthoquinone derivatives and their IC_{50}/24 h values against *T. evansi* at 24 h.

Sr. No.	Naphthoquinone derivative	Abbreviation	IC_{50}/24h
1	**1,4-naphthoquinone**	**NTQ1**	**11.48 μM**
2	2-hydroxy-1,4-naphthoquinone	NTQ2	373.6 μM
3	**5-hydroxy-1,4-naphthoquinone**	**NTQ3**	**12.97 μM**
4	2-bromo-1,4-naphthoquinone	NTQ4	21.97 μM
5	2,3-dichloro-1,4-naphthoquinone	NTQ5	18.19 μM
6	**5-hydroxy-2-methyl- 1,4-naphthoquinone**	**NTQ6**	**5.758 μM**

*Compounds in bold are examined in the present investigation.

2.2. In-vitro drug release analysis

The drug release from nanocapsules was examined in phosphate buffer saline (PBS; pH 7.4) as the dissolution medium using the dialysis membrane (cutoff between 12 kDA and 14 kDA, Hi-Media, India) for 24 h. NNTQs formulations (5 mL) were poured in dialysis bag fixed with dialysis clips and immersed in separate conical flasks which were kept in a shaking incubator maintained at 100 rpm speed and 37 °C. At fixed intervals of time, the samples (1 mL) were withdrawn and replaced with an equal volume of fresh PBS. The samples were analyzed using a UV–visible spectrophotometer at 330, 400, and 410 nm for naphthoquinone content in NNTQ1, NNTQ3, and NNTQ6, respectively. The blank NNTQs in release medium were taken as a reference for the gum damar-loaded NTQ nanocapsules and distilled water was taken as reference for pure NTQs. All experiments were performed in duplicate.

2.3. In-vitro growth inhibitory assay

In-vitro growth inhibitory activity was performed on *Trypanosoma evansi* parasite culture, propagated from cryostabillate (T.ev-India-NRCE-Horse1, Hisar), maintained in Parasitology Laboratory of ICAR-National Research center on Equines (NRCE), Hisar, India. The trypanosomes were cultured in HMI-Medium supplemented with 10% FBS as reported earlier (Kumar et al., 2020). The experiments were initiated with a parasite cell density of 1×10^5 cells/mL and maintained in parasite medium at 37 °C in a 5% CO_2 incubator. Different concentrations of prepared nanoformulation of naphthoquinone derivatives-NNTQ1, NNTQ3 and NNTQ6 were added to the parasite culture at 24, 48 and 72 h. The effect of drug was assessed by counting the parasite numbers using a Neubauer haemocytometer after every 24 h up to three days. The untreated parasites and DMSO (1%)-treated parasites were taken as negative control and solvent control, respectively. The 50% inhibitory concentration (IC_{50}) value for each prepared NNTQs were determined at 24 h using Graph Pad Prism software version 8.4.2.679. The IC_{50} of nanoformulations were estimated from six replicates.

2.4. Microscopic evaluation of drug-treated parasites

The parasites were treated with 1x and 2x (x-IC_{50} value) concentrations of prepared NNTQs and comparatively evaluated with respective NTQs-treated parasites. The slides were prepared after 24, 48, and 72 h of treatment and observed using upright microscope (Nikon Eclipse E200, Tokyo, Japan at 1000 magnification including eye piece-10x, objective lense-100x).

2.5. In vitro cytotoxicity assay

The nanoformulations of selected naphthoquinone (NNTQs) were prepared and varying concentrations of NNTQ (1x, 5x, 10x, and 20x) were tested for their cytotoxicity effect on horse Peripheral Blood mononuclear cells (PBMCs) (Kumar et al., 2020). The PBMCs (100 μL) at a density of 1×10^6 cells were seeded in a 96 well plate and incubated at 37 °C with 5% CO_2 in an incubator with the addition of phytohemagglutinin A (10 μg/mL) for 24 h. Then, different drug concentrations (100 μL) were added and further incubated for 48 h. Only PBMCs cell culture was taken as negative control whereas media was used as background luminescence control. After 48 h of drug incubation, Cell Titer- Glo®

reagent was added and mixed for 2 min and further incubated for 10 min at 37° C. The samples were checked for luminescence using a microplate reader (SpectraMax i3X Multimode Microplate Reader). Percentage of cytotoxicity was calculated from six replicates with reference to the negative control according to the formula given below:

$$\% \text{ Cytotoxicity} = \frac{\text{LUM value of negative control} - - \text{LUM value of test sample}}{\text{LUM value of negative control}} \text{ x } 100 \quad (1)$$

2.6. Measurement of intracellular reactive oxygen species

Parasites (2×10^5) were treated with IC_{50} and IC_{100} value of nanoformulations of selected NTQs for 24 h. The 2′,7′-dichlorofluorescin diacetate (20 μM working solution prepared from 1 mM stock) was added to the parasite culture and incubated for 30 min at 37 °C in a 5% CO_2 incubator. Spectrofluorometer (Spectra Max i3X Multimode Microplate Reader-Molecular Devices) was used to determine the fluorescence intensity at 480 and 520 nm for excitation and emission, respectively. The untreated parasite wells and Hydrogen peroxide (H_2O_2) treated wells were taken as a negative control and positive control respectively in the experiment. The same protocol was followed at 0 h, 2 h, 4 h, 6 h, and 24 h to study the ROS production kinetic. percent ROS levels were measured in treated and untreated cells according to the formula given below:

$$\% \text{ ROS} = \frac{\text{Fluorescence intensity of untreated} - - \text{Fluorescence intensity of treated}}{\text{Fluorescence intensity of untreated}} \text{ x } 100 \quad (2)$$

2.7. Measurement of apoptosis

Annexin V-FITC Apoptosis Detection Kit (Sigma- Aldrich, St Louis, USA) was used for the measurement of apoptosis caused in drug-treated parasite. Parasites (2×10^5 cells/mL) were treated for 24, 48 and 72 h with 1x and 2x (x- IC_{50} value) drug concentrations of prepared NNTQs and then they were harvested and washed with PBS. Further, the parasites were incubated with annexin-V-FITC (AV; 5 μg/mL for 15 min) and propidium iodide (PI; 10 μg/mL for 10 min) and analyzed using a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, Unites States) (Rani et al., 2021). A total of 20,000 events were acquired and the data were expressed as the percentage of parasite in different parasite population phenotype (unstained, stained only with AV, stained only with PI, or stained with both AV and PI).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software 8.02 (San Diego California, USA). The experimental data was analysed by two-way ANOVA followed by Tukey's multiple comparisons test ($P < 0.05$) on NNTQs-treated parasite for anti-trypanosomal activity as compared to negative control. In all cases, a P-value < 0.05 was considered statistically significant between the treated and control group. Results presented with error bars represent mean ± SD.

