Formulation of *Zingiber Officinale* Essential Oil Based Nanoemulsion and Evaluation of Its Neuroprotective Effect Against Hydrogen Peroxide Induced Cytotoxicity in SH-SY5Y Cell Line

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Abstract

The increasing prevalence of neurodegenerative disease underscores the immediately need for effective treatment of neuronal cell death. Free radical scavaning is a major drawback for neurodegenerative disorder. The aim of current study is to preparation of nanoemuslion using surfactant tween 80, water and ginger essential oil rich in zingiberene and also to evaluate the neuroprotective potential of *Zingiber officinale* nanoemulsion against Hydrogen Peroxide induced cytoxicity in SHSY5Y cell line, essential oil renowned for their antioxidant and anti-inflammatory properties. The formulated nanoemulsion has particle size in the nanomeric range and negative zeta potential and low PDI value indicate the stable formulation. The nanoemulsion employed enhances the oral bioavailability and stability of these compounds, offering superior therapeutic potential compared to conventional formula. SHSY5Y cell line from the current study it was resulted out that *zingiber officinale* nanomulsion 50, and 100µg/ml doses significantly reversed the effect of hydrogen peroxide induced toxicity in SHSY5Y cell lines. The result showed that *zingiber officnale* nanoemulsion is a promising way for the treatment of neurodegenerative disorders.

KEYWORD: *Zingiber officinale*, nanoemulsion, particle size, SHSY5Y cell line, neurodegenration.

Introduction

In Western countries, people having age more than 65 years progressively suffering from age likes neurodegenerative disorder. In today's time alzhimer's disease is the main reason approximate greater than 26 million people are suffering from this disease. This number may be expected to increase four times in 2050. Yet today's world there is no effective treatment available for age related neurodegenerative disorders, which tend to move in irreversible way and are associated with social-economic and large personal costs (1-2). Neurodegenerative

disorders are categorized by oxidatice stress, neuroinflammation, and synaptic dysfunction, protein miscoding leads to brain damage, and neuronal apoptosis (3-4). In case of Alzheimer's disease, oxidative stress leads to mitochondrial dysfunction, the intracellularly in the form of of neurofibrillary tangles hyperphosphorylated tau (τ) proteins accumulated and extracelluarly plaques of beta-amyloid (Aβ) excessively accumulated, also genetic and some environmental factors are associated. For the treatment of this type disorders and finding of new herbal drugs is the main task for scientific research (5). The drugs derived from the plant are showing promising antioxidant and anti-inflammatory actions. In last few years ginger and ginger-derived bioactive compounds have been explored for their pharmacological effect. Ginger biological source is *Zingiber Officinale* belonging to family *Zingiberace* is a member of perennial herb. Its rhizomes are much popular due to uses as additive agent and spices in drinks and food items for the purpose of flavour (6-7). It is considered that ginger is originated in India or South-East Asia. The *Zingiber Officinale* varies in different bioactive constituent as growing in different area and also varies with drying technique. Generally, *Zingiber officinale* rhizome mainly contains small amount of essential oils, starch, oleoresins, sugars, mineral salts, mucilage, gum, starch, and organic acids (8). About 40-60% of rhizomes dry weight is starch. *Zingiber officnale* contains many bioactive constitutes that paly valuable role in biological activity and play a crucial role in management of many disease. The key active ingredients are 6-gingerol and 6-shogaol. Along with this ginger also contains 10-gingerol, gingerdiones, gingerdiols, 5-acetoxy-6-gingerol, paradols, 6-dehydrogingerols, 12- gingerol and 3, 5-diacetoxy-6-gingerdioal. In ginger essesntial oil the main constitutents is alpha-zingiberene (9-11). In recent years, it was found that ginger was evaluated for their pharmacological potential. Ginger show potential effect as an antioxidant, antifungal, anti-allergic, antimicrobial, anti-inflammatory, antihyperlipidemic, antiobesity, anticancer, antitumorigenic, immunomodulatory, antiapoptotic, antihyperglycemic, and antiemetic. (12-20). The various health promoting effects have been reported for its essential oil in most previous researches, like strong antioxidant, antifungal, and antibacterial activities. Though, as other functional their absorption into water based formulations are challenging, because of low water solubility. Additionally, it was also noted that these compounds are susceptible to light, oxygen, moisture and heat and also less chemical and structural stability (1). So the essential oil dispersion into water as nanoemulsions, using technique offered by nanotechnology, may be play a key role for above shown problem (21-22). Thus the aim of current study is the preparation characterizations and in-vitro evaluation of Z*ingiber officinale* essential oil derived emulsion based formulation.

