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Chitosan nanoparticle toxicity: A comprehensive literature review of *in vivo* and *in vitro* assessments for medical applications

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ABSTRACT

Topic definition: This literature review aims to update the current knowledge on toxicity of chitosan nanoparticles, compare the recent findings and identify the gaps with knowledge that is present for the chitosan nanoparticles.

Methods: The publications between 2010 and 2020 were searched in Science Direct, Pubmed.gov, Google Scholar, Research Gate, and ClinicalTrials.gov, according to the inclusion and exclusion criteria. 30 primary research studies were obtained from the literature review to compare the *in vitro in vivo* toxicity profiles among the chitosan nanoparticles.

Major highlights: Chitosan nanoparticles and other types of nanoparticles show cytotoxic effects on cancer cells while having minimal toxicity on normal cells. This apparent effect poses some considerations for use in incorporating cancer therapeutics into chitosan nanoparticles as an administration form. The concentration, duration of exposure, and pH of the solution can influence nanoparticle cytotoxicity, particularly in zebrafish. Different cell lines exhibit varying degrees of toxicity when exposed to nanoparticles, and of note are liver cells that show toxicity under exposure as indicated by increased alanine transaminase (ALT) levels. Aside from ALT, platelet aggregation can be considered a toxicity induced by chitosan nanoparticles. In addition, zebrafish cells experience the most toxicity, including organ damage, neurobehavioral impairment, and developmental abnormalities, when exposed to nanoparticles. However, nanoparticles may exhibit different toxicity profiles in different organisms, with brain toxicity and liver toxicity being present in zebrafish but not rats. Different organs exhibit varying degrees of toxicity, with the eye and mouth apparently having the lowest toxicity, while the brain, intestine, muscles and lung showing mixed results. Cardiotoxicity induced by chitosan nanoparticles was not observed in zebrafish embryos, and nanoparticles may reduce cardiotoxicity when delivering drug. Toxicity found in an organ may not necessarily mean that it is toxic towards all the cells found in that organ, as muscle toxicity was present when tested in zebrafish but not in C2C12 myoblast cells. Some of the studies conducted may have limitations that need to be reconsidered to account for differing results, with some examples being two experiments done on HeLa cells where one study concluded chitosan nanoparticles were toxic to the cells while the other seems to have no toxicity present. With regards to LD_{50}, one study has stated the concentration of 64.21 mg/ml was found. Finally, smaller nanoparticles generally exhibit higher toxicity in cells compared to larger nanoparticles.

Scope for future work: This literature review did not uncover any published clinical trials with available results. Subsequent research endeavors should prioritize conducting clinical trials involving human volunteers to directly assess toxicity, rather than relying on cell or animal models.

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1. Introduction

1.1. Chitosan

Chitosan is a linear structural polysaccharide, deacetylated form of chitin, containing fewer acetyl groups [1,2]. The molecular structure of chitosan consists of *N*-acetylglucosamine units (2-acetamino-2-deoxy-β-D-glucopyranose) attached to β-(1,4)-linked glucosamine units (2-amino-2-deoxy-β-D-glucopyranose) [3]. The semi-crystalline chitosan requires at least 60% of residues to contain D-glucosamine [4]. It is derived from chitin, which is found in various sources such as crustaceans (e.g., crabs and shrimp), fungi cell walls, insect cuticles, and green algae [5,3,6].

Chitosan offers several benefits, including its ability to adhere to mucosal surfaces, prolong drug retention at specific body sites, and facilitate enhanced drug penetration [7,8]. Compared to its parent compound chitin, chitosan exhibits water solubility at low pH levels due to its positive charge, allowing it to dissolve in acidic due to amino group protonation enabling electrostatic interactions with negatively charged components, such as sialic acid present in mucus and surface epithelial cells [9,10] or neutral solutions [11].

Chitosan finds applications in various fields, including cosmetics, pharmaceutics, medicine, food, and agriculture [12]. For example, chitosan is used in hair and skin products, as well as for oral health purposes, including delivering herbal extracts, dental varnishes, and buccal tablets [13]. Mohire, Yadav [14-16]. In medicine, chitosan has been incorporated into wound dressings, such as HemCon, approved in the United States in 2003 for controlling hemorrhaging [17]. It has also shown efficacy in reducing bleeding duration and improving healing after tooth extractions [18]. Furthermore, chitosan contributes to food preservation and safety by extending the shelf life of products and exhibiting antimicrobial properties. Studies have demonstrated the antimicrobial effect of chitosan, including its ability to inactivate *Staphylococcus aureus* at low concentrations [19]. Chitosan has also exhibited antifungal properties, inhibiting the growth of *Fusarium solani, C. lagenarium,* and *B. cinerea* in various studies [20-22].

While some studies suggest that chitosan is non-toxic and suitable for drug delivery [23,24], with one study even stating that an FDA Generally Recognized As Safe (GRAS) designation (GRN n° 73, 170, 397 and 443) was given to the chitosan material [25], other studies have reported toxic effects in cell lines and zebrafish exposed to chitosan nanoparticles, resulting in decreased hatching rates, increased mortality, and developmental defects [26,27]. However, some studies have shown toxicities exhibit by cell lines and also in zebrafish, such as the studies presented in Table. Many of these studies showed a decrease in hatching rate and increase in mortality and also defects in development were seen when chitosan nanoparticles were exposed. It is important to consider these findings and the potential toxicities associated with chitosan nanoparticles.

1.2. Nanoparticles

The definition of nanoparticles (NPs) typically refers to particles within the size range of 1–100 nm, as defined by the ASTM 2456–06 Standard Terminology Relating to Nanotechnology and the IUPAC [28, 29] However, some studies have expanded this size range, up to 2000 nm, exceeding the upper limit by 20-fold [30,31].

When nanoparticles are compared against their bulk material counterparts, unique properties that distinguish them from each other. These differences encompass changes in melting point [32], reactivity, and magnetism [33]. These variations are attributed to size-dependent quantum effects and scalable effects, with the former observed in metals and semiconductors [34,35]. Moreover, the reduced size of nanoparticles results in a lower occurrence of point defects compared to bulk materials [36]. However, nanoparticles can still exhibit point defects, as observed using high-resolution electron microscopy [37]. The unique properties of nanoparticles arise from the specific formation of point defects within them. For instance, adhesion, elastic modulus, friction, hardness, stress, and strain exhibit differences when comparing nanoparticles to bulk materials [38].

Furthermore, the stability, self-assembly behavior [39], optical properties [40], and magnetic properties [41] of nanoparticles make them valuable in various fields, particularly in energy technology, information storage technology [42], environment protection [40], and biomedical areas such as imaging [43].

1.3. Chitosan nanoparticles

The usage of chitosan nanoparticles is comparable to that of bulk chitosan in various applications such as food, biotechnology, agriculture, medicine, cosmetics, and drug delivery [44]. The potential of chitosan nanoparticles to enhance active pharmaceutical ingredients or nutraceutical has been demonstrated in several studies listed in Table 1. This aligns with the notion that chitosan nanoparticles can disrupt tight junctions between epithelial cells, thereby increasing drug permeability [45]. Many of the applications listed in Table 1 involve chitosan nanoparticles serving as carriers for active pharmaceutical ingredients, aiming to reduce medication dosage and frequency to minimize side effects or enhance drug absorption. However, some studies explore the inherent properties of chitosan nanoparticles themselves, such as their ability to inhibit biofilm formation on denture materials [46], and their role as a reactive oxygen species modulator in wound healing [47].

Among the various applications of chitosan nanoparticles, some of them prove to be noteworthy for their potential use in the field of medicine, particularly as innovative administration methods compared to established approaches.

A notable example is the utilization of chitosan to enhance the delivery of rifampicin, a drug used in tuberculosis treatment [67]. Rifampicin faces challenges related to its low bioavailability due to enzyme induction and subsequent increased clearance [68]. Incorporating chitosan nanoparticles shows promise in altering the administration route, minimizing the first-pass effect. Although studies have explored pulmonary administration of rifampicin via chitosan nanoparticles, ensuring proper patient technique during inhalation therapy remains a concern lungs [69,70,58,48].

Continuing with the theme of preventing metabolism, insulin administration via the oral route is another venture of using chitosan nanoparticles. Chitosan nanoparticles also hold potential for oral delivery of insulin. Oral administration of insulin is hindered by proteolytic degradation by gastrointestinal enzymes and poor penetration through intestinal walls resulting in poor bioavailability. However, studies indicate that chitosan nanoparticles could mitigate enzyme effects, providing a needle-free method of insulin administration [71]. However, the stability and shelf life of insulin need to be considered as temperature fluctuations can affect its effectiveness.

The study performed by Sudakhar et al. (2020) shows results that seem to be able circumvent the effects of the enzymes, providing a potential novel method of administering insulin that removes the need for needles and swabs. However, many of the insulin products on their own are sensitive to temperature fluctuations, and a corollary of this is that the effective shelf life of the insulin itself will be affected. For example, the vial of human soluble insulin under the brand Actrapid® states on their product literature that once a vial is opened for use, it is recommended that the vial of insulin is kept for only 6 weeks if it is stored below 25°C [72]. However, if left out at 25°C, the recommended duration then would shorten to 4 weeks [72]. Considerations of storage needs to be considered should there be further studies of this new administration route.

Nanoparticle technology can also facilitate the delivery of proteins, such as LSC proteins that carry subunits, which offer protection against gut infections [66,73]. Careful investigation of potential side effects of the vaccination is necessary, as seen with the development of COVID-19 vaccines [74].

Table 1
Table of chitosan nanoparticle types, substance loaded and its usage.