3. Results

Initially, the inhibitory efficacy of six naphthoquinones (NTQs) against *T. evansi* in HMI-Medium supplemented with 10% FBS at 37 °C. Different concentrations of all naphthoquinones were evaluated against *T. evansi* up to three days and parasites were counted on an interval of 24, 48 and 72 h where three naphthoquinone compounds- NTQ1, NTQ3, and NTQ6 stand out with significant antitrypanosomal activity among all. The three active compounds NTQ1, NTQ3 and NTQ6 were selected for the preparation of nanoformulations- NNTQ1, NNTQ3, and NNTQ6 and subjected to comparative evaluation with pure NTQs for anti-trypanosomal activity, morphological changes, cytotoxicity analysis, percent ROS measurement and percent apoptosis measurement.

3.1. Preparation and characterization of NNTQs

The NNTQs were prepared by nanoprecipitation method in which the organic phase was added dropwise to the aqueous phase with vigorous stirring (Rani et al., 2018). All the three nanoformulations were characterized wherein the particle size and zeta potential of the nanoformulations *i.e.*, NNTQ1, NNTQ3, and NNTQ6 was found to be 60.78 nm, 120.9 nm and 119.4 nm, respectively and −27.9 mV, −20.1 mV and −25.2 mV, respectively (Fig. 1A (i, ii) B (i, ii) and C (i, ii)).

3.2. In-vitro drug release analysis of NNTQs

Fig. 1A(iii), B(iii) and C(iiii) depicts the comparative cumulative release of NTQ1, NTQ3, and NTQ6 from respective nanoformulation and compared to pure NTQs drug suspension as a function of time. All the three nanoformulations exhibited a sustained release profile where the% cumulative release was 49.85%, 46.87% and 57.57% for NNTQ1, NNTQ3, and NNTQ6, respectively, whereas the% cumulative release was 71.38%, 65.05% and 69.24% for NTQ1, NTQ3, and NTQ6, respectively; release profiles of all nanoformulation showed a biphasic behavior, with an initial fast release for the first hour, followed by a sustained, slow release up to 24 h.

3.2. In vitro evaluation of growth inhibition efficacy against T. evansi

The parasites were alive, and it showed a significant increase in the parasite population at 24, 48, and 72 h in negative control wells that validates our experiment. Different concentration ranges (1 μM – 20 μM) of three selected NNTQ was evaluated against *T. evansi* in an axenic culture at 24, 48 and 72 h. A contrary picture emerged in NNTQs-treated parasites in comparison to negative control. Fig. 2 depicts the growth inhibitory effects of varying concentrations of NNTQ1, NNTQ3, and NNTQ6 at 24, 48 and 72 h. All the different drug concentrations showed different level of significance that varies with increase in incubation time from 24 h to 72 h. The complete parasite inhibition was exhibited at 15 μM for NNTQ1, 10–15 μM for NNTQ3, and 12.5–15 μM for NNTQ6 at 24 h. DMSO-treated parasites (1%) also revealed a significant increase in parasite growth indicating no inhibitory effect on parasite population and safe for their growth. Moreover, QPS (10 μg/ml or 20 μM) was taken as reference control and exhibited significant ($p<0.0001$) complete

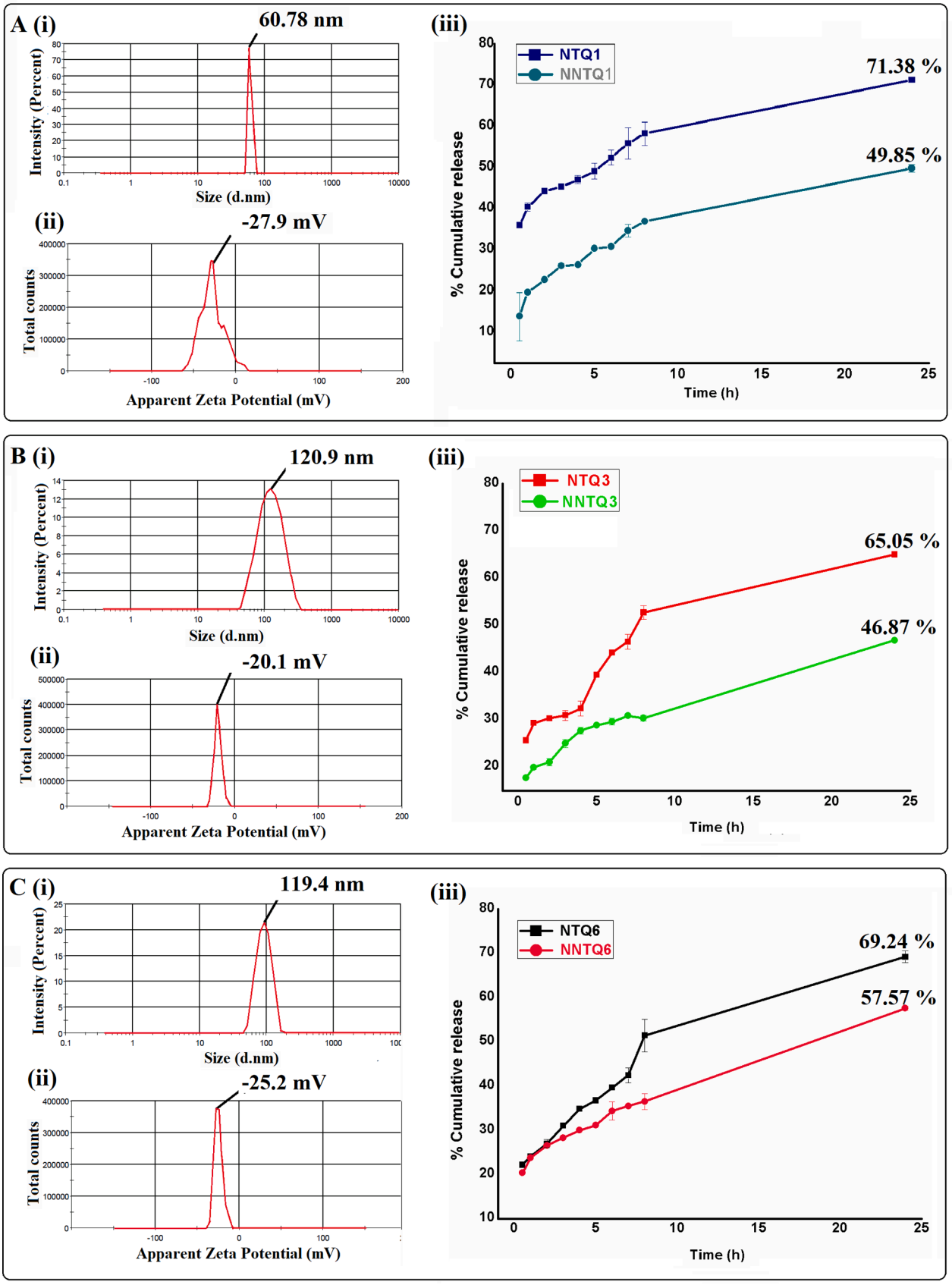

Fig. 1. Particle size image (i), zeta potential (ii), and *in vitro* drug release of image of NNTQ1 (A), NNTQ3 (B), and NNTQ6 (C), respectively.

