ARTICLE

EFFECT OF POLYMER BASED SURFACE COATING ON DROPLET SIZE AND POTENTIAL PHARMACOLOGICAL PROPERTIES OF NEEM OIL NANOEMULSION

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ABSTRACT

Nano emulsion preparations of various phytochemicals are gained increased attention in drug delivery and agriculture than the micro emulsion due to their improved activity, high stability and biocompatibility. In the present study, neem oil based nano emeulsion was prepared under optimum condition by changing oil and surfactant ratio which yield decreased droplet sized particles was further coated with biocompatible polymer chitosan. Distinct effect on droplet size was recorded in chitosan coated neem oil Nano emulsion at the increased concentration of oil; surfactant ratio and increased time of sonication. Anti-bacterial activity was studied against human pathogenic bacterial strains Staphylococcus aureus and Pseudomonas aeruginosa adopting agar cup method and turbidometric method. Chitosan coated neem oil Nano emulsion showed improved spectrum of anti-bacterial activity against tested bacterial strains by showing increase in zone of inhibition and remarkable decrease in optical density under agar cup method and turibometric growth inhibition assay. Minimum Inhibitory concentration (MIC) and Minimum bacteriacidal concentration (MBC) also supported effective anti-bacterial activity by showing least concentration for the growth inhibition than the free Nano emulsion. Biocompatibility was done by determination of cell viability of Vero cells by MTT assay and haemo compatibility study against human peripheral blood cells which reveals less cytotoxicity against Vero cells and blood cells. Distinct effect on the viability was not affected. Further study will helpful to develop polymer based Nano emulsion as an effective pharmacological agent to prevent life threatening diseases.

KEY WORDS
Neem oil, nanoemulsion, Namasivayam, chitosan, droplet size, Professor phytochemical, antibacterial

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INTRODUCTION

Nano emulsion is defined as oil-in-water (o/w) emulsions with mean droplet diameters ranging from 50 to 1000 nm. Usually, the average droplet size is between 100 and 500 nm. Nano emulsions can be prepared using the spontaneous emulsification mechanism which occurs when an organic phase and an aqueous phase are mixed. The organic phase is a homogeneous solution of oil, lipophilic surfactant and water–miscible solvent, the aqueous phase consists on hydrophilic surfactant and water [1,2] Unlike micro emulsions (which are also transparent or translucent and thermodynamically stable) Nano-emulsions are only kinetically stable.

Nano emulsions hold great promise as useful dispersions of deformable Nano scale droplets that can have flow properties ranging from liquid to highly solid and optical properties ranging from opaque to nearly transparent. Moreover, it is very likely that Nano emulsions will play an increasingly important role commercially, since they can typically be formulated using significantly less surfactant than is required for nanostructured lyotropic micro emulsion phases [3]. Unlike micro emulsions (which require a high surfactant concentration, usually about 20% and higher), Nano-emulsions can be prepared using lower surfactant concentration, a surfactant concentration comprised between 3–10% may be enough. The small size of the droplets for cutaneous use allows them to deposit uniformly on skin [4].

Nano-emulsions are suitable for efficient delivery of active ingredients through the skin. The large surface area of the emulsion system, the low surface tension of the whole system and the low interfacial tension of the O/W droplets allow enhancing penetration of actives agents. Due to their small size, Nano-emulsions can penetrate through the “rough” skin surface and this enhances penetration of actives [5,6]. The fluidity nature of the system (at low oil concentrations) as well as the absence of any thickeners may give them a pleasant aesthetic character and skin feel. Nano-emulsions can be applied for delivery of fragrance, which may be incorporated in many personal care products. This could also be applied in perfumes, which are desirable to be formulated alcohol free [7].Nano-emulsions may be applied as a substitute for liposomes and vesicles (which are much less stable) and it is possible in some cases to build lamellar liquid crystalline phases around the Nano-emulsion droplets. Nano-emulsions constitute the primary step in Nano capsules and Nano spheres synthesis using Nano precipitation and the interfacial polycondensation combined with spontaneous emulsification [8].These two techniques require the spontaneous emulsification step in the same optimize conditions. The droplets size and size distribution are depending on the spontaneity of emulsification. The spontaneity of the emulsification is poorly defined, since it should account not only for the rate of the emulsification process, but also for the volume and the particle size distribution of the produced emulsion [9].In the present study, neem oil Nano emulsion stabilized with chitosan was prepared and the prepared Nano emulsion was evaluated against droplet size and anti-bacterial activity has been carried out.