Nanoparticle type	Drug/substance loaded loaded	Use	*in vivo/ in vitro/ ex vivo*	Citation
TPP ionic gelation Chitosan nanoparticles	Rifampicin	It acts as a drug carrier for rifampicin deliver to lungs to treat tuberculosis	*In vivo*	Rawal et al., [48]
TPP ionic gelation Chitosan nanoparticles	DNA/siRNA	It acts as a drug carrier for DNA/siRNA to treat inheritable or acquired diseases	*In vivo and in vitro*	Mao et al., [49]
Lecithin/chitosan nanoparticles	Tamoxifen	It increases the absorption of tamoxifen through intestinal jejunum to treat estrogen-dependent breast cancer	*In vtiro*	Barbieri et al.,[50]
O-Carboxymethyl chitosan nanoparticles	Metformin	Used in the delivery of metformin to pancreatic cancer cells for type 2 diabetes and pancreatic cancer	*In vitro*	Snima et al., [51]
N-(2-carboxybenzyl)chitosan (CBCS)	Timolol maleate	It acts as a carrier for timolol delivery in the eyes to treat glaucoma	*In vitro*	Siafaka et al., [52]
N-(succinyl)-grafted chitosan (CSUC)	Timolol maleate	It acts as a carrier for timolol delivery in the eyes to treat glaucoma	*In vitro*	Siafaka et al., [52]
Chitosan nanoparticles made using double emulsification solvent evaporation using PLGA, acetone and PVA	salmon calcitonin and puerarin	It acts as a carrier for protein and peptide for oral delivery after enhancing bioavailability	*In vitro*	L. Liu et al., [53]
Thiolated chitosan nanoparticles made with chitosan, pentaerythritol tetrakis (3-mercaptopropionate) (PETMP) and acetic acid	Insulin	It allows prolonged release and bioavailability for delivery of oral insulin for diabetes type 2	*In vivo*	Sudhakar et al.,[54]
Chitosan nanoparticles	Interferon-alpha	It is used oral administration of interferon-alpha to treat for cancer and viral infections	*In vivo and in vitro*	Cánepa et al., [55]
Chitosan ionic gelation TPP nanoparticle	Prothionamide	It allows higher drug loading, sustained release, better stability and targeted drug depositing of prothionamide to treat tuberculosis	*In vivo and in vitro*	Debnath et al.,[56]
Chitosan and Fucoidan nanoparticles at different weight ratios (3/1, 4/1 and 5/1 chitosan to fucoidan ratio) prepared by ultrasonication	Gentamicin	It allows gentamicin delivery vial the intratracheal route to achieve higher concentration-time curve AUC and lower toxicity to treat pneumonia	in vitro	Huang et al., [57]
Octanoyl chitosan nanoparticles using double emulsion solvent evaporation	Rifampicin	It has the potential to increase the time of nanoparticles staying in the lungs, thereby enhancing efficiency	*In vitro*	Petkar et al., [58]
Lecithin/chitosan nanoparticles nanoparticle	Amphotericin B	It allowed extended exposure time of the amphotericin B in the eyes for fungal keratitis	*In vitro and in vivo*	Chhonker et al.,[59]
Carboxymethyl chitosan nanoparticles for intranasal administration	carbamazepine	It is used to bypass p-glycoprotein and blood-brain barrier with transporters resistant to multiple drugs so carbamazepine bioavailability is increased in treating epilepsy	*In vitro and in vivo*	[60]
chitosan nanoparticles made with acetic acid and Na_2SO_4	Lithium carbonate	It is used to reduce lithium toxicity by limit the lithium release from the nanoparticles for bipolar disorders	*In vivo*	Narayan[61]
TPP ionic gelation with chitosan nanoparticles	Bedaquiline	It seems to show improved toxicity and lower dosing frequency when compared to conventional dry powder inhalation and oral solution for tuberculosis treatment	*In vivo and vitro*	Rawal et al., [62]
Nanoparticles made with ionic gelation with using sodium TPP and chitosan	Pramipexole dihydrochloride	Parkinson Disease with chitosan nanoparticles showed better antioxidizing activity and treatment group showed better score compared to nasal solution or oral tablets	*In vivo*	Raj et al., [63]
Nanoparticles made with ionic gelation with using sodium TPP and chitosan	Spiramycin	It seemed to show improvements in treating toxoplasmosis caused by *Toxoplasma gondii* (RH and ME49 strain) with the reduction in mortality, and improved pathological status	*In vivo*	Etewa et al., [64]
Nanoparticles made with ionic gelation with using sodium TPP and chitosan	Doxycycline	It allows controlled release of Doxycyline for oral infection caused by bacteria	*In vitro*	Zegan et al., [65]
Low molecular weight chitosan nanoparticles prepared via gelation method with TPP	None	It is used for inhibiting the adherence and development of biofilm of *candida albicans* biofilm on acrylic resin denture base materials	*In vivo*	Gondim et al.,[46]
Chitosan nanoparticles prepared via gelation method with TPP dispersed in hydrogel made using sodium alginate and calcium chloride	None	It is used in expediting wound healing process via the modulation of reactive oxygen species synthesis and consequently promotion of IL-6 secretion in the endothelium	*In vivo*	T. Wang et al.,[47]
Chitosan nanoparticles prepared via gelation method with TPP	LSC protein	It is used in the preparation of vaccinations against Vibrio cholerae, enterotoxigenic *E. coli,* and enterohemorrhagic *E. coli*	*In vivo*	Marandi et al.,[66]

Meanwhile, in the case of fungal eye infections, chitosan nanoparticles show promise in prolonging the contact time of antifungal medication in the precorneal layer. Amphotericin B more generally used for systemic infections [67]. Should amphotericin B be used, it is most often prescribed as an intraocular injection form [75,76]. However, this suggests the potential use of chitosan nanoparticles in eye drop formulations, providing an alternative to intraocular injections. Chhonker et al., [59].

Lastly, there are considerations of use in treating toxoplasmosis with spiramycin [77]. However, there may be some hurdles to the extent of its use as currently it still remains as an unlicensed use in pregnancy, seeing as they are not readily available in some countries, and requires special orders as per the British National Formulary [67]. In addition, there has been some concerns over the increased rate of impurities in one brand of spiramycin reported in Madagascar, where a decision to withdraw the product was made [78]. Future research should then also test if stability is improved with the chitosan nanoparticles and its ability to prevent formation of the impurities. Further research from then on could then assess its appropriateness as a potential treatment of fetal toxoplasmosis using nanoparticles while bearing in mind toxicities that may be imparted by nanoparticles themselves on to the fetus [79].

1.4. Rationale

A recent review performed in 2019 provided an overview of chitosan-based nanoparticles, encompassing their production methods, applications, and toxicity which concluded that chitosan nanoparticles exhibited biodegradability and compatibility with various cell types, suggesting relative safety based on the included studies. Furthermore, it warranted for further research on the safety and toxicity of chitosan

nanoparticles, given the limited current knowledge in this area [44]. Despite the considerable advantages of nanomaterials in the field of biomedicine, additional investigation is essential to comprehensively understand the safety profile of chitosan nanoparticles.

Chitosan nanoparticles are now increasingly prevalent in medicinal research, thus it is important to understand their interaction with organ systems, and the possible implications with different routes of administration. Understanding the safety profile of chitosan nanoparticles is essential for the successful translation into clinical applications and ensuring patient safety. This literature review aims to contribute to the existing knowledge by consolidating and critically evaluating the current research on chitosan nanoparticle toxicity both in *in vivo* and *in vitro* tests. Additionally, the review will provide a comparison of the safety of chitosan nanoparticles in medical applications, in relation to previous literature published before 2019. Moreover, this review will compile a list of cell lines and organisms used in testing. Finally, this review will critically look into some of the implications of the test results of any notable cases found within this review.

2. Method

2.1. Protocol development

Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 guidelines were utilized to design and develop the procedures of this systematic review [80].

2.2. Inclusion and exclusion criteria

The publications between 2010 and 2020 were included in the search. Research articles with *in vitro, in vivo* or *ex vivo*, or any articles that have tested the toxicity of chitosan nanoparticles were included. Non-English articles, gray literature, papers earlier than 2010, studies that do not include the toxicity of the nanoparticles, studies that does not have the chitosan being in the form of nanoparticles, and chitosan nanoparticles used for either diagnostic or non-medical uses were excluded. Additionally, articles that had abstracts only or have unpublished results were also excluded.

2.3. Information sources and search strategy

The literature databases used were Science Direct, Elsevier, Pubmed.gov, Google Scholar, Research Gate, and ClinicalTrials.gov. The listed databases were researched using the keywords for the related articles and as per the inclusion criteria, only papers from 2010 to 2020 were included. Table 2 shows the keywords and the alternative keywords used in performing the search for articles.

The obtained journal articles were screened according to the inclusion criteria. Titles and abstract filters were first searched; later when those were inadequate, the whole paper was screened for the search strategy. After screening the journal articles from the research results as per the inclusion and exclusion criteria, 30 articles were obtained from the literature review. Clinicaltrials.gov was searched for clinical trials however there were no clinical papers published during that period. Fig. 1.

Table 2
Keywords used in search engines.

Keyword	Alternative (s)
Chitosan nanoparticles toxicity	"Chitosan polymer nanoparticles"; "chitosan-based nanoparticles"; "CSNPs" cytotoxicity; hepatotoxicity; ocular toxicity; ocular irritation; inflammation; mortality

3. Results and discussion

Google Scholar returned 10,700 articles with the keywords included for the search. After screening the records duplicate records were removed. The eligible full text articles to be assessed, which adhered to the inclusion and exclusion criteria requirements, were 37. The full text articles were read carefully for assessed eligibility requirements resulting in 30 studies to be included in the qualitative synthesis of this literature review. A list of the articles with pertinent details has been tabulated. Furthermore, articles with the chitosan toxicity studies were critically analyzed to review the parameters influencing the toxicity of chitosan nanoparticles. Consequentially, the parameters size, cell lines, organisms, dose dependence, and organs were found to be the important parameters influencing the toxicity of the chitosan nanoparticles.

3.1. In vitro toxicity of chitosan nanoparticles

Table 3 presents comprehensive information pertaining to the characteristics of chitosan nanoparticles, including their type, size, concentration or dose, mode of administration, testing method, results, and corresponding citations. It is important to note that within each experimental study, diverse parameters were examined across different cell lines, various substances were loaded into the chitosan nanoparticles, and multiple types of chitosan nanoparticles were tested. Furthermore, the column preceding the relevant citation provides insights into the toxicity implications as suggested by the respective research papers. Toxicity grades such as toxic, nontoxic, or undeterminable are assigned, with additional contextual notes provided for experiments where toxicity was observed. In instances where a specific experiment explicitly asserts non-toxicity despite observing cellular effects, the experiment was categorized as "nontoxic" only if the authors have conducted a significance calculation demonstrating the insignificance of the observed toxicity.

3.2. In vivo toxicity of chitosan nanoparticles

Similar to the *in vitro* toxicity, Table 4 displays a comprehensive summary of the chitosan nanoparticles used in the study, encompassing their specific type, size, concentration or dose, mode of administration, testing methodology, resulting outcomes, and relevant citations. In each experimental trial, variations in parameters, cellular models, loaded substances within chitosan nanoparticles, and occasionally multiple types of chitosan nanoparticles were tested. Subsequently, the preceding column prior to the relevant citation will present the toxicity implications as suggested by the respective paper. Lastly, a toxicity classification of toxic, nontoxic, or inconclusive will be assigned, accompanied by supplementary annotations in certain experiments to provide contextual information on the observed toxicity, if any. Nonetheless, if a study has indicated the absence of toxicity of the nanoparticle despite observing cellular effects, it was categorized as "nontoxic". However, this classification was contingent upon the author explicitly stating it, as well as conducting a significance calculation based on the observed toxicity results and demonstrating that the toxicity is statistically insignificant. The organisms used for the experiments included this review are zebrafish, Wistar rats, Sprague Dawley Rats, Mice, albino rabbits, and Swiss male mice.

Lastly, Table 5 shows the explanation for the methods used in table that has presented the results from each study. Each test will have its mechanisms explained briefly to give context to the the method of which each test is being conducted.