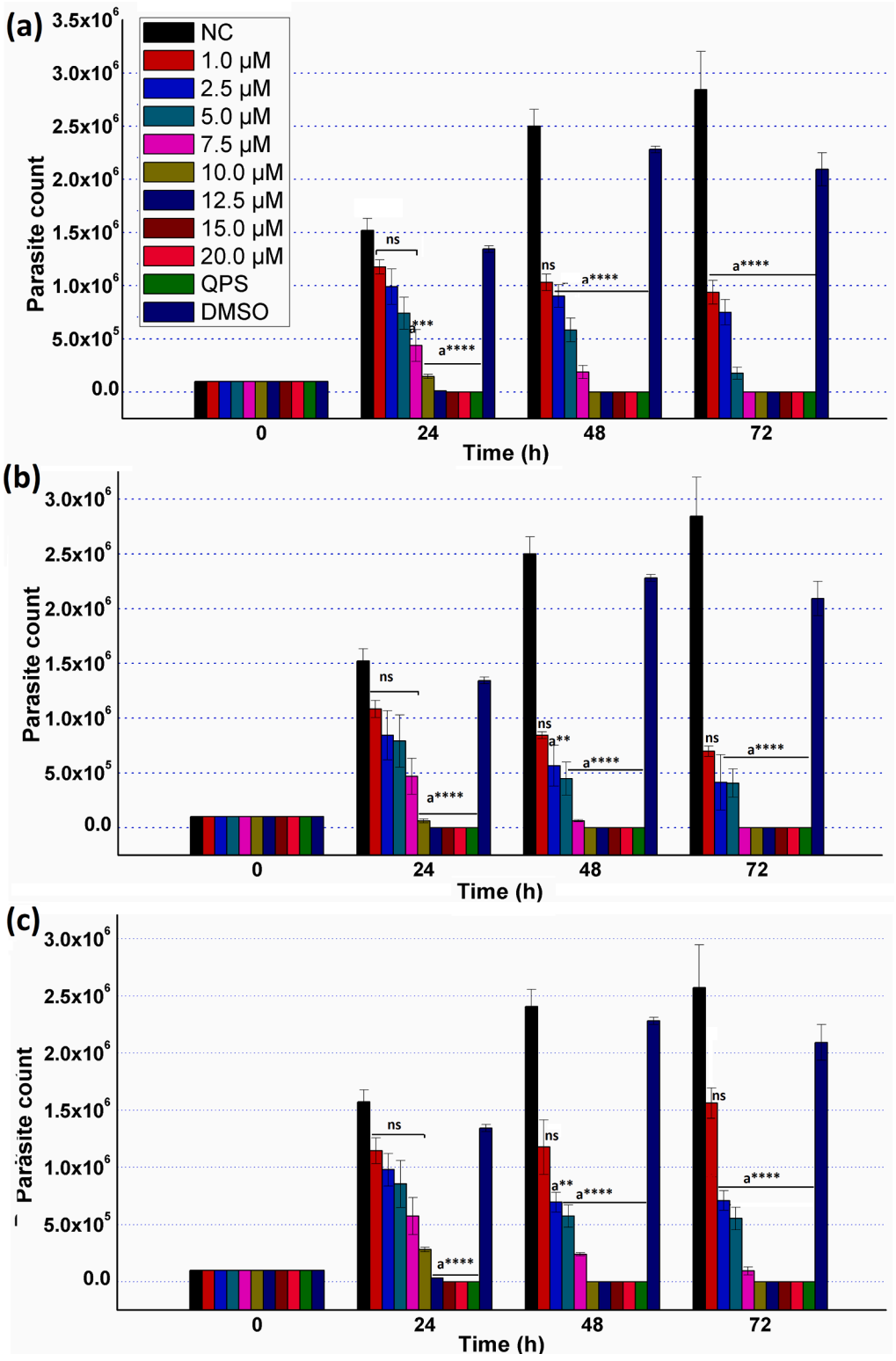

Fig. 2. *In vitro* growth-inhibitory efficacy of NNTQ1 (a), NNTQ3 (b) and NNTQ6 (c) at different concentrations against *Trypanosome evansi*. N.C represents Negative control. Each value in the graphs represents the parasite count (mean ± SD; $n = 3$) observed at different concentrations of the respective nano-formulation. Data were analysed by two-way ANOVA followed by Tukey Post-hoc test (Graph pad prism version 8.4.2.679 software) where, 'a' indicates significant difference as compared to negative control. **** - $p<0.0001$, *** - $p<0.001$, ** - $p<0.01$, * - $p< 0.05$.

inhibition of parasite at 24 h as compared to negative control parasite.

The IC_{50} and log IC_{50} of NNTQ-1, NNTQ-3 and NNTQ-6 -loaded NCs was calculated using Graph Pad prism software. IC_{50} of NNTQ-1, NNTQ-3 and NNTQ-6 was found 3.660 ± 0.640 μM, 3.026 ± 0.668 μM and 3.714 ± 0.786 μM respectively and log IC_{50} was 0.5635, 0.4804, and 0.5699 respectively (Fig. 3). Table 2 depicts the comparison in IC_{50} of selected napthoquinones and their derivatives.

3.3. Microscopic evaluation of drug-treated parasites

Microscopic examination of negative control parasites showed living and dividing parasite with long flagellum and dark stained kinetoplast and nucleus, whereas drug-treated parasites showed dose-dependent and time-dependent degeneration in morphology at 24, 48, and 72 h. Degenerative changes include detached flagella from parabasal body of the parasite, broken flagellum; departed parasite; and debris of parasites

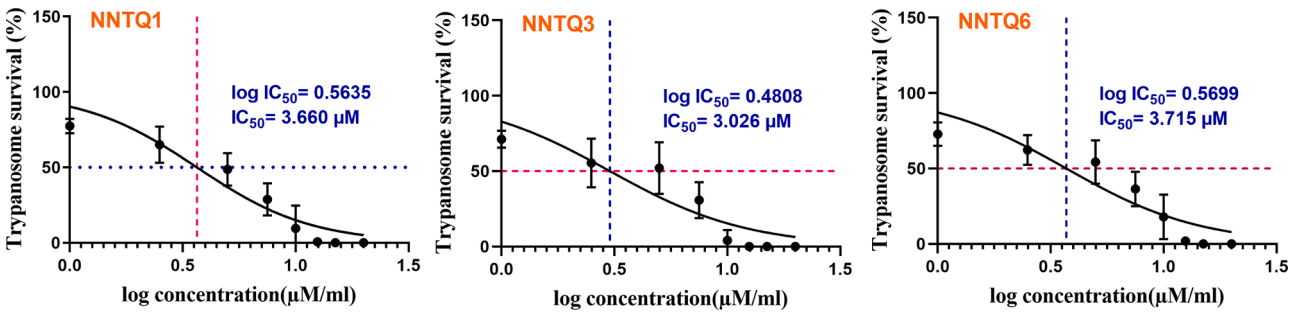

Fig. 3. Growth inhibition curves depict log IC_{50} value and IC_{50} of the NNTQ1 (a), NNTQ3 (b) and NNTQ6 (c) against *Trypanosome evansi*.