In recent times, micro-emulsion or nanoemulsion formulations have received a lot of attention due to their potential effect in different variety of applications, including food, cosmetic and pharmaceutical industry. Nanoemulsions are ultrafine dispersion of oil-in-water have particle size range of 10–600 nm (23). Nanoemulsions with extremely small diameters are kinetically stable and may have a relatively high kinetic equilibrium for many years, which has distinguished them from other micro-sized dispersion systems. The high energy homogenization technique is used for the preparation of nanoemulsions. According to various previous researches, both processing and formulation parameters have considerable effects on characteristics of produced nanoemulsions, considerably (24-26, 36). Thus, the most desired

nanoemulsions with optimum characteristics such as minimum mean particle size and size distribution or maximum net zeta potential and antioxidant activity can be gained by tuning and modification of these parameters (27).

Material and Method

Essential oil extraction and compound analysis

Fresh rhizomes *Zingiber officinale* were collected from a local market of Rohtak, Haryana, India-124001. The essential oils were extracted from these rhizomes by clevenger appratus as previously described (29). The essential oil compositions were analysed by gas chromatography mass spectroscopy (GC-MS).

GC–MS analysis

The oil compositions were analyzed by gas chromatography mass spectroscopy (GC–MS) in Central Instrument Laboratory, Central University of Punjab, Bathinda, Punjab. using an Shimadzu QP 2010 Ultra GCMS chromatography device (Shimadzu Japan) the equipment is equipped with MS, ECD and FID detector, fitted with fused silica analytical column. The analytical conditions were; carrier gas: helium (ca. 1.0 mL/min), injector temperature: 250°C; oven temperature: 40°C, injection mode: split; flow control mode: pressure; pressure: 49.5kPa; total flow: 14.0ml/min; column flow: 1.0ml/min; split ration:10.0. The programmed temperature retention indices (RI), area curve and % area were obtained by GC–MS analysis of an aliquot of the volatile oil spiked with an n-alkanes mixture containing each homologue from n-C11 to n-C27. The identification of the compounds was based on a comparison of their mass spectra database (NIST) and spectroscopic data. The percentage amount of each component was calculated based on the total area of all peaks obtained from the oil. The data obtained were show the different kinds of bioactive constitute presents in essential oil.

Nanoemulsion Preparation

Zingiber officinale essential oil, nanoemulsion was preapered using a non-ionic surfactant Tween 80 and distilled water. The oil-in-water type nanoemulsion was formulated. Because of high hydrophilic-lipophilic balnce value of Tween 80 it was considered that this surfactant is best suitable for the preparation of oil-in-water nanoemulsion. Tween 80 also a small molecules surfactant. So it effectively decreases the droplet diameter as compared to other type of polymer. Initially, coarse emulsion was formulated by mixing water to organic phase consisting of oil and surfactant in varying range 1:1, 1:2, and 1:3 (v/v) by using homogenizer at 1000 rpm for 30 min (26,29). Furthermore the coarse emulsion was then subjected to ultrasonic emulsification using Sonicator. *Zingiber offiinale* essential oil based coarse emulsion. Sonication generates disruptive forces that reduce the droplet diameter converting coarse emulsion to nanoemulsion. Then, the formulated nanoemulsion was characterized and also stability of the emulsion was investigated.

Table-1 Composition of different *Zingiber officinale* essential oil nanoemulsion

Formulation Code	Oil: surfactant ratio v/v	% composition of different components in formulations		
		Oil	Surfactant	water
ZOEF1	1:1	6	6	88
ZOEF2	1:2	6	12	82
ZOEF3	1:3	6	18	76

Characterization of Emulsion

Characterization of Physico-Chemical Properties

The pH of the emulsion was study by the help of pH meter. All the experiments were done in triplicates.