3.3. Toxicity of chitosan based on different parameters

3.3.1. Size

The impact of size on cellular toxicity has been observed, although the nature and extent of the effect vary. Several studies have

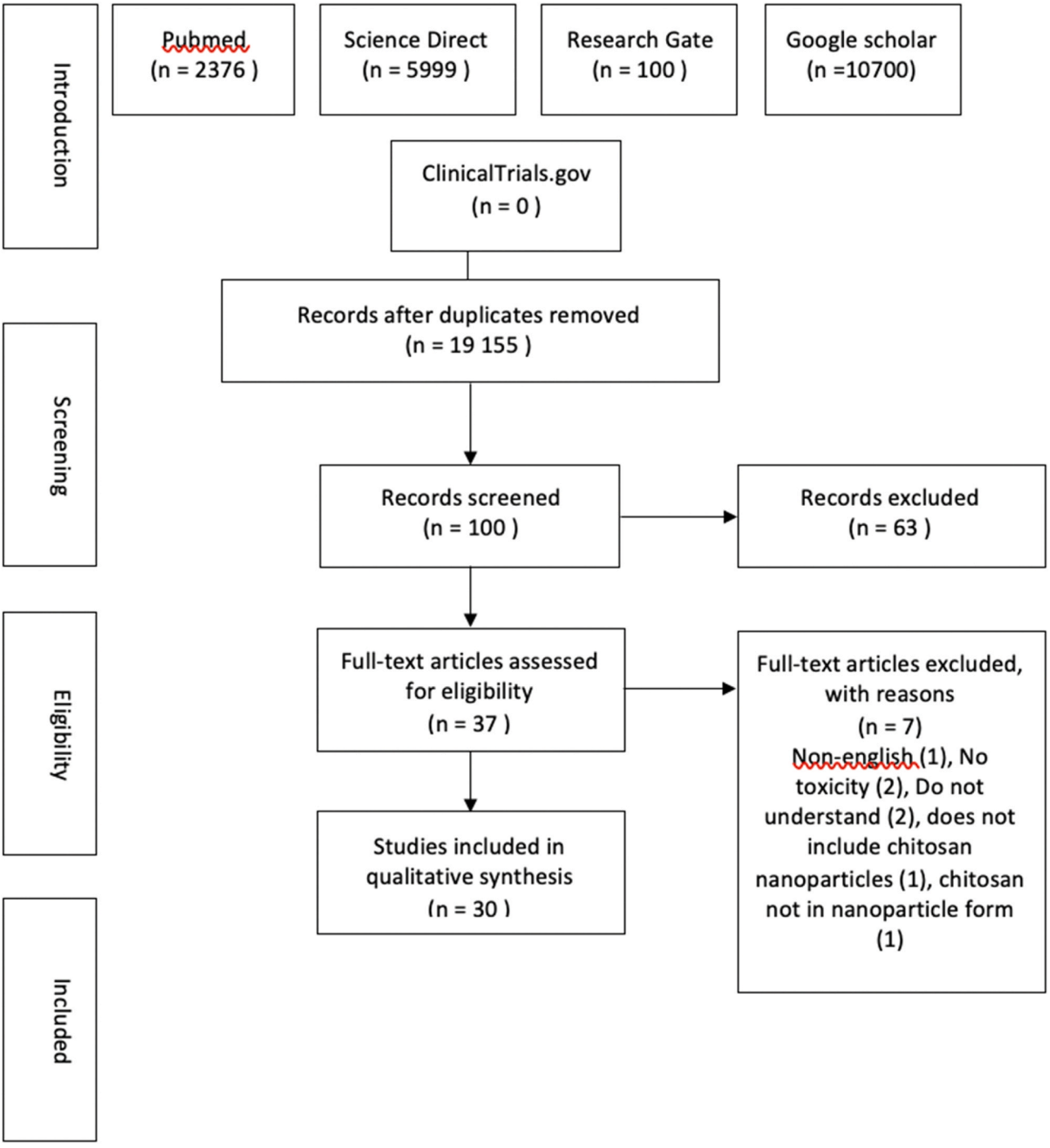

Fig. 1. PRISMA flowchart of article selection and literature review process.

demonstrated that decreasing particle size leads to increased inhibitory effects [26,81,83]. The two studies have shown a cytotoxic effect only on cancer cell lines as opposed to normal cell lines and cancer cell lines both. As seen in one of the studies, no toxicity was seen at all in normal cell lines, namely the MCF12 cell lines [83]. However, other literature suggests that smaller particles are associated with greater toxicity. For instance, a study investigating chitosan/streptokinase on human fibroblast cell lines found that decreasing the size of nanoparticles increased cytotoxicity, while factors such as chitosan concentration, stirring duration, and pH did not directly affect cytotoxicity [139]. Another study on BEL7402 cancer cell lines using chitosan nanoparticles loaded with copper showed higher cytotoxicity for smaller nanoparticles (40 nm) compared to larger ones (70 and 100 nm), although no cytotoxicity was observed in L-02, liver cell lines [140].

Similar observations have been made with other types of nanoparticles, not limited to chitosan. For example, realgar nanoparticles of sizes 100 nm and 150 nm exhibited inhibitory effects on ECV-304 cells, while larger sizes (200 nm and 500 nm) did not [141].

The smaller realgar nanoparticles were suggested for anti-neoplasia treatment due to their ability to suppress angiogenesis with less toxicity compared to bulk arsenic compounds like arsenic trioxide (As2O3). In another experiment, poly(lactic-co-glycolic acid) (PLGA) and titanium oxide (TiO2) nanoparticles of various sizes were tested on RAW264.7 and BEAS-2B cell lines. While no cytotoxic effects were

Table 3
Table of *in vitro* toxicity.

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
Tripolyphosphate (TPP) anion gelatinized chitosan nanoparticles	124.1–402.3 nm	0.125, 0.25, 0.5 mg/ml	Rifampicin	Dry powder incubation/ Murine macrophages J774 cells	CellTiter-Blue Reagent	Nanoparticles showed better cell viability compared to free rifampicin	Non-toxic	Rawal et al., [48]
TPP chitosan NPs	Medium Molecular Weight (MMW) chitosan nanoparticles (autoclaved): 700–1800 nm High Molecular Weight (HMW) chitosan nanoparticles (autoclaved): 2200–3700 nm	0.2, 0.3, 0.4, 0.5, 0.6% w/v chitosan at 10, 100 and 1000 μg/ml 0.2, 0.3% w/v chitosan at 10, 100 and 1000 μg/ml	none	Incubation with nanoparticle formulation/ mouse hematopoietic stem cells (HSC) from ICR strain male mice	MTT assay	Dose-dependent cytotoxicity Smaller particle sizes showed higher toxicity Toxicity was low regardless of particle size when observing concentrations of 10 and 100 μg/ml Molecular weight is shown to be less impactful on the toxicity towards cells	Low cytotoxic effect	Omar Zaki et al.,[81]
TPP chitosan NPs	20–70 nm	50, 100, 150,200 and 300 mg/ml	none	Incubation with nanoparticle formulation / L929 (fibroblast) cells	MTT assay	Dose dependent cytotoxicity is observed LD_{50} is at 64.21 mg/ml	Non-toxic up to LD50	Divya et al., [82]
TPP chitosan NPs	20–30 nm for air dried 4–5 nm for freeze dried samples	10, 20, 40, 60, 80, 100 μg/ml	none	Incubation with nanoparticle formulation/ MCF-7 cancer cells MCF-12 F normal cell line	MTT assay	Dose-dependent toxicity was observed in all nanoparticles prepared Smaller sizes of nanoparticles showed higher cytotoxicity TPP chitosan nanoparticles showed the lowest number of viable cells, thus showed the highest cytotoxicity in this experiment No toxicity shown in MCF-12 F cells	Nontoxic, but can inhibit cancer cell growth	Thandapani et al.,[83]
Sodium Hexametaphosphate (SHMP) crosslinked chitosan nanoparticles	60–80 nm for air dried 20–30 nm for freeze dried			Fresh human blood	Hemolysis Assay	The paper claims that the hemolysis was “slight” after incubation of 2 h Hemolysis that was observed also was deemed as negligible as the results showed a less than 2% hemolysis, where this cut off point was obtained from another experiment in 2002[84].		
TPP chitosan NPs	339 ± 66 nm	0.56 ± 0.06 mg/ml, test was done with 0.025%, 0.0125%, 0.006125%, 0.003% and 0.0015% concentration	None	Incubation with nanoparticle formulation/ Calu-3 cells	MTS assay	0.0015% and 0.0030% showed no significant toxicity Dose dependent toxicity is observed from 0.006% and above Compared to solution chitosan, the nanoparticles overall showed a higher relative cell viability. However, in terms of considering what is toxic, the paper describes a cell viability of less than 50% is used, not in line with the standard given by the ISO993–5, and instead cited two papers from 2009 of chitosan nanoparticles compared to hybrid chitosan nanoparticles[85,86].	Less toxic compared to chitosan solution	Vllasaliu et al.,[87]

(*continued on next page*)

Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
					LDH assay	Dose- dependent toxicity is seen Statistically different toxicity profile is seen at 0.006% and 0.0125% concentrations, where nanoparticles showed notably lower levels of LDH release when compared to just solution		
TPP chitosan nanoparticle	18 ± 1 nm	0.001–1% w/v	None	Incubation with nanoparticles/ bi-potential human liver cells	MTT assay Alanine transaminase (ALT)	Dose dependent cytotoxicity as relative mitochondria dehydrogenase activity decrease as dose increases More notable decrease in pH 6.0. Time dependent toxicity is also observed	Toxic	Loh et al., [88]
carboxymethyl chitosan-2, 2′ ethylenedioxy bis-ethylamine nanoparticles	210 ± 40 nm	5, 10, 15, 20, 25 μg/ml	Folate	Incubation with nanoparticles/HeLa cells	MTT assay	no significant difference compared to control and no dose dependence cytotoxicity	Non-toxic	Chakraborty et al.,[89]
Alginate-chitosan nanoparticle	620 nm	26 μg/ml	Plasmid	Incubation with nanoparticles/ HEK 293	^{3}H thymidine, and count radioactive label activity using beta-counter (Wallac)	No difference when compared with control group When combined with alginate, showed higher cell viability when left for 24 h	Non-toxic	Rafiee et al., [90]
TPP chitosan nanoparticle	TEM diameter (nm) 20–140	1 μg/ml, 10 μg/ml, 50 μg/ml	Methotrexate	Incubation with nanoparticles/ HeLa cancer cells HaCaT cancer cells MCF-7 cancer cells	MTT assay, NRU assay, LDH leakage, and acridine orange apoptosis testing	Cytotoxicity Dose dependent, time dependent and pH dependent toxicity was reported Methotrexate bound to chitosan nanoparticles showed a higher degree of cytotoxicity when compared to free methotrexate Apoptosis Methotrexate bound to chitosan nanoparticles showed a significant and notably higher induction of apoptosis compared to free methotrexate	cytotoxic	Nogueira et al.,[91]
		100, 250, 500 μg/ml		Incubation with nanoparticles/ red blood cells	Hemolysis assay	500 μg/ml showed significant red blood cell agglutination after 1-hour incubation 500 μg/ml showed a notably higher amount of hemolysis, but no significance was mentioned	Toxic, especially at high concentration	
		10, 25, 50 μg/ml	None	Incubation with nanoparticles/ red blood cells	red blood cell agglutination	50 μg/ml induced significant hemolysis at both 10 and 60 min 50 μg/ml causes agglutination of red blood cells		
TPP chitosan nanoparticle	200–300 nm	0.025, 0.05, 0.1, 0.2, 0.4 mg/ml	Rosmarinic acid	Incubation with nanoparticles/ retina pigment epithelium (ARPE-19) Human cornea cell line (HCE-T)	MTT assay	Non-toxic at 1 mg/ml and below, although showed slightly lower cell viability compared with DMEM and cells Non-irritating as no vasoconstriction, hemorrhage, or coagulation in the	Non-toxic	da Silva et al., [92]