Table 2
Comparison of antiparasitic activity of naphthoquinone derivatives and their nanoformulation against *Trypanosoma evansi* at 24 h.

S. No.	Selected Naphthoquinones	IC_{50}/24 h (µM) of native*	IC_{50}/24 h (µM) of nanoformulation
1	1,4-naphthoquinone (NTQ1)	11.48 ± 0.85	3.660 ± 0.640
2	5-hydroxy-1,4-naphthoquinone (NTQ3)	12.97 ± 0.53	3.026 ± 0.668
3	5-hydroxy-2-methyl- 1,4-naphthoquinone (NTQ6)	5.758 ± 0.78	3.714 ± 0.786

*Reference Rani et al., 2021.

were observed during examination. Fig. 4 shows morphological effects due to treatment of NNTQ1 on parasites. Positive control (H_2O_2)-treated parasites also showed similar morphological features as compared to negative control parasites.

3.4. In vitro cell cytotoxicity analysis

Fig. 5 displays the comparative evaluation of percent *in vitro* cytotoxicity by different concentrations (1x, 5x, 10x, and 20x) of NTQ1 *Vs* NNTQ1 (a); NTQ3 *Vs* NNTQ3 (b); NTQ6 *Vs* NNTQ6 (c) on fresh horse PBMCs. All the different tested concentrations showed a dose-dependent cytotoxic effect as compared to negative untreated wells. It was observed that NNTQ1 and NNTQ3 exhibited less cytotoxicity at their 1x, 5x, 10x and 20x doses as compared to their respective NTQ1 and NTQ3 drugs. Similarly, 1x dose of NNTQ6 exhibited less cytotoxic, while 5x, 10x and 20x doses showed comparatively equal cytotoxic effect as compared to pure NTQ6 drug. Additionally, a comparison between nanoformulations and QPS, the NNTQ1 and NNTQ3 showed less cytotoxicity (less than 25%) at 1x and 5x doses as like QPS. The DMSO also showed a dose-dependent cytotoxicity i. e. 2.1%, 4.8%, 24.0% and 44.1% at its 1x, 5x, 10x and 20x dose, showing safe nature as a solvent control.

3.5. Intracellular ROS measurement

In the present investigation, prepared nanoformulations of selected NTQs were comparatively evaluated with respective NTQs to produce ROS in the parasite at 0, 2, 4, 6, and 24 h. Two drug concentrations, IC_{50} and IC_{100} of NNTQs was used for evaluation like NTQs deployed earlier (Rani et al., 2021). Fig. 6(a) shows the percent ROS production due to NTQ1 and NNTQ1 at two concentrations. All the treated well showed dose-dependent and time-dependent increase in percent ROS production as compared to untreated control. IC_{50} value and IC_{100} value of NTQ1 displayed percent ROS level ranges 26.45 to 58.68% and 34.99 to 70.49%, respectively at 0 h to 24 h (Rani et al.,

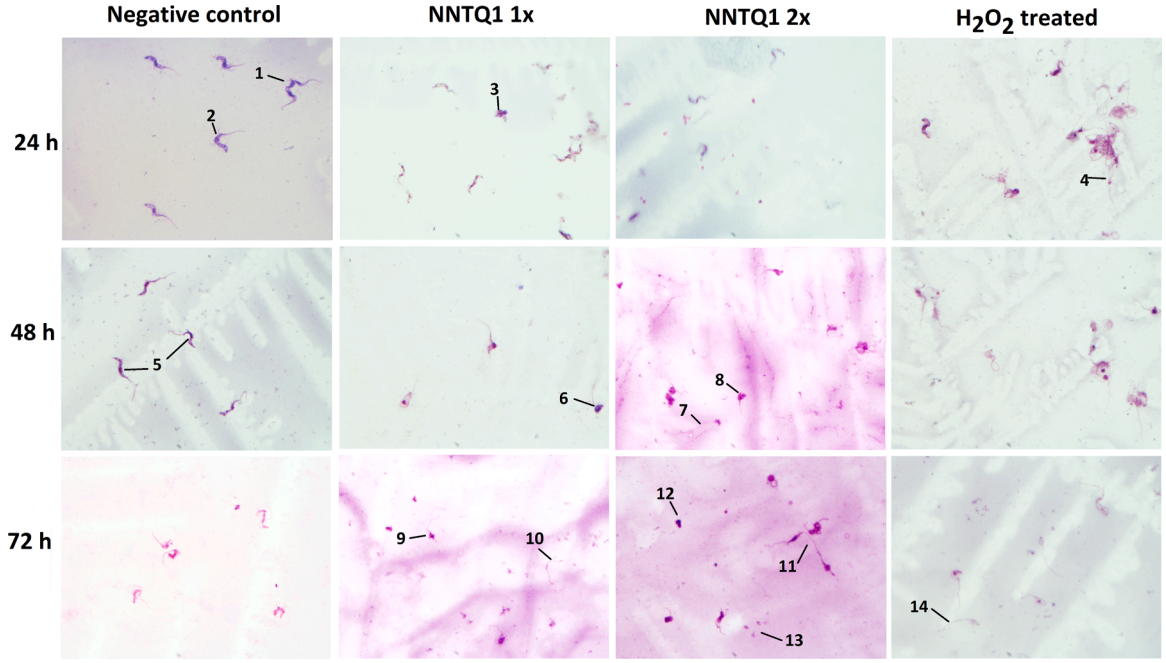

Fig. 4. Microscopic analyses of 1x and 2x NNTQ1-treated *Trypanosoma evansi* after 24, 48, and 72 h. Figure depict dividing parasites with long flagella (1); live parasite (2,5); detached flagella (4, 10); broken flagella (7); departed parasite (3,6,8,9,11,14); debris of dead parasites (12, 13) (Giemesa stain, 100x).