Stability Study

Thermodynamically stability of formulation was done by centrifugation of emulsion formulation at 10,000 rpm for half hour and after that it was checked for any phase separation or not. Heating–Cooling cycle of formulated nanoemulsion was carried out by alternately keeping at 40 C and 4 C for 48 h. This process was repeated for thrice times for checking the nanoemulsion stability at different degree of temperature. Freeze–Thaw stress was also done by keeping formulation at -21C and 25C alternatively for 48 h at each temperature. This cycle was repeated for two times. The experiment was performed in triplicates.

Dispersibility test

This test was conducted using a standard USP-II dissolution apparatus. Approximately 1 mL of each formulation was added drop wise to 500 mL of water at 37 ± 0.5 ºC. A standard stainless steel dissolution paddle rotating at 50 rpm provided gentle agitation. The formulations were visually assessed with the grading system i.e., grade A (rapidly forming nanoemulsion within 1 min, clear and transparent), grade B (rapidly forming nanoemulsion slightly less clear emulsion), grade C (fine milky emulsion), grade D (grayish-white emulsion), and grade E (poor emulsion).

Measurement of Particle Size, Polydispersity index and Zeta Potential

Particle size distribution, polydispersity index (PDI) and zeta potenial of *Zingiber officinale* essential oil nanoemulsion formulation was determined using a Malvern size analyzer (Brookhaven Instruments Corporation, USA). Prior to experiment, formulated emulsion was diluted with double distilled water to do away with the effect of viscosity caused because of ingredients and furthermore to trim down multiple scattering effect.

Cytotoxicity Study of the Nanoemulsion on SHSY5Y Cell Lines

The cell line study was carried in Aakar biotechnologies Private Limited, Lucknow**.**

Neutral Red Uptake Assay

Cytotoxicity of the Nanoemulsion on SHSY5Y cell line (Procured from NCCS Pune) was determined by NRU (Neutral Red Uptake) Assay. The Cells (5000-8000 cell/well) were cultured in 96 well plates for 24 h in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L) supplemented with FBS (Fetal Bovine Serum – HIMEDIA-RM 10432) and 1% antibiotic solution at 37˚C with 5% CO_2. Next day medium was removed and fresh culture medium was added to the defined wells and treated plate. 5 µl of Treatment dilutions (of different concentrations) were added to the defined wells of the plate were incubated for 24 h. 100 µl of NRU (SRL Chem-36248) (40 µg/ml in PBS- Phosphate buffered saline) was added to the defined wells and incubated (Heat Force-Smart cell CO_2 Incubator-Hf-90) for 1h. After that medium was removed, NRU was dissolved in 100 µl of NRU Destain solution. Finally plates were read at 550/660 nm using Elisa Plate Reader (iMark BioRad-USA).

Statistical Analysis

All Results were described as mean ± standard deviation. Statistical analysis was conducted using the One-way ANOVA, followed by Tukey's post-hoc test using Graph pad Prism 5, with $p<0.05$ being accepted as statistically significant value

3. RESULTS AND DISCUSSION

GC-MS Analysis of zingiber officinale essential oil il was identified by GC-MS analysis. Figure 1 shows the gas chromatograph of zingiber officinale essential oil. Zingiberene was found to be the major component of the oil with 33.31 % of total peak area as shown in figure-1 peak 21.