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Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
				Injection into hen egg Chorioallantoic membrane	HET-CAM test)[93,94]	HET-CAM cells when 200 μL of sample was introduced for 5 min		
N,O-Carboxymethyl Chitosan Nanoparticles	150 ± 30 nm	1, 2, 3, 4 and 5 mg/ml	Curcumin	Incubation with nanoparticles/ L929 cell lines Incubation with nanoparticles/ MCF-7 Incubation with nanoparticles/ PC-3 cell line	MTT assay LDH assay Flow cytometry assay	Dose dependent toxicity was found in MCF7 cell line and PC-3 in both free curcumin and curcumin loaded nanoparticles No toxicity was shown in blank nanoparticles	Non-toxic	Yadav et al., [95]
poly methyl methacrylate (pMMA) and chitosan–glutathione nanoparticles	153–264 nm	0.1, 1.0, 10, 100 and 1000 ng/ml	Docetaxel	Incubation with nanoparticles/ Caco-2 cell line MCF-7 cell line	MTT assay	Entrapped Docetaxel in chitosan nanoparticles showed a higher cytotoxicity when compared to free docetaxel	Non-toxic	Saremi et al., [96]
Hyaluronic acid-decorated chitosan nanoparticles	142–211 nm	0, 25, 50, 75 and 100 μM	Raloxifene	Incubation with nanoparticles/ A549 cell line Incubation with nanoparticles/ HepG2 cell line Incubation with nanoparticles/Huh-7 cell line	MTT assay	More cytotoxicity observed in A549 cell line as compared to the other two cell lines The most apoptotic cells were seen in the (RX-HA-CS NP) When compared to just free RX, all nanoparticle formulation showed a significantly higher amount of apoptotic cells RX-CS-NPs showed a higher number of cells undergoing apoptosis in early stages as opposed in later stages RX-CS and RX-HA-CS NP both showed a significantly higher number of necrotic cells as compared to the other treatment groups as well as the control	Toxic	Almutairi et al.,[97]
			None	Incubation with nanoparticles/ A549 cell line		Void showed no significant increase in apoptotic cells	Nontoxic	
TPP chitosan nanoparticle	Size of NP: 500:1–220 ± 7.2 nm 300:1–206 ± 11.8 nm 100:1–164 ± 13.4 nm	10%, 5%, 1% and 0.1% concentrations	Plasmid DNA	Incubation with nanoparticles/ HeLa; ATCC cervical cancer cells MDA-MB-231;ATCC breast cancer cells peripheral blood THP-1; ECACC cancer cells	MTT assay for cell viability Senescence-associated-β-galactosidase activity Oxidative stress using fluorescence detection with 2′, 7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA)	MTT assay No difference in the HeLa cells and MDA-MB-231 cells. However, a difference in the THP-1 cells were revealed to have reduced metabolic activity dose dependent and weight ratio dependent with chitosan nanoparticles with p-DNA Oxidative stress using fluorescence detection with 2′, 7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA) Chitosan nanoparticles group generally showed lower fluorescence when compared to control with the exception on one test with Chitosan-pDNA nanoparticles in HeLa cells	Nontoxic for HeLA and MDA cancer cells but toxic for THP-1 cells	Bor et al., [98]

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Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
						where it showed higher fluorescence Senescence-associated-β-galactosidase activity Stable Chitosan-pDNA particles were shown to have a higher level of the activity and therefore suggested that there is a higher level and faster rate of senescence		
TPP chitosan nanoparticle	0.2% w/v - 164.0 ± 6.3 nm 0.3% w/v- 384.6 ± 4.6 nm 0.4% w/v- 458.9 ± 3.2 nm 0.5% w/v- 475.2 ± 5.3 nm 0.6% w/v- 684.8 ± 4.5 nm	0, 31.25, 62.5, 125, 250, 500, 750, 1000 μg/ml	None	Incubation with nanoparticles/ mouse bone marrow derived (MBMC)	MTT assay	MTT assay for cell viability At 48 h and 72 h, a significant decrease in cell viability % was observed in a concentration of 750 and 1000 μg/ml for the 0.2% w/v chitosan nanoparticles Conversely, a significant increase in cell viability was observed in 750 μg/ml for the 0.3% w/v chitosan nanoparticles	Cannot be determined	Zaki et al., [99]
					Live/dead assay	After 3 days exposure at 750 μg/ml and 1000 μg/ml, a significantly higher number of dead cells were seen in the 0.2% w/v 750 μg/ml 0.3% w/v chitosan nanoparticles showed a higher cell viability as opposed to the control, and it was reported to be "possibly due to promotion of cell proliferation"		
					ROS assay	No significant changes of ROS levels were seen in all treatment groups		
TPP chitosan nanoparticle	0.2% w/v - 164.0 ± 6.3 nm 0.3% w/v- 384.6 ± 4.6 nm 0.4% w/v- 458.9 ± 3.2 nm 0.5% w/v- 475.2 ± 5.3 nm 0.6% w/v- 684.8 ± 4.5 nm	0, 31.25, 62.5, 125, 250, 500, 750, 1000 μg/ml	None	Mouse Hematopoietic stem and progenitor cells (HSPC)	Flow cytometry Immunophenotypic analysis	$Sca\text{-}1^{+}$ surface antigens on hematopoietic stem cells 62.5 μg/ml of 0.3% w/v showed a significant decrease in the number of hematopoietic stem cells Progenitor cells Myeloid committed progenitor cells Both $Gr\text{-}1^{+}$ and $CD11b^{+}$ cells to have revealed size-dependent cytotoxicity $CD11b^{+}$ has revealed that at 0.4% w/v, 250 μg/ml, 0.5% w/v, 750 and 1000 μg/ml, and 0.6% w/v, 1000 μg/ml, chitosan nanoparticles has shown to reduce the percentage of cells $Gr\text{-}1^{+}$ has shown cytotoxicity at 0.5% w/v 750 and 1000 μg/ml and 0.6% w/v at 500 μg/ml and 750 μg/ml Other readings showed no significant difference Lymphoid-committed progenitor cells Neither $CD45^{+}$ nor $CD3e^{+}$ showed any cytotoxicity at any w/v and at	Toxic to hematopoietic stem cells and myeloid committed progenitors	

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Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
						any concentration when compared to control		
Chitosan and polylactic acid nanoparticles (CS-PLA NPs)	Mass of anthraquinone 10 mg – 2.8 ± 2.2 nm 15 mg – 19.6 ± 0.9 nm 20 mg – 23.8 ± 1.7 nm	5, 10, 20, 30, 40, 50 μg/ml, 200 μL	anthraquinone	Incubation with nanoparticles/ HepG2 cancer cells	MTT assay	Dose dependent cytotoxicity was observed for all formulations (blank CS-PLA NP, CS-PLA NP containing anthraquinone, and free anthraquinone) In concentrations of 5, 10, 20, 30, 40 μg/ml, free anthraquinone showed the lowest percentage of cell viability, whereas in the 50 μg/ml, the CS-PLA NP containing the anthraquinone showed the lowest cell viability percentage	Toxic	Jeevitha, Amarnath [100]
					Annexin-V assay and flow cytometry	A notably larger number of cells are detected to be apoptotic in the CS-PLA anthraquinone when compared to free anthraquinone and control No significance score was accompanied with this test		
					DNA fragmentation detected in DNA agarose electrophoresis	DNA degradative smearing that is normally seen in cells that are necrotic is seen clearly in the 3rd lane of the agarose gel and the first lane that is treated with the nanoparticles showed increased fragmented DNA		
Low molecular weight Chitosan TPP nanoparticles	Size of NP: 80% deacetylation degree: In water 127 ± 5 nm DMEM 1 h: 109 ± 29 nm 24 h: 133 ± 22 nm RPMI 1 h: 116 ± 29 nm 24 h: 368 ± 141 nm	312–5000 μg/ml	None	Incubation with nanoparticles/ Human peripheral blood monocytes	MTT assay	MTT assay In general, nanoparticles were more toxic than polymer counterparts Chitosan 80% DDA showed a higher toxicity compared to chitosan 93%, with the IC_{50} for Chitosan 80% is at around 720 μg/ml whereas for chitosan 93%, it is notably higher at a level of 2104 μg/ml	Not specified	Jesus et al., [25]
					IL6 and TNF-a	No single test group showed any significant stimulation when compared to positive controls		
Low molecular weight Chitosan TPP nanoparticles	93% deacetylation degree: In water 292 ± 52 nm DMEM 1 h: 106 ± 20 nm 24 h: 147 ± 74 nm RPMI 1 h: 321 ± 48 nm 24 h: 327 ± 131 nm			RAW 264.7 cell line	MTT assay	Both types of nanoparticles showed toxicity at high concentrations; above 2500 μg/ml for chitosan 80% and above 3000 μg/ml for chitosan 93% nanoparticles Nanoparticles showed higher toxicity when compared to polymer counterparts	toxic	
					ROS and oxidative stress	ROS production was shown to have increased in chitosan 80% DDA for both nanoparticle and polymer formulations but not in both chitosan 93% DDA formulations Induction of cellular death due to the chitosan was not observed in both nanoparticles and polymers		

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Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
						No reversible effects of reducing ROS production was seen when chitosan was allowed to incubate with cells that have LPS-induced ROS production		
					Nitric oxide production	No NO production was induced by any of the test substances All concentrations of the test substances were able to slightly inhibit LPS induced NO production, at a significant level		
Low molecular weight Chitosan TPP nanoparticles		0.1, 1, 2 mg/ml		Human Blood cells	Hemolysis assay	None of the test groups showed a hemolysis superior to 5%, which is the threshold for significant hemolysis according to the ASTM E2524–08 standard	Nontoxic	
		0.1, 1 mg/ml			Coagulation assay	1 mg/ml 80%DDA chitosan showed extended APTT and PT values The rest of the test groups did not show any significant effect	Nontoxic	
		0.1, 1 mg/ml			Platelet aggregation assay	Chitosan 93% DDA nanoparticles showed platelet aggregation whereas 80% DDA nanoparticles did not when compared to positive controls	toxic	
Low molecular weight Chitosan TPP nanoparticles	not stated	0.5, 1, 5, 10 μg/ml	None	Incubation with nanoparticles *Danio rerio*	MTT assay	Significant and notable decease in cell viability in 5 and 10 μg/ml	Toxic	Chou et al., [101]
		5, 10, 50 and 100 μg/ml		Zebrafish liver cells	Trypan blue staining	Dose dependent cytotoxicity as more cells take up dye in higher concentrations		
Octenylsuccinic anhydride modified chitosan (OSA-CS) nanoparticles	Dependent on the drug:OSA-CS ratio	0, 0.01, 0.05, 0.1, 0.2, and 0.5 g/L	none	Incubation with 8-week-old male Kunming mice macrophage	Cytotoxicity assay CCK-8	General increase in cell viability when concentration increased	Nontoxic	Yu et al., [102]
		10, 30, and 50 μM	curcumin			Cell viability increased in 30 μM of curcumin loaded in nanoparticles when compared to free curcumin which has decreased cell viability at the same concentration; due to the slower release of nanoparticles thereby reducing the cytotoxicity Cell viability decreased in 50 μM for both test samples, however no significant difference is shown between the two groups		
		10, 50, and 100 μM	Quercetin			Neither free nor nanoparticle group showed significant toxicity when compared to control		
Octenylsuccinic anhydride modified chitosan (OSA-CS) nanoparticles	Dependent on the drug:OSA-CS ratio	1,2,3 g/L	none	Incubation/ red blood cells from 8-week-old male Sprague Dawley rats	Hemolysis	Nanoparticles showed a lower percentage of hemolysis when compared to chitosan solutions	Nontoxic	Yu et al., [102]
		Not stated			Platelet adhesion rate	Nanoparticles showed a lower percentage of platelet adhesion rate when compared to the chitosan solution, with a *p*-value of less than 0.001.		