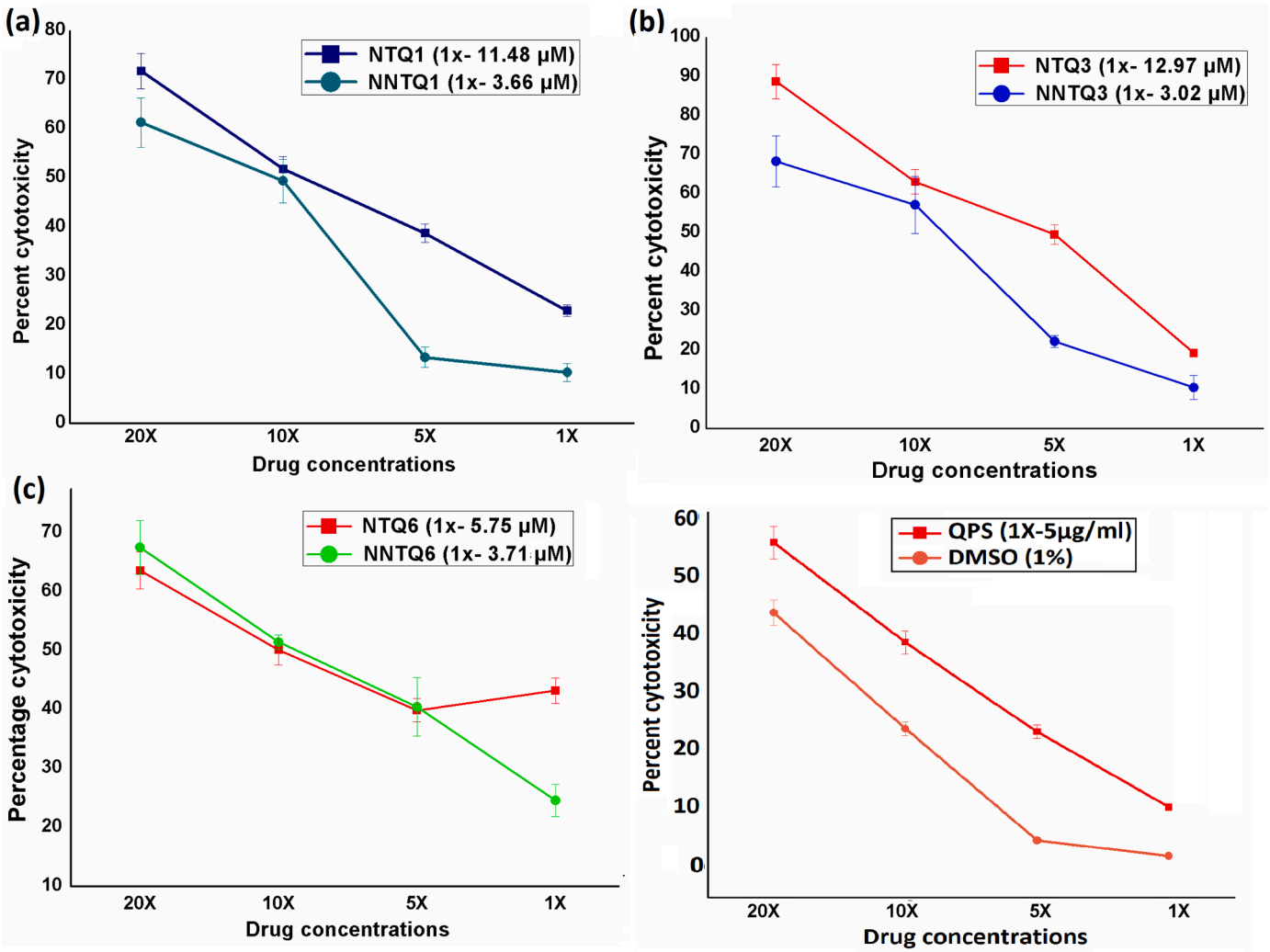

Fig. 5. Comparative evaluation of *in-vitro* cytotoxicity analysis of different concentration of NTQs and NNTQs on horse PBMCs.

2021). When, the ROS production level was evaluated at IC_{50} dose of NNTQ1, it was found that the ROS was increased from 19.63% (at 0 h) to 69.03% (at 24 h), while the IC100 dose showed more ROS from 40.93% (at 0 h) to 92.50% (at 24 h).

Fig. 6(b) displays the percent ROS production due to NTQ3 and NNTQ3 at IC_{50} and IC_{100} concentrations. The NTQ3 at its IC_{50} dose displayed 12.61 to 61.88% ROS generation and IC_{100} dose displayed 17.26 to 71.81% ROS generation as compared to negative control at 0 h to 24 h (Rani et al., 2021). However, its NNTQ3 displayed 17.48 to 71.73% ROS generation at IC_{50} dose and 32.24 to 91.31% ROS generation at IC_{100} dose as compared to negative control at 0 h to 24 h.

Fig. 6(c) displays the percent ROS production due to NTQ6 and NNTQ6 at IC_{50} and IC_{100} concentrations. The NTQ6 at its IC_{50} dose exhibited 11.04% to 66.07% ROS generation, while its NNTQ6 exhibited 17.15% to 71.36% ROS generation as compared to negative control at 0 h and 24 h, respectively. Similarly, the NTQ6 at its IC_{100} dose displayed 13.18% to 69.02% ROS generation, while its NNTQ6 displayed 29.04 to 93.04% ROS generation as compared to untreated at 0 h to 24 h, respectively. The reference drug, QPS also showed a time-dependent ROS generation in the range from 5% to 58% as compared to un-treated from 0 h to 24 h. Positive control, H_2O_2 also showed up to 100% ROS from 0 h to 24 h (Fig. 6).

3.6. Measurement of apoptosis

In the present investigation, we performed a flow cytometry analysis of NNTQs-treated parasite to check the cell death mechanism and the comparative evaluation of the effect shown by the NNTQs with respective pure NTQ. Different phenotypic responses - AV^-PI^- (live cells; LC) and AV^+PI^- (early apoptotic cells, EAC) were observed from PI^- (-ve) parent cells and other two- AV^-PI^+ (late apoptotic cells; LAC) and AV^+PI^+ (necrotic cells; NC) were observed from PI^+ (+ve) parent cells. Table 3 portrayed the four phenotypes of two parent populations observed at 1x and 2x NNTQs-treated parasites labelled with annexin V and propidium iodide. The experimental data of flow cytometry data, revealed consecutive increased cell death in parasite treated with NNTQs. At lower concentration (1x) of NNTQs, the percentage of non-apoptotic/live cells (AV^-PI^-) was greater than the parasites treated with 2x-NNTQs at 24, 48, and 72 h in comparison to negative control cell (Table 3).