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MATERIALS AND METHODS

Preparation of free neem oil nano emulsion

Initially, free neem oil Nano emulsion was prepared by emulsification process by changing the concentration of oil, water and surfactant ratio. Tween 80 (Hi media, India)was used as the surfactant The reaction mixture contains oil, surfactant and distilled water with the ratios of 1:1.5, 1:2.5, 1:5, 1:7.5,Reaction mixture thus obtained was kept under ultra-sonication for 15, 30, 45, 60 minutes. Droplet size was studied after every treatment using DLSI device.

Preparation of polymer coated nano emulsion

Chitosan was used in the study as the stabilizer. Chitosan (extra pure) was obtained from SRL, India Chitosan (0.5%) dissolved in distilled water containing 1% acetic acid. Dissolved chitosan suspension was kept under magnetic stirrer for 2 hours. After stirring, one ml of homogenized chitosan solution was added to the respective reaction mixture (prepared in the respective ratio), kept for sonication at different time periods as described earlier.

Evaluation of biological activities

Anti-bacterial activity

Anti-bacterial activity of Nano emulsion was studied against human pathogenic bacterial strains *Pseudomonas aeuroginosa* and *Staphylococcus aureus* adopting well diffusion assay. Both the strains were obtained from Microbial type culture collection (ATCC) and maintained on Tryptic soy agar (TSA) slants. A loopful of slant culture was inoculated into tryptic soy broth and incubated at 37°C for 12-16 hrs.to reach mild log phase. The respective broth culture was uniformly spread with sterile cotton swabs on sterile Mueller Hinton (MH) Agar Media (Hi-media, India). The wells were made using cork borer and aliquots (50 and 100 µl) was loaded into the wells. The plates were incubated at 37°C for 24 hrs.

Determination of Minimum Inhibition Concentration (MIC)

Anti-bacterial activity was also studied by determination of minimum inhibitory concentration micro dilution calorimetric assay using the chromogenic reagent 3-(4, 5-dimethyl thiazol-2-yl)-2-5-dephenyl tetrazolium bromide (MTT) concentration [10]. Minimum inhibitory concentration (MIC) value was defined as the lowest sample concentration that inhibited visible growth of the test bacterium, as indicated by MTT straining. Only living microorganisms can convert MTT to formaldehyde and a blue color appear.

Biocompatibility studies

Biocompatibility of chitosan coated neem Nano emulsion was studied by determination of cytotoxic effect on Vero cell line by MTT assay. Heamo compatibility against human peripheral blood was also used in this present investigation to evaluate biocompatibility.

Cytotoxicity assay

Chemicals

RPMI1640, fetal bovine serum (FBS), Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Hi media & Sigma Aldrich Mumbai.

Cytotoxicity assay

Inhibition of cell growth of Vero cell line using a tetrazolium dye (MTT) assay and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the untreated ones. Cell line was obtained from National center for cell sciences (NCCS), Pune, India. RPMI1640 was used as the source of cell growth medium and a humidified atmosphere (d 5% CO 2) was maintained for cell culture. Cells harvested in a logarithmic growth phase were seeded on 96 wells at a cellular density of 5x103 cells / ml followed by the addition of different concentrations, incubated for 24hrs at 5 % CO2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl–tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The effect of the Nano emulsion on the proliferation of cells was expressed as the % cell viability.

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In vitro blood cell compatibility

Further confirmation of biocompatibility was carried out by haemo compatibility under laboratory condition. Peripheral EDTA blood sample was used in the study. Collected blood samples were diluted with saline suspension. 0.9 ml of diluted blood sample and 0.1 ml of free Nano emulsion and chitosan coated Nano emulsion was taken in a centrifuge tube, incubated for 3 h at 37° C under shaking. After the incubation, plasma was collected by centrifuging the samples at 4500 rpm for 10 minutes. The concentration of hemoglobin in the plasma was quantified by spectrophotometry; plasma hemoglobin concentration directly correlates the percentage of lysed blood cells. Plasma hemoglobin concentration was quantified spectro photometrically.