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Table 3 (*continued*)

Type	Size	Concentration/ dose	Substance Loaded	Mode of administration/ cell line	Method of testing	Results	Toxic or non-toxic	Citation
Low molecular weight chitosan TPP nanoparticles	86.11 ± 3.76 nm 91.23 ± 5.21 nm	3% w/w chitosan	none pravastatin	Incubation/ with red blood cells from a 45-year-old male human volunteer in normal plasma and hypercholesterolemic plasma	Hemolysis	Generally, the elevated cholesterol groups showed a higher degree of hemolysis All elevated cholesterol groups that received some of treatment showed a lower amount of hemolysis compared to the group that has red blood cells suspended in elevated cholesterol plasma without any treatment Results also shows that there is a difference between the hemolysis percentage as the pravastatin chitosan nanoparticles showed a lower percentage of hemolysis compared to free pravastatin and chitosan solution	No conclusion on toxicity	Harisa et al., [103]
TPP chitosan nanoparticles Hyaluronic acid covered TPP chitosan nanoparticles	142.80 ± 2.22 nm 170.80 ± 0.14 nm	1, 5, 10, 25, 50, 75, 100, 200 μg/ml	S-nitroso-Mercaptosuccinic acid Mercaptosuccinic acid S-nitroso-Mercaptosuccinic acid	Incubation with human uterine cervix carcinoma (HeLa) cell lines	MTT assay	Dose-dependent cytotoxicity is seen S-nitroso-mercaptosuccinic acid nanoparticles showed the lowest overall cell viability of all three groups Compared to the non-covered group, the nanoparticles with the hyaluronic acid covered S-nitroso-mercaptosuccinic acid showed higher cytotoxicity MSA containing HA-coated CS NPs have lower cytotoxicity compared with S-nitroso-MSA containing HA-coated CS NPs against HeLa cell line in the range of 0–100 μg/ml	Toxic above 25 μg/ml	Pelegrino et al.,[104]
TPP chitosan nanoparticles Hyaluronic acid covered TPP chitosan nanoparticles	142.80 ± 2.22 nm 170.80 ± 0.14 nm	1, 5, 10, 25, 50, 75, 100, 200 μg/ml	S-nitroso-Mercaptosuccinic acid Mercaptosuccinic acid and hyaluronic acid	Incubation with human prostatic carcinoma (PC-3)	MTT Assay	Dose-dependent cytotoxicity is seen		

Table 4
Table of *in vivo* toxicity.

Type	Size	Concentration/dose	Substance Loaded	Mode of administration	Method of testing	Results	Toxic or non-toxic	Citation
Tripolyphosphate (TPP) anion gelatinized chitosan nanoparticles	200 nm and 340 nm	-Dose 200 nm: 5, 10, 20, 30, 40 mg/L -Dose 340 nm: 10, 20, 40 mg/L	None	Incubation with Zebrafish embryos	Acridine orange staining Intracellular ROS Heat shock protein Hatching rate and mortality	Increased acridine orange uptake at 20 and 30 mg/L Increased ROS levels were observed in 5 mg/L Heat Shock protein increased at 20 and 40 mg/L Hatching rate and mortality increased in a dose dependent manner Smaller nanoparticles showed a higher toxicity than larger nanoparticles	Toxic	Y.-L. Hu et al., [26]
TPP chitosan NPs	124.1–402.3 nm	313.56 mg/kg	Rifampicin	Wistar rat inhalation of dry powder chitosan nanoparticle formulation	Acute toxicity testing checked by conducting histopathology on lungs	Nanoparticles showed better cell viability compared to free rifampicin	Non-toxic	Rawal et al., [48]
TPP chitosan NPs	222.00 ± 6.30 nm to 248.50 ± 23.50 nm Optimum particle size reported in abstract as 248.50 nm	0.045 mg/kg/day and 0.250 mg/kg/day	Piperine	Intra nasal administration to male Wistar rats	Caspase 3 assay measurement TNF-α ELISA	PIP-nanoparticles at 0.25 mg/kg/day showed to have the lowest activity for both caspase 3 activity and the lowest concentration TNF-α. None of the treatments were able to revert either levels that is shown by the negative control group None of the formulations dosing at 0.045 mg/kg/day could show significant effect on neural apoptosis and inflammation	Non-toxic	Elnaggar et al., [105]
Complex of chitosan and o-carboxymethyl chitosan nanoparticles	250–300 nm	4 mg/kg	Doxorubicin	Oral administration on Sprague Dawley rats	Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA)	Showed higher activity of SOD and CAR compared to IV doxorubicin Reduced toxicity of nanoparticle was shown	Non-toxic	Feng et al., [106]
Trimethyl chitosan lipid nanoparticles	162.8 nm	100 μL, no exact concentration was reported	Baicalein	Eye irritation test on Albino Rabbits	Eye irritation test via modified Draize Test	Scores for the cornea, iris and conjunctivae were all 0 and were considered to be non-irritating Histology of saline and nanoparticle eye was comparable	Non-toxic	Li et al.,[107]
TPP chitosan NPs	84.86 nm	100, 150, 200, 250, 300, 350 and 400 mg/L, 200 μL of each concentration	None	Zebrafish embryo	Malformation to zebrafish body Mortality percentage Lethal effects such as Yolk coagulation Inactivated gastrula Somites formation	Common malformations included malformation to the zebrafish body axis, deflated swim bladders, pericardial swelling, and swelling to the yolk sac. Dose-dependent toxicity was observed in the present study for all chitosan samples Nanoparticles showed lower toxicity when compared with normal chitosan particles at the same concentrations With the exception of the 200 mg/L concentration, all chitosan nanoparticles showed higher hatching rate as compared to normal chitosan particle. Statistically significant difference is seen in 300 and 400 mg/ml Toxicity of chitosan is dose dependent and is lower in nanoparticles compared to normal particles	Toxic	Y. Wang et al., [27]
Spray dried TPP chitosan NPs	600–1000 nm	High dose: 450 mg/kg Low dose: 225 mg/kg	None	Orally fed nanoparticles to Sprague Dawley rats	Record mortality rate of rats	0 rate mortality	Nontoxic	(Hong-liang [1108])
carboxymethyl chitosan-2, 2′ ethylenedioxy bis-ethylamine nanoparticles	210 ± 40 nm	5, 10, 15, 20, 25 μg/ml	Folate	Intraperitoneal injection to male Swiss Mice	Record mortality rate of rats If 2 out of 3 animals were dead at given does, dose considered as toxic If 1 animal showed mortality, repeat dose to confirm	No mortality Grouped as unclassified and considered as safe	Non-toxic	Chakraborty et al.,[89]

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Table 4 (*continued*)

Type	Size	Concentration/dose	Substance Loaded	Mode of administration	Method of testing	Results	Toxic or non-toxic	Citation
TPP chitosan NPs	120–646 nm	25, 50, 100, 200 mg/l for acute toxicity 200 mg/l for other toxicities	none	Incubation with Zebrafish embryos	Acute toxicity assay measured by teratogenic abnormalities: Neurobehavioral toxicity Heart dysfunction Hepatotoxicity assays using to check liver size, yolk retention and steatosis	Failed to show acute toxicity Mild neurobehavioral impairment is observed when nanoparticles are compared to DMSO Cardiac functions remain generally unaffected Liver size impairment was observed in zebrafish embryos	Toxic	Abou-Saleh et al.,[109]
TPP chitosan NPs	172.6–479.65 nm	2 g/kg	Docetaxel	Orally administration of nanoparticles to Wistar rats	Acute oral toxicity Measured with Mortality rate Physical appearance Body weight of animals Biochemical analysis of blood Liver and kidney function tests Histopathological examination	No mortality for both groups No signs of irritation, ocular toxicity and illness No significant difference in biochemical blood analysis or liver and kidney function tests reported Lack of abnormal changes, pathological change, lesion, or deformation within both groups Concluded to be non-toxic according to results	Non-toxic	Mahmood et al.,[110]
TPP chitosan nanoparticle	247 ± 20 nm	5, 10, 20, 30, 40, 50 mg/L	None	Incubation with Zebrafish embryos	Neurobehavioral assessments Spontaneous embryonic contractions Tactile sensitivity test Free swimming activity and in response to light-dark stimulation ROS detection within cells Apoptosis 24 h after fertilization (hpf) and 96hpf Histopathological examination Transmission electron microscopy of muscles	Embryonic contractions Control initiated at 20hpf and reached maximum of 4.1 bends/min at 24 hpf Tactile sensitivity test Significantly Lower number of larvae swam out of the circle as compared to the control Results seems to point to a 72hpf motor defects No significant difference between treatment groups Free swimming activity No obvious changes Swimming activity in response to light-dark stimulation Generally, more movement was recorded in dark periods than in the light period Chitosan nanoparticles increased movement in the dark periods significantly as opposed to the control and tween 80 modified counterpart ROS Higher than control, lower than tween 80 modified Acridine orange and Annexin V/PI No obvious difference to control group Transmission election microscopy (TEM) examination of skeletal muscles No signs of inflammatory in any groups Space between muscle fibers where larger in treated groups as opposed to control, suggested deterioration of muscle TEM revealed many differences in muscle fibers, such as dissolving, larger spaced muscle fibers, karyolysis, condensed nuclei, swelled mitochondria were seen in both the chitosan nanoparticles and tween modified counterpart	Toxic	Z. Yuan et al., [111]

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Table 4 (*continued*)

Type	Size	Concentration/dose	Substance Loaded	Mode of administration	Method of testing	Results	Toxic or non-toxic	Citation
TPP Tween 80 modified chitosan nanoparticle	251 ± 15 nm	5, 10, 20, 30, 40, 50 mg/L	None	Incubation with Zebrafish embryos		Embryonic contractions Decreased tail bend rate was significantly lower during the same time for Tween modified chitosan nanoparticles Tactile sensitivity test See TPP chitosan nanoparticles Swimming activity in response to light-dark stimulation See TPP chitosan nanoparticles Free swimming activity No obvious changes ROS Showed higher ROS levels compared to chitosan nanoparticle and control Tween modified showed even higher levels of ROS as compared to positive control group Acridine orange and Annexin V/PI No obvious difference to control group Transmission election microscopy (TEM) examination of skeletal muscles See TPP chitosan nanoparticles		
Low molecular weight Chitosan TPP nanoparticles	not stated	100 μg/ml	none	Incubation with nanoparticles *Danio rerio*	Analyze locomotion	Dysphoria and Hypoxia was seen in zebrafish before dying in chitosan nanoparticle treated test	Toxic	Chou et al., [101]
		10, 50, 100 μg/ml		Zebrafish larvae	Survival rate	Higher concentrations showed lower survival rate Longer incubation decreased survival rate however only pre 24 h Purified Low molecular weight chitosan nanoparticles showed a much higher survival rate when compared with unpurified		
		100 μg/ml			Hematoxylin and eosin staining	Intestine, epidermis and muscles are damaged when compared to control		

Table 5
Table of the test description for the studies included.