The percentage of AV^-PI^- decreased while the percentage of AV^+PI^- increased with increase in incubation time in case of NNTQs-treated parasites. Moreover, the treated parasites showed decreased percent LC and increased percent EAC, LAC, and NC in case of QPS-treated parasites, but when the responses were compared with NNTQs-treated, they were less pronounced in comparison to NNTQs. At twice the concentration of IC_{50} values of NNTQs, an increase of AV^+PI^-, AV^-PI^+, and AV^+PI^+phenotypes were observed and the effect was more noticeable at 72 h resulting in intense cell death. Fig. 7 depicts the

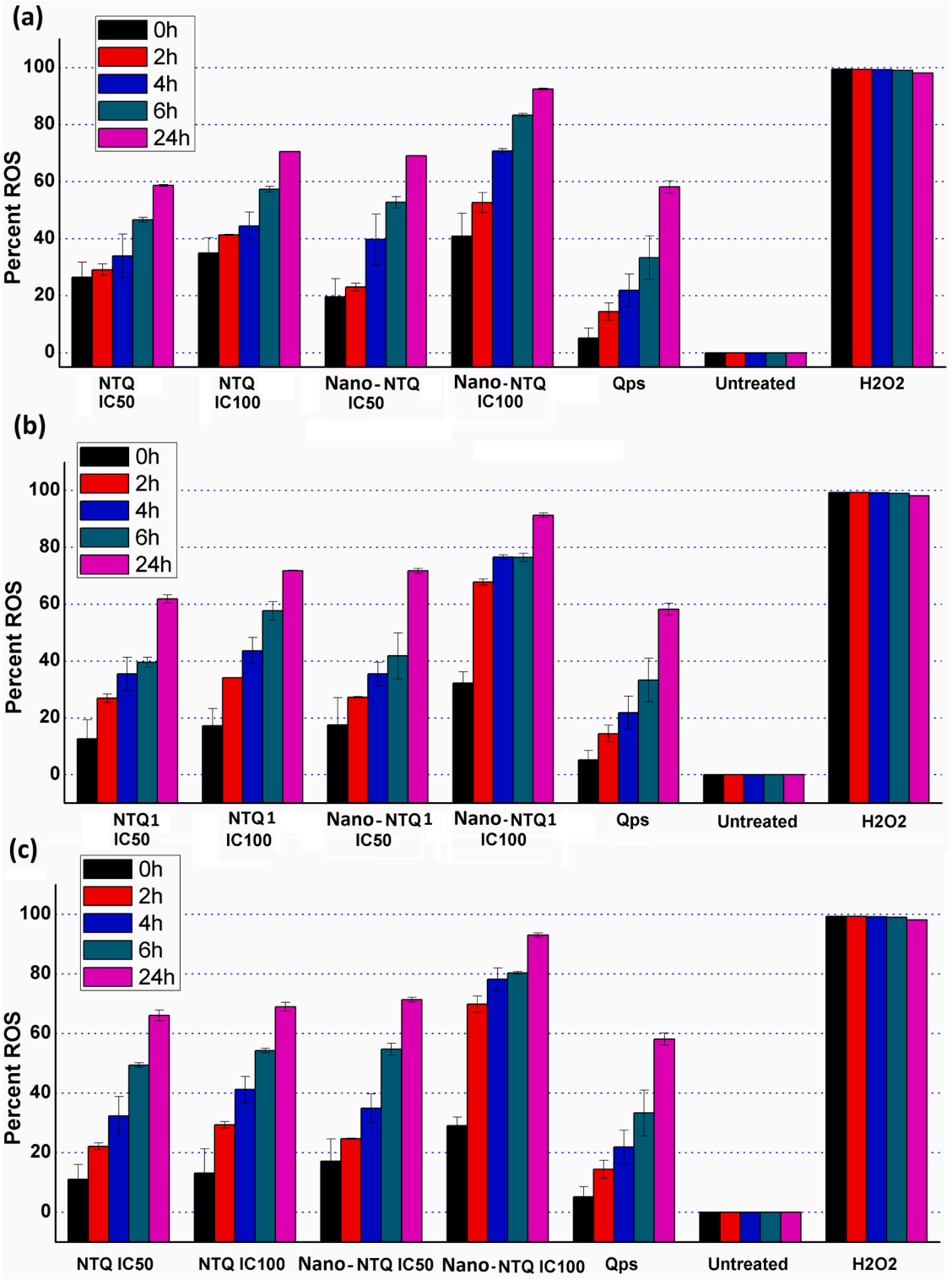

Fig. 6. Comparative ananlysis of intracellular percent ROS level generated by NTQ1 and NNTQ1 (a), NTQ3 and NNTQ3 (b); and NTQ6 and NNTQ6 (c) at IC_{50} and $1C_{100}$ concentrations incubated with *Tryopanosoma evansi*.

histogram view of different stages of apoptosis of NNTQ1-treated parasites at 24 h, 48 h and 72 h.

4. Discussion

Three naphthoquinone compounds, NTQ1 (1,4-naphthoquinone), NTQ3 (5-hydroxy-1,4-naphthoquinone- juglone) and NTQ6 (2-methyl-5-hydroxy-1,4-naphthoquinone- plumbagin) were selected as they showed good antitrypanosomal activity among six compounds against *T. evansi* from our previous study (Rani et al., 2021). The polymeric nanocapsules of selected naphthoquinones (NNTQ1, NNTQ3, and NNTQ6) were prepared via nanoprecipitation method with the aim to further enhance the efficacy against the parasite. The size and zeta potential of all nanoformulations *i.e.*, NNTQ1, NNTQ3, and NNTQ6 was in

Table 3
Flow cytometric analysis of *T. evansi* treated with 1x and 2x concentrations of NNTQ1, NNTQ3, and NNTQ6 labelled with Annexin V/propidium iodide at 24, 48, and 72 h.

