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A Comprehensive Study on the Phytochemical Mediated Green Synthesis of Potassium Nano Particles for Use in Implication in Inflammatory Cancer Therapy and Immunity

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ABSTRACT

Potassium Chloride and Curcuma longa rhizome extract were used for the synthesis of Eco-friendly Potassium Nanoparticles for application in various fields. Ethanolic extract of curcuma longa was used for the synthesis of potassium nanoparticles. The potassium nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19 to know the kinetic behavior of Potassium nanoparticles. The phase evolution of calcined powder as well as that of sintered samples was studied by X-ray diffraction technique (Philips PAN analytical, The Netherland) using Cu Kα radiation. The morphology of PNPs was investigated by scanning electron microscope (XL30, Philips Electronics, Amsterdam, The Netherlands) with the ability to perform elemental analysis by EDX. Malvern Zetasizer 3000 HSA (Malvern Instruments, Worcestershire, UK) was used to determine the distribution of particle size and zeta potential of PNPs. The cytotoxicity property of the nanoparticles was evaluated by MTT assay on MCF-7 and HepG-2 cell lines, and the cytotoxic concentration 50% values were determined for 24 h and in vitro anti-inflammatory activity Inhibition of albumin denaturation & human red blood cell membrane stabilization method (HRBC) methods.

KEYWORDS: Green Synthesis, Nanoparticles, Potassium, Cancer

INTRODUCTION

Nanotechnology has yielded various dependable nanomaterials synthesis over a diverse range of chemical constitution and sizes (1) and is gaining more relevance as nanofertilizer in agriculture (2). A decrease in crop yield has been observed due to factors such as limited rainfall, drought, inadequate bush fallowing resulting in soil fertility decrease, and insufficient organic manure among others (3). Despite chemical fertilizer application to complement soil fertility and to maximize crop yield, an imbalance between climate regulation, food and feed production, carbon storage and water retention in the ecosystem contribute to soil degradation (4). To improve soil quality and increase productivity, fertilizer was the solution. Their continuous and intensive usage in crop farming which the crop only ends up using less than 50 % of the applied amount while the other remaining unutilized by the crop is lost through hydrolysis, photolysis, leaching and immobilization of microbial and degradation, thus impacting soil microorganisms, slowing down soil microbial activities, threatening human health and the ecosystem, thereby limiting the profit margin of farmers. For example, 40–60% of nitrogen (N), 70–90% of phosphorus (P), and 50–80% of potassium (K) fertilizers are lost and/or fixed in soils, leading to great economic losses. (5).

The method of nanoparticles (NPs) synthesis mediated by plants provides an added advantage of increased life span of NPs that overcome the shortcomings of conventional chemical and fertilizers (6). Plants metabolites and biomolecules contain carbonyl, hydroxyl and amine functional groups mainly considered responsible for metal ions reduction into NPs (7). These molecules not only assist in bioreduction of the ions to the nano scale size, but they also play a crucial role in capping the nanoparticles which is salient for biocompatibility and stability. Reducing agents such as, alkaloids, phenolic compounds and sterols can reduce metal ions into NPs in a single reaction. (8). No literature detailed the synthesis of potassium nanoparticle using aqueous extract of Curcuma longa extract for over a decade, and none of the synthesized nanoparticle was for cancer. This study aimed to synthesize potassium nanoparticles (K-NPs) and to determine its efficiency as a cancer and inflammation.

MATERIALS AND METHODS

Collection and Authentication Of Plant: The rhizomes of Curcuma longa were collected from the open fields in the month of January-February around Bareilly district. The plant was identified and authenticated by Dr. Alok Srivastava, Associate Professor, Department of Plant Science, MJP Rohilkhand University, Bareilly, Uttar Pradesh-India. The plant voucher specimen no. was RU/PS/2501/15.

Preparation of Extracts: The powered plant materials of *C. longa* were separately extracted with 95% v/v ethanol in Soxhlet extractor (hot extraction). The extracts were evaporated under reduced pressure using a Rotary flash evaporator. The extracts thus obtained were stored separately in airtight containers and maintained at 4 °C for further use. (9, 10)

Preliminary Phytochemical Screening: The extracts obtained from successive solvent extraction were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, phenolics and tannins, proteins and amino acids, saponins and phytosterols using reported methods. (11, 12)