Type of test	Description of test	Citation
Acridine orange staining	As a dye that is selective to nucleic acids, apoptotic cells allow it to permeate into the cells whereas normal cells do not. Once DNA and the dye intercalate, green fluorescence will be emitted	Asharani et al., [112].
CellTiter-Blue Reagent (Promega)	The mechanism behind this reagent is the transformation from resazurin to resofurin via reduction reaction. The signal is from fluorescence, of which in most cases will increase when the number of viable cells is higher. It is claimed on the product information that the relationship between fluorescence and cell number is linear	Promega Corpora [113].
MTT assay	MTT is also known as (3-(4–5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide) tetrazolium. This assay relies on the reduction process into another product called formazan, where it is first formed as an insoluble crystal inside the media of which the MTT is added and reduce. It can be dissolved by acidified isopropanol, where a purple colored mixture is made. Metabolically active and viable cells will show a relationship in which as its numbers increase, the purple dye's concentration also increases	Riss et al.,[114].
Caspase-3 Assay	Apoptosis has some relationship with caspase-3 as the enzyme itself is activated in the events that are associated with apoptosis itself[115,116]. By conjugating p-nitroalanine (pNA) to peptides that were able to detect caspase specifically, the peptides were able to measure protease enzyme activity in the components that are present after the lysis of cells and cell components obtained after homogenizing the cells. Caspases are detected as due to their ability to cleave the peptide from the p-nitroalanine, which in itself can give a yellow or orange colour[117]. This was then measured by using a spectrophotometer at a 405 nm wavelength. Within the study, the activity of the enzyme is directly proportional to the reaction.	Ghavami et al.,[115, 117,116]
TNF-α ELISA	TNF-α, or tumor necrosis factor-alpha, is a cytokine that is produced by many leukocytes, which not only serves as the basis of killing tumour cells, but also inflammation and fever[118,119]. Enzyme systems are utilized to measure the concentration of an antibody or antigen that is found in blood whenever an ELISA, or enzyme linked immunosorbent assay, is performed[120]. Once samples are added in, it is left to allow any TNF-α molecules to bind to the antibodies coated in the wells. Afterwards, the samples are removed and then a substrate solution is added, with the washing off of unbound substances in between the two. Once that is done, an enzyme-linked antibody specific for the rats TNF-α were added in, left to bind to the TNF-α that is in the wells that is bound to the antibody in the wells, thus the name "sandwich technique"[121]. A substrate for the enzyme linked on the second antibody is then added, which will give a blue product that converts to yellow. To stop the reaction, a stop solution is combined. TNF-α levels are directly proportional to the intensity of colour measured.	Engelmann et al., [118,120,119,121]
Modified Draize test	Acclimated Albino rabbits are evaluated to ensure they are normal, and the cage should not cause any accidental injuries Designate one eye to be the test and the opposite eye is left untreated. Usually, the former being the right and the latter being the left. A scoring system is used to detect Ocular changes that includes changes to the parts of the eye Involve corneal opacity after treatment, iris irritation severity, redness of conjunctiva, swelling and discharge A higher score indicates a higher irritating effect to the eye Test such as fluid levels of test compound and histology checking of the eyes can be made, of which the latter is reserved for severe reactions Right eye of each rabbit was treated with nanoparticles whereas left side used saline as control for 1 week, 4 times a day After euthanizing the rabbits, the eye was removed and fixed with formaldehyde Light microscope analysis of the cornea, iris and conjunctiva cells was performed	Wilhelmus[122]
MTS assay	3-(4,5-dimethylthiazol-2-yl)− 5(3-carboxymethonyphenol)− 2-(4- sulfophenyl)− 2 H-tetrazolium, or also known as MTS, is used in a colorimetric assay. The reading can be done in anywhere in between 450 and 540 nm, 490 nm being the absorbance peak.	Promega Corpora [123]
LDH assay	LDH, or lactate dehydrogenase, reduces NAD to NADH, where the NADH will be utilized in converting a tetrazolium dye, in this case using the dye solution mentioned, L2277, to give a colour change. Once this change is observed, spectrophotometry is conducted at 490 nm. This tests for the damage that a cell has undergone by measuring the amount of LDH being released. If there is more LDH, the higher the intensity of the colour and therefore, signify more damage to the cell	Sigma-Aldrich[124]
^{3}H thymidine, and count radioactive label activity using beta-counter (Wallac)	^{3}H is a radioactive isotope of hydrogen and can undergo decay to release electron under the process of beta decay. The electrons that are released are detected to by a scintillation counter or beta-counter. The higher the count, the more the electrons were detected.^{3}H thymidine is used to measure DNA synthesis in different studies. In the study presented here, it is used as a measure to cell replication, the higher the level of counts, the more the cell proliferation[90].	[125-127]
NRU assay	Also known as Neutral red assay, any viable cells will take up the red dye after incubation of 3 h Dye accumulates in lysosome of viable cells, whereas it cannot accumulate in cells that are dead The higher the absorbance reading, the higher the number of viable cells	Borenfreund, Puerner[128,129]
Chorioallantoic membrane (HET-CAM test)	Hen's Egg Test (HET) is done on the embryonic vasculature inside a hen's egg as means to test for toxicity. For 10 days, fertile hen's eggs were incubated at 37 °C. For each nanoparticle solution has had 5 eggs assigned, each egg having 200 μL of nanoparticle solution applied. Vasoconstriction, hemorrhage, or coagulation were the signs of toxicity in this test Using equation: ((301-time for hemorrhage within 5 min in seconds) x 5 + (301-time for vasoconstriction to occur within 5 min in seconds) x 7 + (301- time for coagulation to occur within 5 min in seconds) x9) /300 After applying formula to calculate the extent of toxicity, the score obtained will quantify irritation potential that is observed 1–4.9 slight irritation 5–8.9 moderate 9 and above severe	Berger et al.,[93,94]

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Table 5 (*continued*)

Type of test	Description of test	Citation
Senescence-associated-β-galactosidase activity	Senescence, or loss of reproductive ability of cells via division however the cell still being alive[130], has markers that are present when the process has occurred such as beta-galactosidase along with $p16^{INK4a}$, a tumor suppressor protein[131] In the cell lines used, after 3 days of incubation with the pDNA containing chitosan nanoparticles, cells were fixed using formaldehyde, glutaraldehyde. At a pH of 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-inolyl-β-D-galactoside (X-gal), and 40 mM citric acid/sodium phosphate staining solution was added Photography under a light inverted microscope, where cells tested positive for the Senescence-associated-β-galactosidase activity were counted per 100 cells	Bor et al.,[98,130, 131]
Oxidative stress using fluorescence detection with 2′, 7′-Dichlorodihydrofluorescein Diacetate (THP)	Neither the original compound 2′, 7′-Dichlorodihydrofluorescein Diacetate itself nor its hydrolyzed polar product is able to fluoresce. However, if the DCHF is instead oxidized by anything from reactive oxygen species (ROS) and other peroxide enzymes, it instead is converted to a product that is able to fluoresce with a high degree[132]. Therefore, the more fluorescence, the higher the amount of ROS and peroxides, thus more oxidative damage[133].	Rajneesh et al., [132]
Live/dead assay	Calcein AM/EthD-1 Live/Dead Viability/Cytotoxicity assay Calcein acetoxymethyl (AM) stains live cells due its transformation to fluorescent calcein[134] EthD-1 binds to DNA inside cells that are dead due to the membrane being compromised. This therefore stains them.	Miles et al.,[134]
Annexin-V assay and flow cytometry	Phosphatidylserine (PS) is a cell surface protein that is presented whenever cells start to undergo apoptosis Annexin V, meanwhile is a protein that binds to phospholipids, has an affinity to PS Living cells do not allow the Annexin V to bind as the PS molecule is not exposed and Annexin V is not able to penetrate the cell membrane's phospholipid bilayer Propidium iodide (PI) or other suitable staining agents are used to compare between dead and apoptotic cells	Jeevitha, Amarnath [100]
Hemolysis assay	Incubate Red Blood Cells with chitosan nanoparticles Suspension centrifuged and measure the liquid that is centrifuged for its optical absorbance at 540 nm $\frac{\text{Absorbanceof sample–Absorbanceof negative control}}{\text{Absorbanceof positive control} - \text{Absorbanceof negative control}} \times 100\%$ equation was used for study "Construction of an environmentally friendly octenylsuccinic anhydride modified pH-sensitive chitosan nanoparticle drug delivery system to alleviate inflammation and oxidative stress"[102].	Jesus et al.,[25,102]
Coagulation assay	Checking activated partial thromboplastin time (APTT) and prothrombin time Prothrombin Prothrombin time assess the activity of activity of factor VII, a clotting factor Time recorded us the time for fibrin strands to appear after adding thromboplastin and calcium to platelet poor plasma APTT APTT measures the activity of the intrinsic and common pathway of coagulations Time recorded for a clot to form after adding calcium to platelet poor plasma	SUCHMAN, GRINER[135]
Platelet aggregation assay	15 min incubation at concentration of 2 mg/ml Count platelet (PC) and apply equation for platelet aggregation Platelet aggregation (%)= $(PC_{\text{negative control}} - PC_{\text{sample}})/PC_{\text{negative}}$ x 100%	Jesus et al.,[25]
Trypan blue staining	Cells that are living will exclude trypan blue whereas dead cells will allow the dye to enter and stain the cell	Strober[136]
Hematoxylin and eosin staining	Hematoxylin stains the nucleus violet after it has been converted into hematein via oxidation processes. The cytoplasm is stained pink by the eosin Shows the morphology of cells	Ozawa, Sakaue [137]
Cytotoxicity assay CCK-8	WST-8 (2-(2-methoxy-4-nitrophenyl)− 3-(4-nitrophenyl)− 5-(2,4-disulfophenyl)− 2 H-tetrazolium, monosodium salt) is reduced to form a dye that is soluble in water The more the living cells, the more reduction reactions occur and therefore the more the water-soluble dye is produced. It is therefore inferred that the amount of dye produced is directly proportional to number of living cells.	Merck KGaA[138]
Platelet adhesion	Form a uniform polymer film on test tube Platelet rich plasma supernatant was placed together with the film Count number of unattached platelets Platelet adhesion % calculation $\frac{\text{Number of unattached platelets in control} - \text{Number in sample}}{\text{Number in control}} \times 100\%$	Yu et al.,[102]

observed with PLGA nanoparticles, TiO2 nanoparticles of sizes 10 nm and 20 nm exhibited significant cytotoxicity, whereas the 100 nm size showed reduced cell viability[142]. The exact mechanism underlying the inverse relationship between nanoparticle size and cytotoxicity is not well-established, although studies suggest that smaller particles may adsorb more biomolecules and enter cells more easily [26,143], This concurs with the toxicity results of the dose dependent toxicity as discussed in section 4.3.3.

In conclusion, smaller nanoparticles tend to exhibit higher toxicity, not only for chitosan nanoparticles but also for other types of nanoparticles. Nanoparticle manufacturers should consider size as a critical parameter. Further research is needed to confirm the proposed mechanisms underlying the increased toxicity of smaller nanoparticles.

3.3.2. *Cell lines*

Both normal cells and cancer cell lines are tested, with the sources coming from different organisms. Most of the tests that are reported in this literature review are done on human cells and have showed little to no toxicity, although some studies such as the ones performed the on the liver cells [88] and platelets in human blood cells [25] exhibit more significant toxicities.

The first test with the human liver cells showed that 0.5% w/v chitosan nanoparticles exposure was tolerable for the liver cells incubated, however only up to four hours. Meanwhile, concentrations above 0.5% w/v chitosan nanoparticles showed cells to have disrupted cell membranes and alanine transaminase leaked. Alanine transaminase (ALT) is an enzyme that is responsible for respiration in that it is converted to

pyruvate via a process called the Cahill cycle or the glucose-alanine cycle [144]. As a measurement, ALT is widely used as a measure of liver function in a series of tests called the liver function test. In terms of ALT, ALT is used as a measure of the extent of liver damage, as the higher the concentration, the more cells that have died and allowed the ALT leaked in the first place [145]. Some diseases that are indicated with a high ALT level include nonalcoholic steatohepatitis and cirrhosis [146, 147]. In this study, nanoparticle concentrations of 0.5% and 1% showed a significant rise in ALT levels when compared to the medium. It is clear with the evidence provided that there is toxicity elicited in the liver cells as the ALT levels in the collected supernatant indicates ALT to have leaked out of the cells which mirror the liver cell death in liver damage. It is also interesting to note that this experiment also looked into the time dependence of the toxicity, in which 4 h was the given time in which liver cells started showing any signs of apparent toxicity. Time dependence is a pertinent consideration as the duration of exposure can result in different results with respect to toxicity. Signs of this can be seen as far back as 1982, where a study conducted on chemotherapy showed that exposure time can impart higher cytotoxicity despite being given at a lower dose when compared to a higher dose in a shorter amount of time [148].