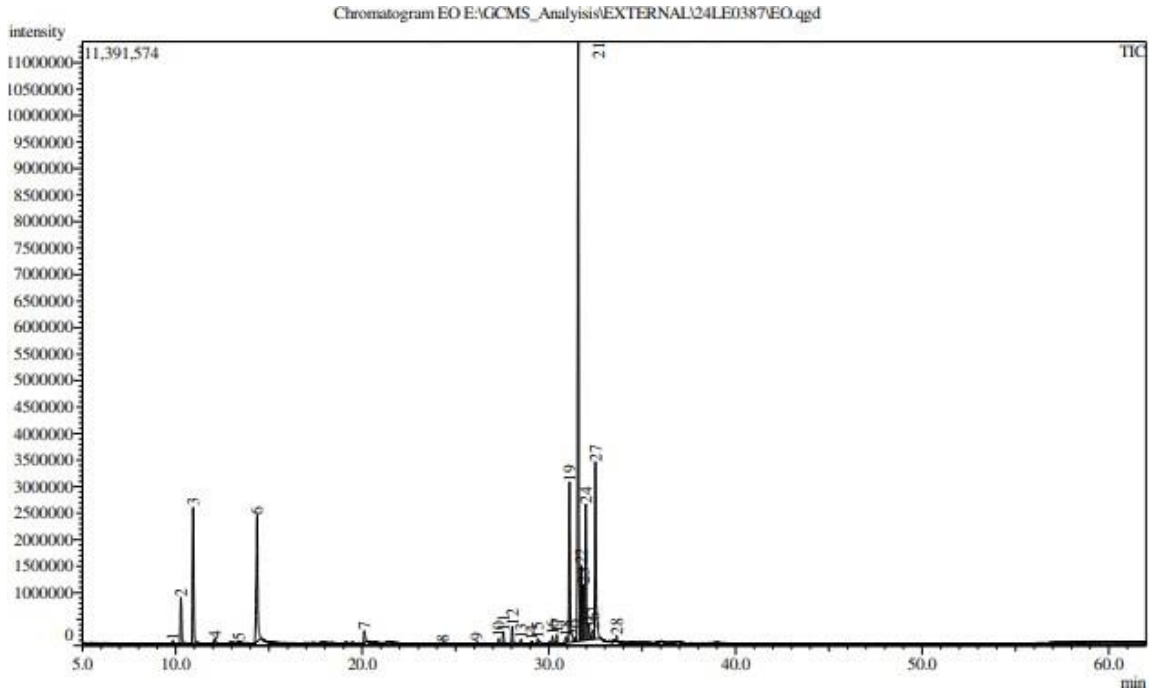

Figure-1 GC-MS of Zingiber Officiale essential oil

3. Physicochemical Characterization

As shown in table-2 higher concentration of surfactant in nanoemulsions indicates the higher percentage of Transmittance as shown in table 2. It was noted that surfactant concentration affects the visual appearance of nanoemulsions as shown from off white to milky white. This increase in percentage Transmission may be due to reduction in droplet diameter with increase in surfactant concentration. The pH of all the formulation varied in range of 6.0 to 6.5.

Stability of Emulsion

Increase in surfactant concentration considerably affected the stability of Zingiber officiale nanoemulsions. Stability of nanoemulsions under study was increased with increasing concentration of surfactant. ZOEF1 fail the freez-thraw cycle where creaming could observed as shown in table 2

Table:2 Physicochemical characterization and stability of Zingiber Officinale Nanoemulsion

Formulation	Dilution with water	Centrifugation	Freeze-thraw cycle	Heating-Cooling Cycle	Visual Appearance	pH	% Transmittance
ZOEF1	**A**	**X**	X	√	Off White	6.4	68.42
ZOEF2	**A**	√	√	√	Milky White	6.5	85.48
ZOEF3	**B**	√	√	√	Milky White	6.5	92.62

ZOEF*- *Zingiber officinale* nanoemulsion

Nanoemulsion Characterization

The physicochemical characteristics of drug-loaded formulations are shown in Table 3. Particle size distribution is an important characteristic affecting the in vivo fate of nanoemulsion. The rate of drug relase is widely influence the particle size of nanoemusion (30, 31). The efficacy of nanoemulsion in neuronal cell also depends on permeability of particle size which influences the treatment. The average particle sizes of all the formulations are in the nanometric range. The surfactant ration varies the particle size increasing concentration of Tween 80 decrease the particle size as shown in table 3 and figure 2. According to previous study it has shown that formulation in nanoscale can enter directly in the systemic blood circulation by the paracellular pathway, which enhances the bioavailability of sparingly soluble drugs (32). Polydispersity index (PDI) is a measure of the uniformity of particle size in the formulation. The PDI values for all Nanoemulsion were shown in (Table 3), the PDI of ZOEF3 was 0.242 and ZOEF2 is 0.298 which indicating the uniformity of droplet size in formulation. Zeta potential play important role in emulsification efficacy of nanoemulsion. Each particle surface has a net charge that causing them to repel each other. In current study nanoemulsion formulated with tween 80 as surfactant showed negative zeta potential value was ZOEF3 (-14 mV), followed by ZOEF2 (-8.58mV) which indicate the greater stability. The zeta potential of ZOEF1 was highest (-2.58) which indicate that the formulation is unstable. However phase separation was occur in formulation ZOEF1 after centrifugation. From all three drug-loaded nanoemulsions, the optimized formulation was ZOEF3, with a desirable size (235.2± 3.35nm) as shown in figure-3, lower PDI value (0.247±0.07), higher negative zeta potential (-14±0.05) as shown in figure-4; this was therefore selected as the final *Zingiber officinale* nanoemulsion formulation (ZOEF3) to be used for assessing its neuroprotective effects on the human neuronal cell lines.