Green Synthesis Of Potassium Nanoparticles: Green synthesis of nanoparticles has gained the momentum over chemical and physical methods due to their distinctive properties, wider applicability and environmental sustainability. The bio-active compounds viz., sugar, flavonoid, protein, enzyme, polymer and organic acid present in the plant extracts acts as a reducing agent and takes charge in bio induction of metal ions into potassium nanoparticles. Plant extract were tried for synthesis of nanoparticles in the present study. (13)

Green synthesis of potassium nanoparticles from plant extracts: In the present study synthesis of potassium nanoparticles through green approach was carried out using plant extract of Curcuma longa (Haldi).

i) Extract of Curcuma longa: Ethanolic extract of curcuma longa was used for the synthesis of potassium nanoparticles.

ii) Preparation of aqueous potassium nitrate (350 ppm) stock solution: Potassium nitrate (KNO_3) procured from the Sisco was used as a precursor in the synthesis of potassium nanoparticles (K NPs). 0.35 g of KNO_3 was dissolved in 1000 mL of deionized water and stored in bottle for future use. (14)

iii) Standardization of protocol for green synthesis of potassium nanoparticles: The plant extract (Curcuma longa) contains many bioactive compounds which acts as a reducing and capping agent that reduces metal ions. Potassium nanoparticles were synthesized using Curcuma longa with different methods viz., heating with stirring, dark condition and autoclave method. Among the different methods tried, one method which gives the size of the nanoparticle less than 100 nm was selected for further synthesis of potassium nanoparticles. (15, 16)

A) Heating with stirring method: The different combinations of precursor (KNO_3) and plant extract were kept on the hot plate @ 70 °C along with magnetic stirring for 400 rpm for 30 and 45 minutes and particle size analyzer observations were recorded.

B) Dark condition method: The different combinations of precursor (KNO_3) and plant extracts were kept under dark condition for 8 and 12 hours and particle size analyzer observations were recorded.

C) Autoclave method: The different combination of precursor (KNO_3) and plant extract were kept in autoclave (121°C temperature and pressure 15 pounds per square inch) for 15 and 30 minutes and particle size analyzer observations were recorded. (17, 18)

Characterization Techniques:

UV-Vis Spectroscopy: The potassium nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19 to know the kinetic behavior of Potassium nanoparticles. The scanning range for the samples was 200-800 nm at a scan speed of 480 nm/min. The spectrophotometer was equipped with "UVWinlab" software to record and analyze data. Base line correction of the spectrophotometer was carried out by using a blank reference. The UV-Vis absorption spectra of all the samples were recorded and numerical data were plotted in the "Origin 6.5". (19)

$$\beta = \text{full width at half maximum}$$

Scanning Electron Microscope (SEM): In this research work, Jeol JSM-6480 LV SEM machine were used to characterize mean particle size, morphology of nanoparticles. The freeze dried sample of K-NP solution was sonicated with distilled water, small drop of this sample was placed on glass slide allowed to dry. A thin layer of platinum was coated to make the samples conductive Jeol

JSM-6480 LV SEM machine was operated at a vacuum of the order of 10-5 torr. The accelerating voltage of the microscope was kept in the range 10-20 kV. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM. The EDX analysis of sample was done by the SEM (JEOLJSM 5800) machine. The EDX normally reveals the presence of phases.

DLS particle size analyzer: A laser diffraction method with a multiple scattering technique has been used to determine the particle size distribution of the powder. It was based on Mie-scattering theory. In order to find out the particles size distribution the Potassium nanoparticles was dispersed in water by horn type ultrasonic processor [Vibronics, model:VPLP1].Then experiment was carried out in computer controlled particle size analyzer [ZETA Sizers Nanoseries (Malvern Instruments Nano ZS)] to find out the particles size distribution. (20, 21)

In-Vitro Anticancer Activity: The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37 °C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week. (22)

Cell Treatment Procedure: The monolayer cells were binding with trypsin-ethylen diaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a heamocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37b°C, 5% CO_2, 95% air and 100% relative humidity (23, 24). The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT Assay: After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using 96 well plate counter (24). The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using GraphPad Prism software.

Anti-inflammatory Activity

In vitro anti-inflammatory activity Inhibition of albumin denaturation: The following procedure was followed by Saleem et al., for evaluating the percentage of inhibition of protein denaturation:

i) Control solution (50 ml): 2 ml of egg albumin, 28 ml of phosphate buffer (pH 6.4) and 20 ml distilled water. (25)

ii) Standard drug (50 ml): 2 ml of egg albumin, 28 ml of phosphate buffer and various concentration of standard drug (Aspirin) concentration of 100, 200, 400, 800, and 1000 µg/ml.

iii) Test solution (50 ml) 2 ml of egg albumin, 28 ml of phosphate buffer and various concentration of K-NP are 100, 200, 400, 800, and 1000 µg/ ml. All of the above solutions were adjusted to pH using a small amount of 1N HCl. The samples were incubated at 37º C for 15 minutes and heated at 70º C for 5 minutes. After cooling the absorbance of the above solutions percentage inhibition of protein denaturation was calculated using the following formula (26, 27).