RESULTS

Free and chitosan stabilized neem oil nano emulsion

Droplet size of neem oil Nano emulsion was greatly influenced by the amount of oil -surfactant ratio used and time of sonication Among the different condition, decreased droplet size was recorded in increase in surfactant concentration and time of sonication (Figure 1).The smallest droplet size of Nano emulsion was reported in 1:7.5 for oil and surfactant ratio and 60 minutes of sonication and the average droplet size was found to be 60.10 nm. .In the present study, emulsion with the mean droplet size as low as 169 nm was obtained in the presence of high oil-water concentration and maximum sonication time (60 minutes).

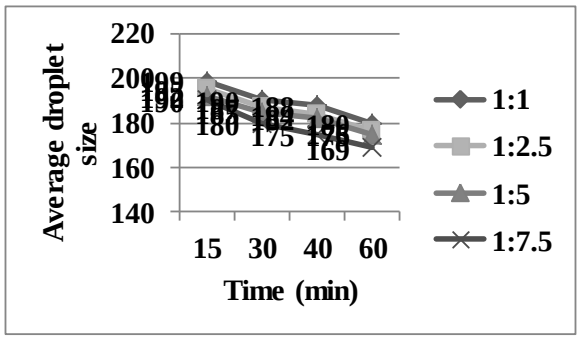

Fig. 1: Effect of sonication on droplet size of 1:7.5 ratio of oil-water; surfactant ratio.

Chitosan stabilization on Nano emulsion showed distinct effect on the droplet size. Droplet size was found to be decreased in all the tested parameters [Fig. 2]. As in free Nano emulsion, surfactant concentration and ultra-sonication influenced droplet size. Less droplet size was recorded in 1:7.5 oil-water; surfactant concentration at maximum ultra-sonication time. But in all the tested conditions (oil-water; surfactant ratio, ultra sonication time), lesser droplet size has been interfered from chitosan stabilized Nano emulsion than free neem oil Nano emulsion.

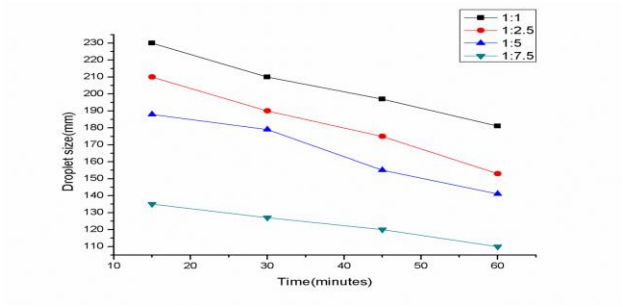

Fig. 2: Effect of chitosan coating on droplet size of neem oil Nano emulsion at different time of sonication.

Anti-bacterial activity

Anti-bacterial activity of chitosan stabilized neem oil Nano emulsion was studied against *P. aeruginosa* and *S. aureus* by well diffusion assay and micro dilution colorimetric MTT growth inhibition assay [Table 1].

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Table 1: Effect of chitosan stabilized neem oil Nano emulsion on antibacterial activity of pathogenic strains by agar diffusion assay

Treatment	Zone of inhibition (mm)	
	P.aeruginosa	*S. aureus*
Free neem oil Nano emulsion (50 µl)	12.0	11.0
Free neem oil Nano emulsion (100 µl)	14.0	12.5
Chitosan stabilized neem oil Nano emulsion (50 µl)	21.0[a]	19.0[a]
Chitosan stabilized neem oil Nano emulsion (100 µl)	23.0[a]	20.0[a]
Negative Control	0.0	0.0