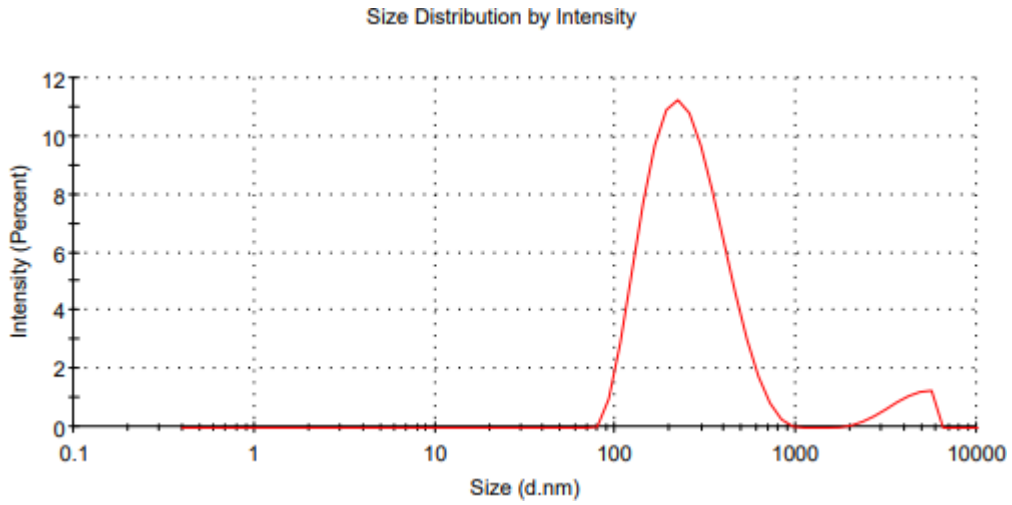

Figure: 2 Average Particle size of *Zingiber officinale* nanoemulsion

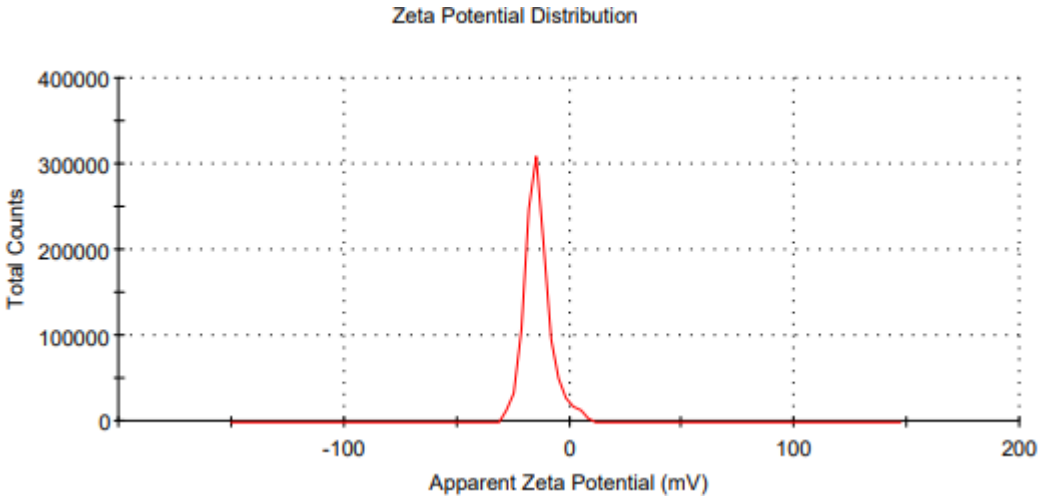

Figure: 3 Zeta potential of *Zingiber officinale* nanoemulsion

Table: 3 Characterization parameter of Nanoemulsion

Formulation	Oil: Surfactant	Particle Size (nm)	Zeta Potential (mV)	PDI
ZOEF1	1:1	303±17.52	-2.58±.020	0.412±0.12
ZOEF2	1:2	268±9.65	-8.38±0.05	0.298±0.06
ZOEF3	1:3	235.2±3.35	-14.00±0.08	0.247±0.07

Assesment of Hydrogen Peroxide (H_2O_2) and Zingiber Officinale Nanoemulsion cytotoxicity on SH-SY5Y Cell Viability

H_2O_2 induced cytotoxicity is a well-known model for various adversarial effects, counting decrease cell viability, increase production of reactive oxygen species, impaired enzyme scvanging activity, inflammation, and apoptosis. In this neurotoxic model, the SH-SY5Y cells were treated with different concentrations of H_2O_2 (0, 25, 50, 100, 200, 400, and 800 µM) for 24 h, after that evaluation of cell viability using the NRU assay. According to previous study it was concluded that the treatment with 200 µM H_2O_2 was the exact condition for subsequent study.