Percentage inhibition = [Vt / Vc - 1] x 100

Where, Vt = Absorbance of test sample Vc = Absorbance of control

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Human red blood cell membrane stabilization method (HRBC): The percentage of hemolysis was estimated by assuming the hemolysis produced in the presence of distilled water as 100% (28, 29, 30). The percentage of HRBC Membrane stabilization or protection was calculated by using the following formula:-

Percentage protection = [100 - (optical density sample / optical density control) x 100.

RESULTS AND DISCUSSION

Evaluation Of Extract: The ethanol extract obtained was light to dark yellow in colour, greasy with an indistinct odour and astringent in texture. The extracts were soluble in an organic solvent and were considered in water to be insoluble. The percentage yield was measured and set at 8.48% w/w.

Figure 1: Extraction of Curcuma longa rhizome

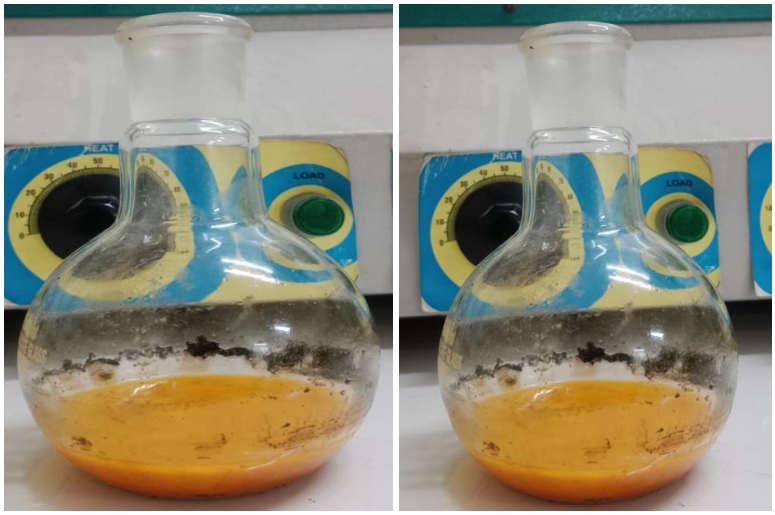

Figure 2: Extract of Curcuma longa rhizome

Pre-Phytochemical Analysis: The crude petroleum ether and ethanol extract were screened the present alkaloids, flavonoids, glycoside, carbohydrate, protein, starch, amino acid, steroid, tannins and saponins using the simple chemical test as reported in a standard reference book.

Table 1: Pre-phytochemical Screening of ethanol extract

Class of Drug	Chemical test	Ethanol
Carbohydrate	Fehling Molisch Benedict	Positive
Alkaloids	Dragendroff test Hagers Mayer Wagners	Positive
Flavonoid	Alkaline Lead acetate	Positive
Glycoside	Legal test Killer Killiani	Positive
Terpenoid and steroid	Salkowski	Negative
Protein and amino acid	Millons test Ninhydrane	Positive
Saponin	Foam	Negative
Tannin	$FeCl_3$	Positive

Green Synthesis Of Potassium, Nanoparticles From Plant Extract: Laboratory experiments were conducted to standardize the protocol for green synthesis of potassium nanoparticles using plant extract. The synthesized nanoparticles were characterized using UV-Vis spectrophotometer, PSA, SEM and EDX techniques.

Standardization of the protocol for green synthesis of potassium nanoparticles: Based on the different research articles and association with scientists who focused on synthesis of nanoparticles through green approach, the present study was carried with plant extract of Curcuma longa. The bio-active compounds present in the plant extract of Curcuma longa acts as a reducing agent and have been tried in multiple ways to synthesised the nanoparticles in different methods viz., heating with stirring, autoclave and dark condition methods to standardize the protocol.