[a] -Column carries alphabet is statistically significant at 5 % level by DMRT

Results showed that both the tested bacterial strains were susceptible to the Nano emulsion. But chitosan stabilized Nano emulsion brought about enhanced activity against the both tested strains. An increase in zone of inhibition was recorded in chitosan coated Nano emulsion than the free Nano emulsion. Improved anti-bacterial activity was also supported by colorimetric liquid MTT assay which reveals effective growth inhibition of chitosan coated Nano emulsion against both the tested bacterial strains as dose dependent manner. In *P.aeruginosa*, free Nano emulsion brought about 37 % of inhibition at high dosage and the inhibition was increased in chitosan coated Nano emulsion as 85 % [Fig. 3]. Similar finding was also recorded in *S. aureus*. High rate of growth inhibition was found in chitosan coated Nano emulsion treatment [Fig. 3, 4].

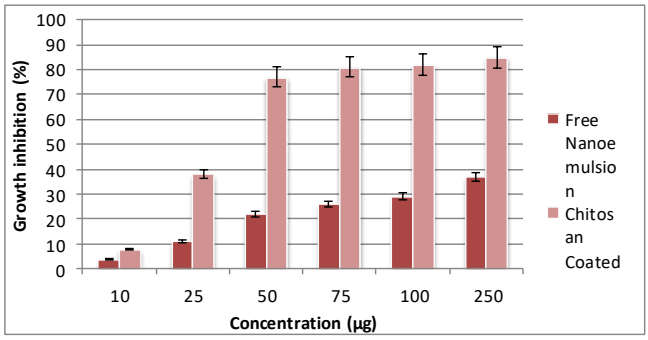

Fig. 3: Effect on free and chitosan coated Nano emulsion on growth inhibition (%) of *P.aeruginosa*.

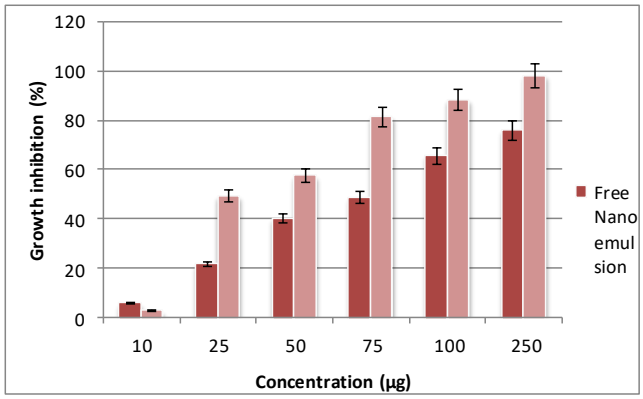

Fig. 4: Effect on free and chitosan coated Nano emulsion on growth inhibition (%) of *S. aureus*.

Biocompatibility studies

Nontoxic effect of tested Nano emulsion was also studied in the present study. Cytotoxicity tests against Vero cell line and peripheral cells (haemo compatibility) were carried out to determine biocompatibility. Colorimetric micro titre plate MTT assay was used to determine cytotoxicity which shows less toxic effect of both the free and chitosan coated Nano emulsion [Table 2].Cell viability was retained in maximum concentration. Above 99 % of cell viability was noticed in least dosage of Nano emulsion. Similar results have been observed in peripheral blood cells (hemo compatibility test. [Table 3] depicts hemolysis of tested emulsion against human peripheral blood which indicates both free and chitosan coated Nano

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emulsion exhibited very less toxic effect in all the dosages. Interestingly, hemolysis was low as chitosan coated Nano emulsion showed as The present findings would suggests the possible utilization of chitosan based Nano emulsion as an effective anti-microbial agent against life threatening disease causing organisms.