The neurotoxicity of *Zingiber officinale* nanoemulsion was examined by exposing the SHSY5Y cells with different concentration (0, 1, 10, 50, 100, 250, 500, 1000 µg/ml) of formulation as given in **figure-4**. The results indicates that the cell viability decrease with the concentration in dose dependent manner. Significantly reduction of cell viability was seen in

50 and 100µg/ml dose ($p < 0.01$, 0.001, 0.001, 0.001, and 0.001, respectively compared to the control group). Hence it was resulted that dose of 50 and 100µg/ml were maximum non-toxic dose for SHSY5Y cell Line. For the evaluation of protective effect of *Zingiber officale* nanoemulsion these dose were selected for further identification against H_2O_2 induced neurotoxicity

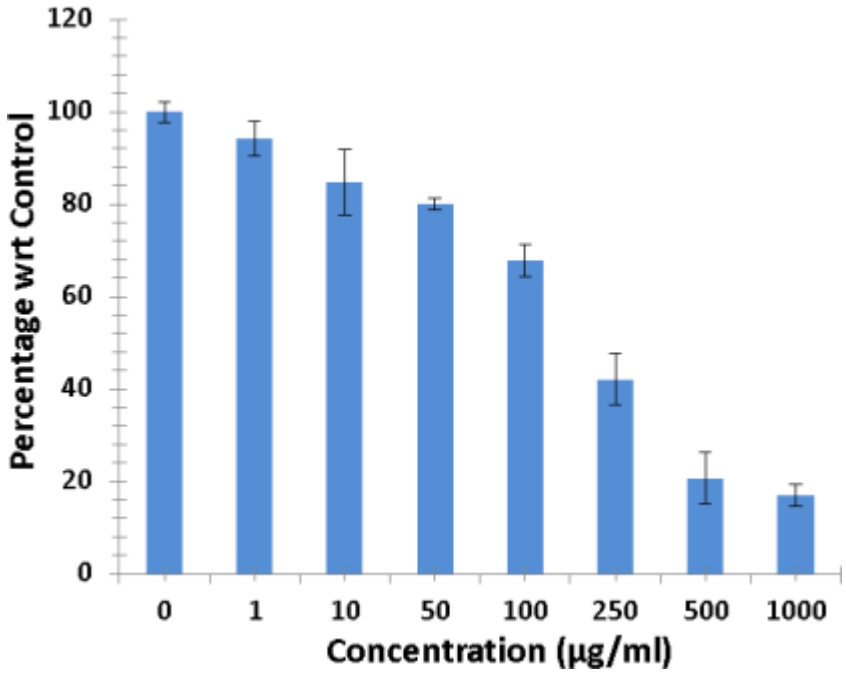

Figure: 4 Effect of Zingiber Officinale Nanoemulsion on the cell viability of SHSY5Y Cells.