Hot plate heating with stirring methods: The varied quantities of Curcuma longa rhizome extract with different combinations of precursor solution i.e., potassium nitrate (350 ppm) were used for synthesis of potassium nanoparticles through hot plate heating @ 70 °C along with stirring for 400 rpm for 30 and 45 minutes. PSA observations recorded that, the particle size ranged between 357.4 to 484.8 nm for Curcuma longa (Table 1). The size of the particles were decreased with increasing the quantity of rhizome extract as well as the precursor solution and decreased with heating at 70 °C along with stirring at 400 rpm for 45 minutes compared to 30 minutes, but the size of the synthesized particles were not in the nano range i.e., less than 100 nm.

Dark condition method: The varied quantities of Curcuma longa rhizome extract with different combinations of precursor solution i.e., potassium nitrate (350 ppm) were used for synthesis of potassium nanoparticles through dark condition method which was incubated in dark at room temperature for 8 and 12 hours. PSA observations recorded that, the particle size ranged between 522.9 686.4 nm for Curcuma longa extract (Table 1). The size of the synthesized particles were not in the nano range i.e., less than 100 nm.

Autoclave method: The varied quantities of Curcuma longa extract extract with different combinations of precursor solution i.e., potassium nitrate (350 ppm) were used for synthesis of potassium nanoparticles through autoclave method. After reaching 121 °C temperature and 15 psi pressure in autoclave, kept for 15 and 30 minutes, PSA results showed that, the particle size ranged between 237.2 to 322.4 nm for Curcuma longa.

Standardized protocol for green synthesis of potassium nanoparticles by using the Curcuma longa rhizome extract: The extract of Curcuma longa of about 0.5 mL was added to 15 mL of potassium nitrate (350 ppm). Then the reaction mixture was autoclaved for 30 min at 121 °C temperature and pressure of 15 pounds per square inch. A change in colour from initial transparent to light brown color (Plate 3), indicated the reduction of K^+ ions to potassium nanoparticles (89.1 nm). This was further confirmed by UV-Visible Spectrophotometer.

Table 2: Standardization of the protocol for green synthesis of potassium nanoparticles

KNO_3 350 ppm (ml)	Time	C. longa extract (mL)	PSA (nm)
	Method: Heating @ 70°C along with stirring for 400 rpm		
10	30 min	0.1	484.8
10	30 min	0.5	449.2
10	30 min	0.8	424.4
15	30 min	0.1	422.5
15	30 min	0.5	389.6
10	45 min	0.1	411.3
10	45 min	0.5	403.5
10	45 min	0.8	387.2
15	45 min	0.1	376.9
15	45 min	0.5	357.4
	Method: Dark condition		
10	8h	0.1	686.4
10	8h	0.5	669.4
10	8h	0.8	613.5
15	8h	0.1	592.4
15	8h	0.5	538.6
10	12h	0.1	656.3
10	12h	0.5	616.6
10	12h	0.8	607.8
15	12h	0.1	587.4
15	12h	0.5	522.9
	Method: Autoclave		
10	15 min	0.1	322.4
10	15 min	0.5	301.6
10	15 min	0.8	267.8
15	15 min	0.1	282.3
15	15 min	0.5	273.1
10	30 min	0.1	312.8
10	30 min	0.5	261.6
10	30 min	0.8	257.6
15	30 min	0.1	246.9
15	30 min	0.5	237.2

Characterization Of Potassium Nanoparticles

UV–Vis spectral analysis: The UV-Vis absorption spectra of the K-NP were shown in Figure. Absorption spectra of Ag nanoparticles formed in the reaction media has absorbance maxima at 398 nm. A remarkable broadening of peak at around 350 nm to 480 nm indicates that the particles are polydispersed. It was observed that the peak was blue shifted in the absorption spectrum from 350nm to 480 nm with increasing reaction time.

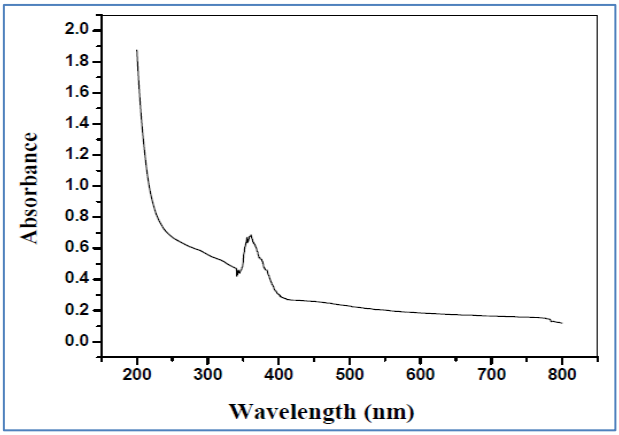

Figure 3: K-nanoparticles prepared of Curcuma longa

SEM Analysis of K- Nanoparticles: The SEM image of potassium nanoparticles synthesized by chemical reduction method and green synthesis process by using 0.5% extract and 1mM AgNO3 concentration was shown in Figure 4. The SEM image showing Potassium nanoparticles synthesized using curcuma longa extract confirmed the development of potassium nanostructures.