Treatments to*T. evansi*		Time	% Parent cells %PI^- cell AV^-PI^-Live cell (LC)	AV^+PI^-Early apoptotic cells (EAC)	%PI^+ cell AV^-PI^+Late apoptotic cells (LAC)	AV^+PI^+Necrotic cell (NC)
Negative control		**24 h**	97.84 ±0.32		0.93 ± 0.02%	
			93.32 ±0.19	1.16 ± 0.45	81.79 ± 0.18	14.62 ± 2.05
		48 h	94.95 ±0.00		3.66 ± 0.22	
			90.49 ±0.31	4.20 ± 0.06	1.04 ± 0.29	64.32 ± 4.45
		72 h	95.68 ±0.81		0.45 ± 0.18	
			82.62 ±2.49	9.36 ± 2.16	3.98 ± 2.54	94.31 ± 2.54
QPS control		**24 h**	85.78 ± 0.22		8.15 ± 0.41	
			85.20 ±0.41	6.85 ± 0.89	0.07 ± 0.07	42.43 ± 1.62
		48 h	79.67 ± 1.13		15.08 ± 2.23	
			74.08 ±2.23	17.08 ± 2.17	0.31 ± 0.00	78.15 ± 4.04
		72 h	75.41 ±1.57		18.51 ± 0.61	
			54.89 ±1.52	31.89 ± 3.68	1.31 ± 0.01	90.95 ± 1.58
NNTQ1	**1x**	**24 h**	95.86 ±0.76		2.29 ± 0.62	
			85.27 ± 2.66	11.83 ± 2.59	3.64 ± 1.17	94.84 ± 0.54
		48 h	95.23 ±0.78		2.22 ± 0.18	
			79.21 ± 1.45	17.46 ± 1.59	2.24 ± 1.42	95.46 ± 1.80
		72 h	87.34 ±2.19		7.14 ± 1.62	
			48.92± 10.35	46.73 ± 10.22	0.03 ± 0.03	99.94 ± 0.06
	2x	**24 h**	87.92 ±1.47		8.91 ± 1.32	
			50.05 ± 5.04	44.87 ±4.56	0.20 ± 0.00	98.99 ± 0.40
		48 h	74.91 ±1.11		13.12 ± 1.03	
			25.27 ±5.24	70.94 ±5.43	0.00 ± 0.00	100.00 ± 0.00
		72 h	74.14 ±0.12		21.83 ± 0.07	
			40.45 ±0.20	59.07 ±0.65	2.62 ± 1.31	93.19 ± 1.55
NNTQ3	**1x**	**24 h**	96.53 ±0.24		1.58 ± 0.16	
			93.94 ±0.42	2.77 ± 0.41	10.39 ± 0.00	71.19 ± 0.86
		48 h	95.51 ±0.13		2.52 ± 0.26	
			91.88 ±0.47	5.51 ± 0.38	7.75 ± 1.69	81.28 ±2.40
		72 h	92.24 ±1.43		5.53 ± 1.19	
			62.40 ±1.21	31.84 ±0.62	0.33 ± 0.17	99.16 ± 0.35
	2x	**24 h**	95.27 ±0.42		2.42 ± 0.24	
			92.32 ±0.33	3.87 ± 0.25	3.95 ± 1.06	76.46 ± 0.03
		48 h	74.20 ±0.13		20.72 ± 0.33	
			33.79 ±0.61	59.18 ±1.16	0.40 ± 0.05	88.79 ± 0.65
		72 h	72.33 ±0.75		22.34 ± 0.31	
			36.35 ±3.36	55.09 ±0.56	0.14 ± 0.05	87.56 ± 4.50
NNTQ6	**1x**	**24 h**	97.06 ±0.51		0.91 ± 0.28	
			88.41 ±1.86	8.00 ± 1.87	7.27 ± 2.49	92.73 ± 2.49
		48 h	96.53 ±0.12		2.09 ± 0.08	
			61.46 ± 3.42	34.89 ± 3.45	0.52 ± 0.52	99.52 ± 0.36
		72 h	47.67 ±7.80		24.24 ± 3.89	
			21.61 ±1.21	70.30 ± 4.82	0.01 ± 0.01	99.48 ± 0.52
	2x	**24 h**	89.01 ±0.64		7.87 ± 0.62	
			52.71 ± 1.46	41.79 ± 1.41	0.31 ± 0.19	99.03 ± 0.26
		48 h	71.81 ±7.78		22.54 ± 6.91	
			33.36 ± 2.56	63.96 ±2.80	0.77 ± 0.26	99.74 ± 0.26
		72 h	35.71 ±7.36		0.04 ± 0.04	
			14.51 ±1.46	82.37 ±5.60	0.27 ± 0.04	95.80 ± 0.44

nanoscale and stability range. Moreover, zeta potential values with negative zeta potential showed higher cellular uptake as compared to the nanoformulation having positive zeta potential (Patil et al., 2007). The preparation of nanoparticles is based on the different in surface tension of two liquids. The aqueous phase has high surface tension that pull the surrounding liquid more powerfully as compared to the organic phase that has low surface tension. This difference in the surface tension of two liquids led to interfacial turbulence and thermal inequalities that cause a continuous whirl of the solvent at the liquid interfaces. This further resulted in mutual miscibility between the solvents and formation of nanoparticles (Mora-Huertas et al., 2010).

This is the first study to investigate the therapeutic potential of nanoformulated naphthoquinone against *T. evansi*. The efficacy of NNTQs was tested against trypanosomes and IC_{50} was calculated. The IC_{50} value of NTQ1, NTQ3, and NTQ6 was 11.48 μM, 12.97 μM, and 5.758 μM respectively (Rani et al., 2021), while the NNTQ1, NNTQ3, and NNTQ6 was found 3.660 μM, 3.026 μM, and 3.715 μM, respectively. The IC_{50} of nanoformulations was ~ 3.1 times, 4.2 times and 1.5 times less when compared with respective NTQ derivative *i.e.*, NTQ1, NTQ3 and NTQ6. Only drug molecules are responsible for diminishing the