The second test with the blood cells tested for multiple parameters, however only one parameter successfully showed any toxicity that is induced by the chitosan nanoparticles, whereas the other parameters failed to show any toxicities. The reason why platelet aggregation can be considered a toxicity is due to the fact that when platelet aggregates, thrombi can form and can cause episodes of thrombosis as a result of the thrombi forming from the aggregation of platelets [149]. The toxicity that is observed in the nanoparticles seems to be due to the deacetylation degree. One study does show that in rabbit red blood cells, the chitosan samples with a moderate deacetylation degree showed a shorter coagulation time compared to chitosan nanoparticles that had the lowest and the highest deacetylation degree [150]. However, in other studies looking in cell viability rather than platelet aggregation, the relationship between the degree of deacetylation and toxicity can affect the relationship between the concentration and molecular weight of the chitosan nanoparticles [151]. What this then entails for future considerations is that if nanoparticles are to be introduced into patients, there may be a need to check if there are any contraindications of nanoparticle use in patients with high risk of venous thromboembolism. This is a concern as already other existing medication are usually avoided in patients with high venous thromboembolism risk, such as some oral contraceptives and tricyclic antidepressants [67]. Conversely, there could also be considerations of whether there may be interactions with antiplatelets and anticoagulants in terms of affecting blood clotting balance and any incompatibilities in terms of using chitosan nanoparticles as a vehicle containing anticoagulants. For example, degradation has occurred in smoking cessation medication, varenicline, has shown to react with the oxidative degradants of polyethylene glycol (PEG) [152].

On the other hand, cancer cell lines exposed to chitosan nanoparticles have shown to have toxicity. For example, MTT assays, reactive oxygen species and senescence assays were conducted and THP-1 white blood cell cancer cells showed to have suffered the most toxicity as all results from all the results has shown that there is a significant decrease in cell viability in the MTT assay, and showed an increased amount of cells undergoing senescence when stable chitosan nanoparticles loaded with plasmid DNA where incubated with the THP1 cells [98]. While the HeLa cervical cancer cells and MDA-MB-231 breast cancer cells also showed similar results in the test for senescence, neither of the cells showed significant decrease in cell viability for the MTT assay [98]. As a footnote, the senescence that was induced in the cells were unaffected by the gene loaded into the chitosan nanoparticles and was considered to be inert and does not contribute to the toxicity observed [153]. A second example to this would be experiments conducted on A549 lung cancer cells, Hep G2 and Huh-7 liver cancer cells with chitosan nanoparticles adorned with hyaluronic acid which were further encapsulated with hyaluronic acid [97]. The lung cancer cells were shown to undergo apoptosis when the levels of nitric oxide were increased while exposed to the loaded chitosan nanoparticles. Blank nanoparticles, also named as "Nano-void", exposed to the apoptotic cells showed no significant increase in the apoptotic cells for A549 cells. However, when the free raloxifene drug was compared to the nanoparticle groups, the nanoparticles showed a significant and larger proportions of apoptotic cells, most notably late apoptotic cells. Additionally, the proportion of necrotic A549 cells are also much higher in the groups that were exposed to raloxifene chitosan nanoparticles, both adorned and unadorned with hyaluronic acid when compared to the free drug, blank chitosan nanoparticles, raloxifene loaded in hyaluronic acid nanoparticles.

3.3.3. *Organisms*

All of the toxicity studies that were performed on the zebrafish cells showed some form of toxicity and were concluded to be toxic. For example, the study on zebrafish liver cells have shown that the membranes of the cells were destroyed, which thereby caused the death of both the zebrafish larvae and mature fish [101]. Another example would be the gill lamella damage, which suggests oxygen starvation before death and is also referred to in the study as its major cause. One more point that can be observed from the study is that the study also mentioned that larval zebrafish epithelial cells also experienced toxicity with signs including epithelial cell protrusion, yolk extension breaking, and tissue fluid bubbles forming. It is mentioned, however, that the toxicity of these chitosan nanoparticles may be limited as the toxicity was exclusively observed in acidic pH due to the fact that chitosan can dissolve only in acidic pH levels when compared to a neutral or alkali environments. Other *in vivo* experiments also show the same results. For example, one study done on TPP nanoparticles [26] showed an increase in the levels of ROS, Heat shock protein, mortality, and hatching rate, and another one showed deformations in not only the development of the zebrafish from larvae to adult fish, but also in their neurobehavior as evidenced by the swimming behavior that is observed in one of the studies [109]. The neurobehavioral impairment is also observed in another study, where the contraction of the embryo, a test for the tactile sensitivity, and the free-swimming activity when exposed to darkness or light has been recorded, and the groups treated with chitosan showed a notable decrease in terms of tactile sensitivity as more larvae failed to swim out of the circles that were prepared for the larvae [111]. Within the same study, a modified version of the nanoparticles was being observed, and that was the Tween-80 modified chitosan nanoparticles, which showed a similar result compared to the chitosan nanoparticles. Meanwhile the contractions from the embryo, the tween modified chitosan nanoparticles did showed a significant drop in the times of tail-bends after 1 min when the embryos 20 and 21 h after fertilization. Both of these results were claimed to be proof of movement disorders, which is postulated as a possible corollary of swim bladder defect as it is an important part of zebrafish locomotion.

In spite of this, toxicity that is found within zebrafish is not shared by most if not all the animal studies. For example, the Wistar rats that were used to test piperine-loaded chitosan nanoparticles showed that the authors concluded that the chitosan nanoparticles were safe to use and that the chitosan nanoparticles could significantly help treat nasal irritation caused piperine and showed no brain toxicity [105]. This contrasts the results obtained in the zebrafish as the zebrafish showed significant toxicity in the neurobehavioral development of zebrafish larvae, which also mentioned that both nanoparticles regardless of the presence of the tween 80 modification made to the chitosan, showed primary and secondary motor neuron development were both inhibited [111]. Aside from the neurobehavioral toxicity, muscle structure was affected, in which it is speculated that the deterioration of muscle was the reason that the space between muscle fibers became enlarged. Last but not least, the developmental toxicity of the nanoparticles was observed as it decreased the rate of zebrafish larvae hatching, increasing the death rate and malformation incidences among the zebrafish larvae,

similar to other studies already mentioned within this paper [26,27].

Notably, the tests with zebrafish listed in this review all had the nanoparticles being exposed to the fish at their embryo and larval stages and not during the adult stages of the fishes' life cycle. One study on nanoparticles, though not on chitosan nanoparticles, has revealed that nanoparticle exposure to embryo results in a higher mortality when compared to adult zebrafish [154]. The paper did mention in the discussions that while this is the case, the regulations being used as a reference for toxicity testing showed that the toxicity found in the embryos were the same in most of the nanoparticles tested. Notwithstanding this, the paper also has stated that for the tests with magnesium oxide nanoparticles being less toxic to the embryos than to the adult fish. As this review could not find a comparative study within the search parameters, future studies with chitosan nanoparticles tested on zebrafish may need to consider testing in adults as there may possibilities that toxicity studies in embryos, while can produce comparable toxicity results seen in adults for other compounds [155,156], it is not always the case [157].

The findings that are produced from experiments using Wistar rats and Zebrafish are important because both show contrasting results and are both being extensively used in experimentation. If these contrasting results are not resolved or explained, complications in terms of supporting evidence can occur, especially in planning future trials of the chitosan nanoparticle in humans.

3.3.4. Dose dependence

By far, dose dependence toxicity seems to be notably consistent characteristic to be observed, with nine studies in this literature confirming this. However, one study does state that there is no dose dependence observed. The study in question in particular is the one studying vancomycin [89]. The study failed to show any dose dependence because the experiment not only failed to show any toxicity, all concentrations used in testing against HeLa cells showed no significant change between each of the concentrations [89]. However, one study that performed experiments on HeLa cells showed there was a toxicity associated with HeLa cells being incubated with the chitosan nanoparticles [104] and contradicts the finding to the former study. This may indicate that there were some limitations in the 2012 study that severely affected the results when compared to the more recent results.

Dose dependence toxicity is also observed in different nanoparticles, with some examples being iron oxide nanoparticles [158], zinc oxide and silicone dioxide nanoparticles [159].

3.3.5. Organs

In this literature review, different organs exhibited different ranges of toxicity and the toxicities to be discussed are the toxicities to the liver, intestine, muscles, heart, lungs, eyes and mouth.

Chitosan is metabolized by lysozymes and enzymes from bacteria present in the colon [160]. However, this does not discount the liver for being a source of potential toxicity. For example, an experiment conducted on zebrafish embryos to observe the hepatotoxicity that may be present revealed that liver size impairment was present in the embryos, while other toxicities that were observed such as neurobehavioral impairment and cardiotoxicity were slight and have no significant effect observed [109]. This seems to concur with the result shown by the *in vitro* experiments conducted on the liver cells [88,101]. However, these results are not seen when Wistar rats are used instead. One such experiment is when Wistar rats are fed with docetaxel loaded chitosan nanoparticles, no changes to liver function were observed, alongside the absence of other toxicities such as mortality, irritation, ocular toxicity and kidney function disruptions. It was also then concluded to be non-toxic to the Wistar rats [110]. Additionally, another experiment has reported that the chitosan nanoparticles being antioxidants and protects the liver against hepatotoxicity that is induced in albino rats by using diethylnitrosamine [161]. It is stated that the chitosan nanoparticle treatment was able to decrease liver enzymes such as ALT, AST, GGT, alkaline phosphatase after the chitosan nanoparticles were fed to the albino rats. One more experiment showed that within Wistar rats given chitosan nanoparticles, the liver kidney tests which not only includes the ALT, alkaline phosphatase, and AST but also the bilirubin, urea and creatinine [110]. In this third experiment, both the control group and the test group showed similar levels of the parameters tested and the authors have concluded that the chitosan nanoparticles showed good safety and biocompatibility [110].

Meanwhile, toxicity is also observed in the intestines of zebrafish larvae when they were exposed to low molecular weight chitosan nanoparticles after a hematoxylin and eosin staining [101]. However, other studies seem to show somewhat conflicting results. For example, one experiment showed that when Wistar rats were fed chitosan nanoparticles loaded with hydrochlorothiazide, it showed less destruction to intestinal epithelium when compared to free hydrochlorothiazide administration [162]. Nevertheless, this study also showed some damage from the chitosan nanoparticles, with some examples of the damages to the intestine include loss of epithelium in some areas, desquamation of epithelial cells, decreased microvilli present on microvilli and an opposite effect of a higher number of goblet cells. An experiment that puts the result of the zebrafish experiment into doubt is an experiment that was performed on rainbow trout fish, where the fish were treated with nanochitosan/zeolite composite would improve the structure of the cells within the intestine of rainbow trout as opposed to damaging them [163].

Muscle toxicity reported in the zebrafish embryos is another example of organ toxicity reported in this review (Z. [111]). Once again, some experiments have results that seem to suggest otherwise. A study that was completed with C2C12 myoblast cells showed that after a treatment lasting for 12 h, no cytotoxicity was induced to the cells, with all of the samples showing a cell viability of more than 80% and that any difference observed from the control group was not significant [164]. Cell proliferation inhibition and heightened levels of apoptosis were shown in another experiment done on human smooth muscle cells from human umbilical cords, where the cells are treated with hydroxybutyl-chitosan nanoparticles loaded with small interfering RNA (siRNA) [165]. However, the second experiment actually reveals a usefulness in this finding, in that vascular smooth muscle cells replicating represents one of the occurrences in cardiovascular conditions developing [166,167], so by increasing the apoptosis and inhibiting replication of cells via tissue factor, it will mitigate the process of the cardiovascular diseases arising [165].