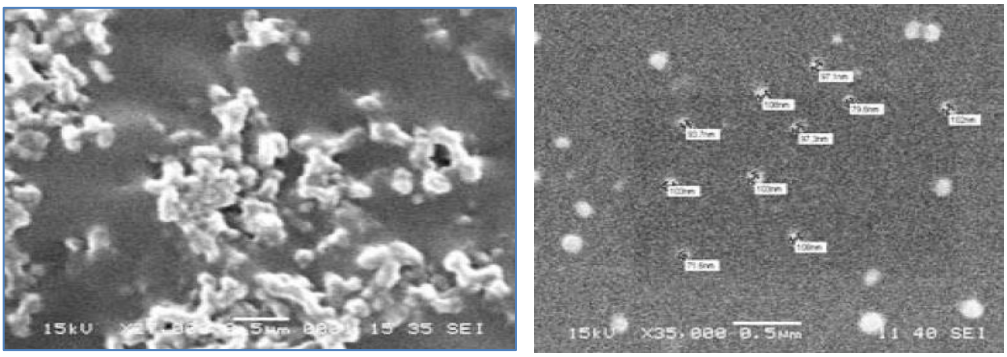

Figure 4: SEM image of potassium nanoparticles

Dynamic light scattering particle size analyzer: Figure 5 shows the graphical representation of average particle size distribution of K nanoparticles. They were in a range : of 20-140 nm. However, beyond 100 nm range the percentage of nanoparticles present is very less. The highest fraction of K-NP present in the solution was of 50nm. From the plot it was evident that the solution was consist of nanoparticles having various sizes which are indeed in agreement of the result obtained by SEM analysis.

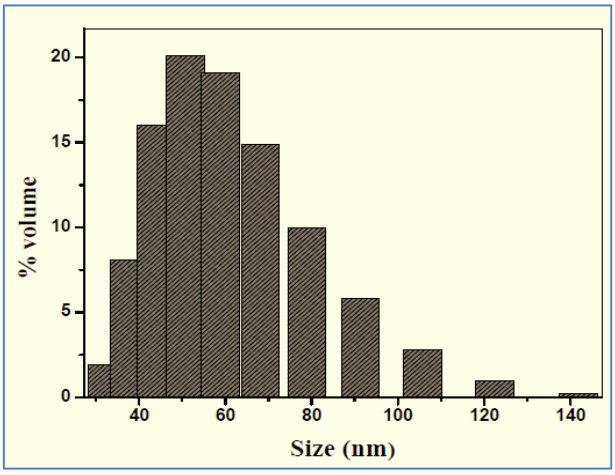

Figure 5: Particle size distribution of K nanoparticles synthesized.

In-Vitro Anticancer Activity: The invitro anticancer activity of the Curcuma longa extract and Potassium nanoparticles of Curcuma longa were given in Table 3 and 4. The K-NP and extract were screened for its cytotoxicity against HeLa cell line at different concentration to determine the IC_{50} value. The percentage growth inhibition was found to be increased with the increasing concentration of test compound. The IC_{50} value of K-NP and extract on the HeLa cell line were found to be 62.587 and 297.9 µg/ml and R^2 values were 0.9993 and 0.9953 respectively. The K-NP of C. longa showed significant invitro anticancer activity against HeLa cell line when compared to the extract.

Table 3. MTT Assay of K-NP of Curcuma longa and Curcuma longa extract

Nanoparticles	Conc. µg/ml	Absorbance	% inhibition	IC_{50} µg/ml	R^2
K-NP of Curcuma longa	18.75	0.4053	2.1721	62.587µg/ml	0.9993
	37.50	0.3633	12.3089		
	75	0. 1376	66.7739		
	150	0.2366	94.2880		
	300	0	100		
Extract	**Conc. µg/ml**	**Absorbance**	**% inhibition**	**IC_{50} µg/ml**	**R^2**
Curcuma longa extract	18.75	0.4110	0.8045	297.7µg/ml	0.9953
	37.50	0.40833	1.4481		
	75	0.4016	3.0571		
	150	0.3836	7.1600		
	300	0.2040	50.764		

Inhibition of Albumin denaturation method: The inhibitory effect of different concentration of K-NP C. longa and C. longa extract on protein denaturation as shown in Table 4 K-NP C. longa and C. longa extract at a concentration range of 100, 200, 400, 800, 1000 µg /ml and standard 100, 200, 400, 800, 1000 µg /ml showed significant inhibition of denaturation of egg albumin in concentration dependent manner. Both membrane stabilization activity and effect on protein denaturation contribute to the in vitro anti inflammatory activity of K-NP C. longa and C. longa extract used in our study.