Effect of *Zingiber officinale* Nanoemulsion on H_2O_2 Induced Cytotoxicity in SH-SY5Y Cells

In Figure 5 neuroprotective properties of *Zingiber offcinale* Nanoemulsion against H_2O_2 induced cytotoxicity in SH-SY5Y cells was shown. Alone H_2O_2 exposure significantly reduced cells viability ($p<0.01$) as compared to control. On treatment with *Zingiber offcinale* nanoemulsion at dose of 50, and 100µg/ml reverse the decrease in cell viability effect as compared to H_2O_2 treated cells. These results indicate potential effect of *Zingiber offcinale* nanoemulsion to protect the cell viability under oxidative stress.

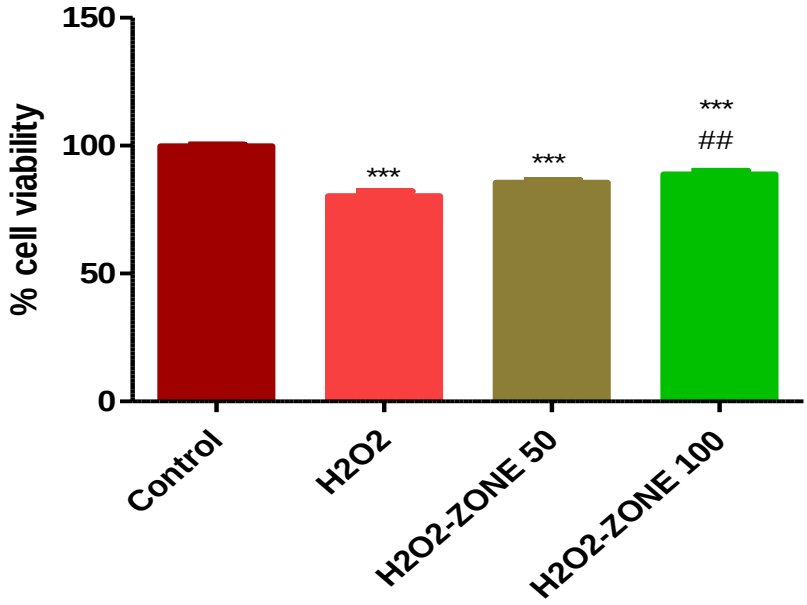

Figure 5- Effect of *Zingiber officinale* Nanoemulsion on H_2O_2 induced cytotoxicity in SHSY5Y cell lines. **p<0.001, ***<0.0001as compared to control and ## p<0.001, ### p<0.0001 compared H_2O_2 and vehicle treated cell. H_2O_2: Hydrogen Peroxide at a dose of 200µM and vehicle treated group, ZONE-*Zingiber officiale* nanoemulsion at dose of 50 and 100µg/ml respectively.

Conclusion

According to previous study *Zingiber officinale* is widely used for its different applications in food product and traditional medicine. Its bioactive compound exhibit antioxidant, anti-inflammatory, and neuroprotective effects. These compounds have shown promise in addressing neurological conditions like Alzheimer's, Multiple sclerosis, Parkinson's, and Huntington's disease by protecting the nervous system from free scavenging or oxidative stress, one of the main factors in neurodegeneration. Antioxidant properties of *Zingiber officnale* extract help neutralize free radicals which causes cell damage (33-35). However, challenges such as poor bioavailability and stability often limit the therapeutic potential of natural bioactive compound. To overcome these challenges the formulation was developed which have higher stability, solubility and higher bioavailability.

In the current study, we successfully developed *zingiber officinale* essential oil nanoemulsions. The developed nanoemulsion exhibited a small particle size, low PDI, and negative zeta potential. A neuroprotective effect of the *zingiber officinale* nanoemulsion was used against hydrogen peroxide induced neurotoxicity by in vitro. Our study showed that the *zingiber officinale* nanoemulsion at a dose of 50, and 100µg/ml was used out of which 100µg/ml had a better neuroprotective activity. As the current study was performed using an in vitro cell line, further studies are needed to confirm the efficacy of this nanoemulsion in an

In-Vivo model. These results suggested that this drug delivery carrier could offer a promising approach for the treatment of neurodegenerative disorders.

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Disclosure statement

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Additional information

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