The fourth domain that has toxicity observed in it is the nervous system, where two studies have shown some signs of neurobehavioral impairment [109,111]. Meanwhile in contrast, one study failed to show any toxicities in the brains of Wistar rats that were intranasally given the nanoparticles [105]. For the case of nanoparticles being toxic to the brain, one study showed that in the cerebellum and frontal cortex of male Sprague-Dawley rats to have chitosan nanoparticles modified with polysorbate-80 concentrating in those parts when introduced into the rats [168]. Not only did the Sprague Dawley rats lost weight, affected areas exhibit cell apoptosis, inflammation, and oxidative stress [168]. However, another experiment showed that instead of the destruction of cells, chitosan nanoparticles seem to help in increasing the concentration of acetyl-11-keto-β-Boswellic (AKBA) when it is loaded into O-carboxymethyl chitosan nanoparticles, which then enhances the protective effect to Sprague-Dawley rat cortical neurons when exposed to the nanoparticles [169]. In the experiment, the nanoparticles were shown to be significantly more effective in reaching in the plasma as compared to the free AKBA [169] which was soluble in fat instead of water [170,171]. Other than in the plasma, other organs such as the brain and liver showed a significantly higher concentration of the nanoparticles loaded with the AKBA when compared against the free AKBA, whereas the opposite was true when organs such as the kidney and the spleen was observed [169]. The most important finding relevant to the neural toxicity in the paper, however, will be the cell viability and

the LDH levels after the cortical neurons were incubated with the free AKBA and the nanoparticles loaded with the AKBA. Here, two models to simulate ischemia, oxygen glucose deprivation (OGD) and middle cerebral artery occlusion (MCAO) were utilized. To summarize the OGD process, cells would be placed in a medium that is lacking in oxygen, and instead have nitrogen and carbon dioxide culture medium with no glucose [172]. On the other hand, middle cerebral artery occlusion is performed by inserting a suture from an isolated external carotid artery to the middle cerebral artery to occlude it [173]. Evidently, with the higher levels in the brain, the nanoparticles loaded with the AKBA showed a significantly higher percentage of cell viability when compared to the free AKBA with a *p*-value of less than 0.05 within the OGD model [169]. The MCAO model also showed the AKBA nanoparticles showed a lower score of neurological deficit scores, and also a lower volume of infarct [169].

Nanoparticles and lung damage have been recorded on multiple occasions. For example, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention has stated that with nanoparticles made from titanium oxide (TiO_2) have the potential to cause cancers and that it can happen to workers that have to work with these nanoparticles at the relevant occupations [174]. Other types of nanoparticles that can cause lung cancers include carbon nanotubes [175], cerium oxide (CeO_2) nanoparticles, and silver nanoparticles [176]. One concern of inhaling nanoparticles causing toxicity is that it can accumulate as The International Commission on Radiation Protection (ICRP) has stated that 50% of nanoparticles can reach to the alveoli of lungs in people, and that if the nanoparticles are insoluble, 25% of remains in situ indefinitely [177]. Chitosan nanoparticles, meanwhile, have shown mixed results. For example, one experiment that had Wistar rats inhaled rifampicin filled chitosan nanoparticle showed that the nanoparticles performed favorably to free rifampicin in that the nanoparticle group showed a higher number of surviving and viable cells [48]. However, results that contrast the rifampicin experiment are found in another experiment using A549 lung cancer cells treated with raloxifene, where the nanoparticles showed a higher toxicity when compared to raloxifene administered without the use of any nanoparticle system [97]. Meanwhile, a comparison of A549 treatment with uncoated poly(lactic-co glycolic acid) nanoparticles to chitosan coated nanoparticles revealed that both groups showed similar cell viabilities in a cytotoxic assay and stated that chitosan is not the only parameter for the extent of cytotoxicity in these cells [178].

Ocular toxicity studies of chitosan nanoparticle seem to show minimal toxicity. For example, a modified Draize test performed on albino rabbits showed that there are no toxicities observed in the eyes [107], the description of the Draize test is given in Table. Another experiment using TPP chitosan nanoparticles also failed to show ocular toxicity when Wistar rats were administered 2 g/kg of nanoparticles while they were simultaneously being tested for oral toxicity [110]. Additionally, another method of using chicken hen eggs, called the HET-CAM method, a score of 0 was recorded in one study and suggested that it was appropriate to use chitosan nanoparticles to deliver antibiotics, with the added boon of less frequent dosing and longer exposure to the drug applied in the eye [179]. This benefit of not only having a non-toxic drug delivery system and also increasing the duration of drug release has been recorded in one experiment which showed this property in chitosan grafted polyethylene glycol methacrylate nanoparticles loaded with a monoclonal antibody, bevacizumab [180]. Additionally, chitosan nanoparticles with rosmarinic acid showed no toxicity to retinal and corneal cell lines ARPE-19 and HCE-T cells respectively [92]. In general, the ocular toxicity of chitosan results from the different experiments appear to agree with one another and shows to be a promising delivery method that is safe for the eyes and can also be used as a method of either sustained or controlled drug release.

Cardiotoxicity induced from chitosan nanoparticles seem to be not present according to the experiment performed on the zebrafish embryos [109]. Other studies seem to have suggested by introducing drugs via nanoparticles instead of the free drug seems to be beneficial in terms of the toxicity experienced. One such example of that is an experiment conducted with cancer cells of the liver when albumin-chitosan nanoparticles were adorned with retinoic acid was used to deliver doxorubicin hydrochloride in a targeted manner [181]. This experiment showed that because the concentration at which doxorubicin loaded in chitosan nanoparticles was recorded to have its inhibitory effect on growth of HepG2 cells was higher when compared to than when the same concentration of the drug was not put in a system and free [181]. It was also postulated that in spite of the need to have *in vivo* tests to verify the potential benefit the nanoparticles can give, this formulation can decrease the amount of doxorubicin required to give to a patient requiring the medication, which then consequently reduces the cardiotoxicity of the drug via the chitosan nanoparticles [181]. This result seems to be also seen in another experiment with another two types of chemotherapeutic drugs simultaneously, paclitaxel and doxorubicin [182], loaded into magnetic chitosan nanoparticles with red blood cell membrane coating, also showed an increased duration of the drug's circulation and release, augmenting the cumulation and intake of the nanoparticles in tumors when compared to a more traditional method of using polyethylene glycol to modify the chitosan nanoparticles [183].

Lastly, oral toxicity is to be discussed. In general, toxicity in the oral region remains largely absent. For example, one study that experimented with Wistar rats checked their rate of mortality, physical appearance and also weighed them as the experiment is conducted [110]. The results of the chitosan nanoparticles as compared to the control used in the experiment showed that both groups did not show any illness signs, both have comparable body weights recorded at all days where the measurement was taken, that is on day 1, 7 and, 14, both did not show one rat with irritation or toxicity to the skin and eyes, and both groups did not have any mortality recorded [110]. Moreover, another experiment that also tested on Wistar rats showed similar results [184]. This second experiment used an immunosuppressant called 6-mercaptopurine, which can also be used as a treatment for cancer such as some forms of cancers of the white blood cells (leukemia) [182]. Toxic adverse effect reduction and lethal dose improvement by two times relative to the treatment of using unmodified 6-mercaptopurine are two of the highlights of the study [184],. However, unlike the first experiment where neither group showed any fatalities in rats, this experiment did show some rats dying, where on the 14th day of the experiment using 300 mg per kg body weight had one died in the traditional 6-mercaptopurine group whereas none in the nanoparticle treated group. The next step that was taken was then to dose three of the rats at 2000 mg for every kilogram of the rats' body weight for each group. Two out of the three rats tested in the increased dose of nanoparticles died whereas all three of the rats tested with the increased dose of normal 6-mercaptopurine. When a necropsy was performed, however, no irregularities were found in any of the organs of the rats.

To summarize, the organs that showed the least amount of toxicity amongst all that were discussed are eyes and the mouth and the results of the studies presented seems to point that chitosan is a promising delivery that not only is non-irritating to the eyes but also proven to be able to act as a modified release formulation. This seems to be the case with the heart as well, however, the evidence found for this toxicity are mainly *in vitro* experiments as opposed to the eyes where *in vivo* results were provided. Whereas for the liver, intestine, muscles, lungs and brain, results obtained for these organs are conflicting between different organisms and therefore needs more research to elucidate the trend in toxicity.

Chitosan nanoparticles exhibit minimal cardiotoxicity based on zebrafish embryo experiments, showing potential benefits in targeted drug delivery. Oral toxicity studies on Wistar rats confirm the safety of chitosan nanoparticles, with no adverse effects observed. The results highlight the suitability of chitosan nanoparticles for ocular and oral applications, while further research is needed to investigate toxicity trends in the liver, intestine, muscles, lungs, and brain.

3.4. Recommendations for future research

Many of the results that contradict each other seem to stem from when Wistar rats are compared to Zebrafish models in this report. This may be simply due to the fact now more zebrafish experiments are conducted as it is now a model that is being used more in the present timing and setting [185], thus increasing the amount of studies that have zebrafish models in the first place to be searched. Nevertheless, with such contrasting results in both the Wistar rats and Zebrafish models, one possibility of research is to conduct a study on the differences in the organisms when the same type of chitosan nanoparticle is being administered in terms of looking at the not only the mortality rate of the organisms but also the differences in the development of the organisms as is done in studies that experimented on zebrafish alone. This will allow a direct comparison of the two organisms in terms of knowing the sensitivity of each of the organisms toward chitosan nanoparticles.

At least as of writing this literature review, not one clinical research paper on chitosan nanoparticle toxicity in humans has been published with results. One suggestion for future research would be to research the toxicities of the chitosan nanoparticles in human participants, using different methods of administration.

3.5. Limitations

A limitation of this study is that it does not consider any non-English articles in the review and can miss valuable information on the toxicities of chitosan nanoparticles. With the limited number of staff and no official translator to properly transcribe the non-English articles, they were excluded.

Another limitation of this study is that it did not consider any organisms in which excretory systems are impaired, that is, no tests were done on organisms that had pre-existing liver damage or kidney damage. This is important with respects to any of the systemic formulations as there are important implications in which these nanoparticles are used with potential patients should any of these formulations reach that point of development and use in the medicinal field.

The long-term effects of chitosan nanoparticles on organ systems are crucial to assess their safety. Short-term studies may not capture potential delayed or cumulative toxicity effects that could occur with prolonged exposure.

A larger sample size would provide more robust data and increase the generalizability of the results. Most studies in the research articles have not provided a comprehensive analysis of other potential toxic effects. Thus, the limited scope of toxicity assessment may not capture all potential risks associated with chitosan nanoparticles.

The studies mentioned primarily use animal models, such as albino rabbits and Wistar rats, to assess ocular and oral toxicity. While animal models provide valuable insights, the results may not always directly translate to human responses. Differences in anatomy, physiology, metabolism, and genetic factors between animals and humans can influence the toxicity outcomes. There is lack of clinical trials; without data from human trials, it is challenging to draw definitive conclusions about the safety of chitosan nanoparticles in a clinical setting.

In summary, while the research mentioned in the suggests that chitosan nanoparticles have minimal toxicity in various organs on application, it is important to acknowledge the limitations of the studies. Further research, including larger sample sizes, longer-term studies, comparative assessments, human clinical trials, and a broader range of toxicity evaluations, is needed to comprehensively evaluate the safety and potential risks of chitosan nanoparticles.

4. Conclusion

While toxicity is present in some of the tests given, most of the tests described in this paper showed relatively low to no toxicity. Nevertheless, the exact toxicities in humans are not reported. Furthermore, the results of the different organs and cell lines vary, some of which appear to contradict one another. Moreover, the method of testing the cytotoxicity of each nanoparticle for each cell line and organs differ in terms of not only the reagents, but also the guideline on determining toxicity as well. Potential areas of safe nanoparticle use seems to be favoring oral, ocular and cardiac system for drug delivery, however blood clotting and other organ systems will require further investigation. Recommendations for future studies will have to research the chitosan nanoparticle toxicities in human test subjects so that clinically relevant decisions such as choosing the correct formulation in drug production can be made.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Rajan Rajabalaya reports administrative support and writing assistance provided by the Universiti Brunei Darussalam.

Data availability

No data was used for the research described in the article.

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