Table 4: In Vitro Anti-Inflammatory activity of K-NP C. longa and C. longa extract and Aspirin of Protein Denaturation Method

Conc. (µg/ml)	% inhibition		Aspirin Standard
	K-NP C. longa	C. longa extract	
100	65.65 ±0.24	50.11 ±0.42	111.34± 0.22
200	115.61 ±0.36	83.65 ±0.67	143.05± 0.29
400	204.76 ±0.98	129.11 ±0.95	197.67± 0.82
800	289.45 ±1.01	172.43 ±1.24	251.51± 0.98
1000	315.65 ±1.34	238.65 ±1.36	318.87± 1.43

Values are expressed as Mean ± Standard deviation

HRBC Membrane Stabilization Method: In the study of membrane stabilization activity of K-NP C. longa and C. longa extract at concentration range of 100, 200, 400, 800, 1000 µg /ml protected significantly in a concentration dependent manner the erythrocyte membrane against lysis induced by hypotonic solution. Aspirin in the concentration of 100, 200, 400, 800, 1000 µg /ml used as standard also offered protection of HRBC membrane against damaging effect induced by hypotonic solution. The membrane stabilization action and inhibitory effect of different concentration of K-NP C. longa and C. longa extract is presented in Table. 5.

Table 5: In Vitro Anti-Inflammatory Activity of -NP C. longa and C. longa extract And Aspirin of HRBC Membrane Stabilization method

Conc. (µg/ml)	% inhibition		Aspirin Standard
	K-NP C. longa	C. longa extract	
100	49.11 ±0.12	42.56 ±0.15	80.24± 0.11
200	55.47 ±0.29	46.23 ±0.25	83.25± 0.23
400	59.76 ±0.43	51.87 ±0.33	89.62± 0.45
800	63.11 ±0.53	54.74 ±0.37	95.45± 0.63
1000	69.11 ±0.57	58.31 ±0.52	98.11± 0.74

Values are expressed as Mean ± Standard deviation

Protein denaturation is a process in which protein lose their tertiary structure and secondary structure by application of external stress or compound such as strong acid or base a concentration inorganic salt , an organic solvent or heat most biological protein lose their biological function when denaturated. Denaturation of protein is a well documented cause of inflammation. As a part of the investigation on the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied.

The HRBC membrane stabilization has been used as a method to study the in vitro anti- inflammatory activity because the erythrocyte membrane is analogues to the lysosomal membrane and its stabilization implies that the extract may well stabilize the lysosomal membrane. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacteria enzyme and protease, which causes further tissue inflammation and damage upon extracellular release. The lysosomal enzyme released during inflammation produce a various disorders. The extracellular activity of these enzymes are said to be related to acute to chronic inflammation. The non steroidal drugs act either by inhibiting the lysosomal enzymes or by stabilizing the lysosomal membranes. The HRBC method was selected for the in vitro evaluation anti-inflammatory property because the erythrocyte membranes is analogues to the lysosomal membranes and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membranes is the important in limiting the inflammatory response by preventing the release of lysosomal constituents of activate neutrophils, such as bactericidal enzymes and protease, which causes further tissue inflammation and damage upon extracellular release.

CONCLUSION

The Curcuma longa rhizome extract used for the synthesis of nanoparticles by different methods viz., heating with stirring, autoclave and dark condition methods. These different methods were unable to reduce the particles below 100 nm and obtained particle sizes range of 237.6 to 686.1 nm. The K-NP and extract to carry out the invitro anticancer activity. The K-NP and extract were subjected to invitro anticancer studies using HeLa cells line by MTT assay. K-NP showed significant anticancer activity by MTT assay. In-vitro anti- inflammatory activity of K-NP and extract showed a significant effect by the two different methods like Inhibition of Albumin denaturation method and HRBC Membrane Stabilization Method.

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