Table 2: Effect of free Nano emulsion (FN) and chitosan coated Nano emulsion (CS-NE) on hemolysis of peripheral blood

	Free Nano emulsion	Chitosan coated Nano emulsion
Concentration (µg)	Cell viability (%)	Cell viability (%)
1000	15.2	7.2
750	10.2	4.2
500	7.2	2.2
250	3.4	1.1
125	1.3	0.6
62.5	0.6	0.2
31.2	0.2	0.07
15.6	0.09	0.03

Table 3: Effect of free Nano emulsion (FN) and chitosan coated Nano emulsion (CS-NE) on the cell viability (%) of Vero cell line

	Free Nano emulsion	Chitosan coated Nano emulsion
Concentration (µg)	**Cell viability (%)**	**Cell viability (%)**
1000	87.4	91.2
750	89.2	92.1
500	92.2	94.2
250	94.1	96.2
125	96	97.2
62.5	97.1	99.2
31.2	98	99.4
15.6	98.5	99.5

DISCUSSION

Nano emulsions are emulsions with droplet size on the order of 100 nm. A typical Nano emulsion contains oil, water and an emulsifier. Nano emulsions have lot applications in the field of medicine as drug delivery agent, food sector and agriculture [2]. Success of Nano emulsion is primarily based on its stability and the various factors. There are various factors which control the stability. Nano emulsions are kinetically stable and given sufficient time, will separate into different phases. Different destabilization mechanisms of Nano emulsions namely flocculation, coalescence, Ostwald ripening and creaming/ sedimentation lead to reduce the uses of Nano emulsion [12].Destabilization can be controlled by various factors which have been improving the stability. In the present study, chitosan stabilized neem oil Nano emulsion was prepared by changing oil-water; emulsifier ratio under sonication at different time periods which exhibits distinct changes on the droplet size and biological activities. The present study, neem oil nano emulsion prepared under specific condition by changing oil-water surfactant ratio at different time of sonication which reveals decrease droplet size of emulsion was observed at increasing time of sonication, oil-water surfactant ratio. Similar finding has been reported by Ghotbi et al [13] Rather than surfactant concentration, ultra sonication play a vital role on the droplet size determination of Nano emulsion. Kentish et al [16] observed droplet size of flaxseed oil Nano emulsion was found be decreased in maximum ultra-sonication speed and time. Emulsion stability is dependent on role of surfactants, its composition and the drop size distribution. Nano emulsions exhibit stability against sedimentation or creaming due to the small size of droplets. Diffusion rate and Brownian motion exhibited by these droplets. Predominates over sedimentation/creaming rate is found due to gravity. Flocculation does not occur in Nano emulsions prepared by using nonionic surfactants as no attractive forces are created. Nano emulsions may remain stable for a short span to years depending on how they are formulated and other process parameters involved in formation. They are sometimes referred to as “approaching thermodynamics Coating of nano materials by polymer is an effective strategy for functionalization. In this study, chitosan was selected. Chitosan is an important plant based polymer Because of the biocompatibility, biodegradability, nontoxicity and adsorption properties of chitosan, it is recommended for stabilization [17].In this study, distinct reduction of particle size was recorded in chitosan coated neem oil nano emulsion which was observed in all the tested concentration of oil-surfactant water ratio and the sonication time. Anti-bacterial activity of both free and chitosan coated neem oil emulsion was studied

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against human pathogenic bacterial strains by well diffusion assay and MTT micro dilution tube assay. Maximum growth inhibition was recorded in chitosan coated Nano emulsion treatment. In general the droplet size reduction cause increased surface area, the number of active groups and determine the increase in antibacterial activity. Chitosan coating reduced the droplet size of the tested Nano emulsion known to improve the anti-bacterial activity as reported in the earlier studies [13]. Biocompatibility was studied by determination of cell viability and hemolysis against vero cell line and human peripheral blood cells which shows both the free and polymer coated nano emulsion did not induce any toxicity. Further study using animal model will be helpful to utilize nano emulsion based on chitosan coated neem oil as an effective anti-bacterial agent.

CONCLUSION

Functionalization of nano materials by polymers is an attractive field of nano science and nanotechnology which have a lot of application in the biomedicine field. The present study, preparation of chitosan coated neem oil nano emulsion prepared by changing oil-water-surfactant concentration under different time of sonication reveals reduced droplet sized particles which brought about enhanced antibacterial activity against human pathogenic bacterial strain and less cytotoxic effect against vero cell line and human peripheral blood cells .This study findings would suggest the possible utilization of polymer coated nano emulsion as an effective and biocompatible antibacterial agent.

CONFLICT OF INTEREST
There is no conflict of interest.

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None

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