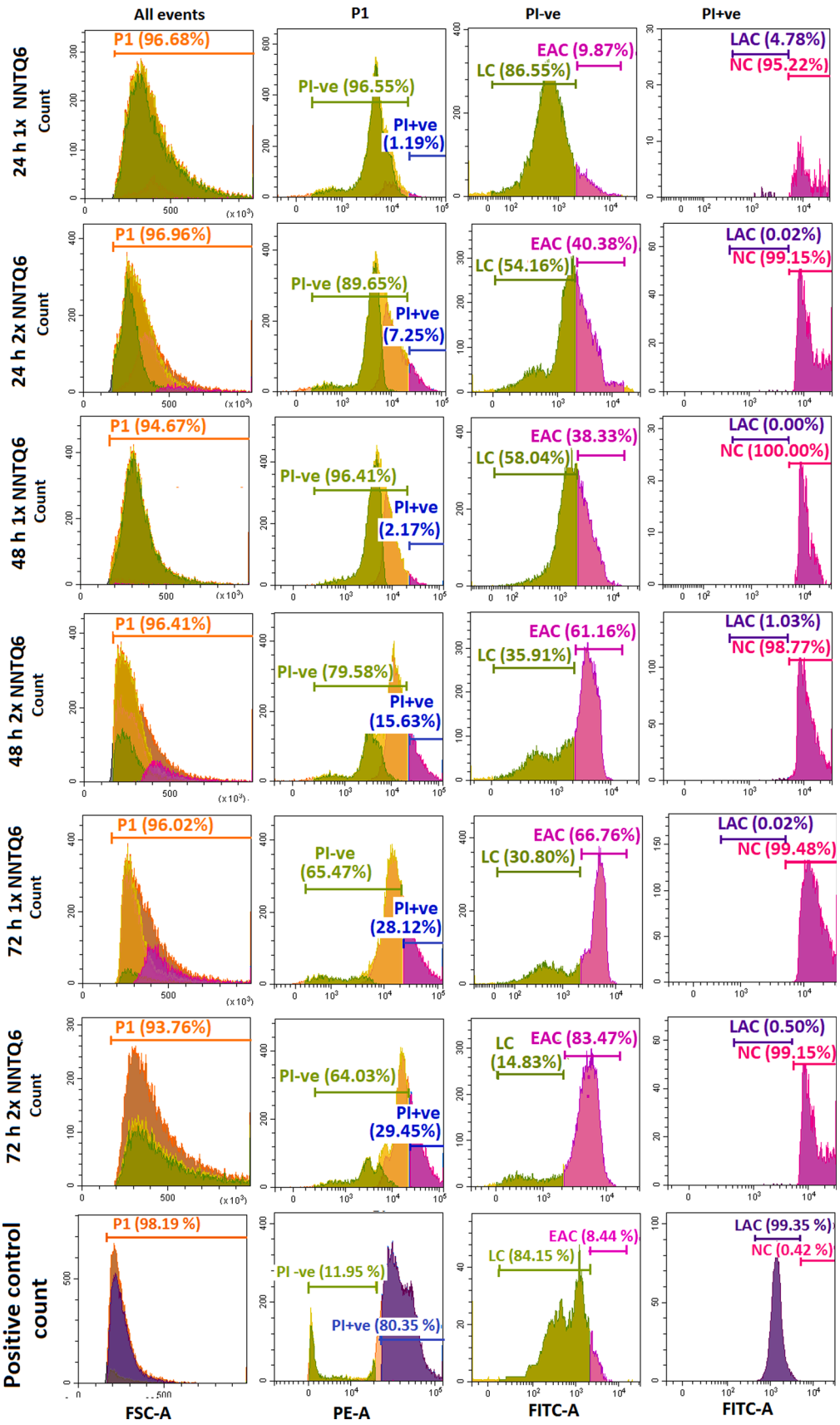

Fig. 7. Flow cytometry apoptosis analysis of NNTQ1-treated *Trypanosoma evansi* using Annexin V/propidium iodide staining. Flow cytometric histogram of 1x and 2x-NNTQ1-treated cells at 24 h, 48 h, and 72 h showing (i) all events; (ii) P1 cell population having PI -ve cells and PI +ve cells; (iii) PI -ve cells including live cells (LC, AV^{-} PI^{-}) and early apoptotic cells (EAC, $AV^{+}PI^{-}$); and (iv) PI + ve cells including late apoptotic cells (LAC, AV^{-} PI^{+}) and necrotic cells (NC, $AV^{+}PI^{+}$) with respect to% parent population. x represents the IC_{50} value of the nanoformulation.

growth of parasite, solvent have no role in the antitrypanosomal activity. The increased antitrypanosomal efficacy enhancement in the case of nanoformulation might be owing to their nanosize range, more cellular intake, and more availability at the site of action (Baldissera et al., 2016; Kimani et al., 2019)

Furthermore, varying concentrations of NNTQs were found safe in cytotoxicity terms on horse PBMCs as compared to their respective pure derivatives. The NNTQ1 and NNTQ3 showed less cytotoxicity, while NNTQ6 showed more cytotoxicity even at lower IC_{50} dose. From the experimental evaluation, it was concluded that all the drugs are cytotoxic at some extent, but nanoformulations helped to reduce the effective doses and hence the cytotoxic effect relative to pure drugs.

The selected naphthoquinone showed dose-dependent (IC_{50} and IC_{100}) and time-dependent generation of ROS in NTQs-treated *T. evansi* as demonstrated by DCF-DA assay (Rani et al., 2021). Herein, a comparative evaluation of ROS production between the IC_{50} dose of pure NTQ and its nanoformulation, the level of ROS production was nearly same at all the different time interval by both. Nevertheless, the IC_{100} dose of NNTQs produced more percent ROS when compared with IC_{100} dose of pure NTQ. The NNTQs produced approximately equal or more ROS percent in comparison to their respective pure NTQs even at lower concentrations. The IC_{50} of nanoformulations was less ~1.5–4.0 times, still produced equal or more ROS than their respective NTQs. Moreover, NNTQs could produce more level of ROS as compared to QPS, a commercial drug for animal trypanosomosis, thus representing the enhanced efficacy of nanoformulation against *T. evansi.* It has been established that generation of ROS contribute to anti-parasitic action of the naphthoquinone (Pieretti et al., 2013; Salomao et al., 2013; Rani et al., 2021) and more ROS generation by nanoparticles as supported by the literature (Saini et al., 2016; Adeyemi et al., 2017).

In this study, the effect of one-time and twice the IC_{50} value of NNTQs on *T. evansi* was measured by incubating drug-treated parasite with annexin V-FITC using flow cytometry so as to differentiate the parasites based on different phenotypes they acquired and to delineate the mechanism of action for nano-naphthoquinones. Our result show that the incubation of parasites with NNTQs at concentrations corresponding at one-time and twice the IC_{50} values exhibited transition of live cells (AV^-PI^-) to early apoptotic cells (AV^+PI^-), late apoptotic cells (AV^-PI^+), and necrotic cell (AV^+PI^+). A dose-dependent and time-dependent effect was observed for 2x concentration of NNTQs as compared to 1x concentration of respective NNTQs and apoptotic effect was increased with respect to incubation time respectively. All the three NNTQs showed the pronounced effect with varying degree of consequences noticed in the following sequence - NNTQ6 > NNTQ3 > NNTQ1. The enhancement in trypanocidal activity might be due to more cellular uptake of nanoformulations that further increased the percentages of EAC, LAC, and NC in NNTQs-treated parasites in comparison to NTQs-treated parasites. In conjunction with our previous experimental observations, naphthoquinone increases the apoptotic-like effect, while the present investigation reveals that the nanoformulation enhanced the apoptotic-like effect that boost up the antitrypanosomal activity against *T. evansi* more than naphthoquinones even at ~1.5–4.0 times lesser doses.

5. Conclusion

This unprecedented report illustrates the use of gum-based nanocarrier used for the encapsulation of naphthoquinones and enhanced potential for their antitrypanosomal potential against *Trypanosoma evansi.* The ensued NNTQs exhibited significant growth inhibition of trypanosomes even at ~1.5–4.0 times less drug concentrations and were safer on horse PBMCs as compared to native drug. Moreover, the NNTQs were found to induce generation of more ROS that contributed inhibitory effect on the growth of *T. evansi.* Our findings suggest that NNTQ-treatment induces *T. evansi* cell death by enhanced apoptotic and necrotic effects in time-and dose-dependent manner. Therefore, it was concluded that NNTQs showed significant therapeutic potential against the parasite and could be exploited as a future therapeutic alternative for the commercial drugs after some more studies including *in vivo* experiments.

CRediT authorship contribution statement

Ruma Rani: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Balasubramanian Narsiman:** Resources, Writing – review & editing, Visualization, Supervision. **Rajender S. Varma:** Conceptualization, Writing – review & editing, Visualization, Supervision. **Rajender Kumar:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

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