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SYNTHESIS AND CHARACTERIZATION OF OPTIMIZED NANO-EMULGEL MICELLES AS A VEHICLE OF PLANT EXTRACTED CURCUMIN: DRUG RELEASE AND *IN VITRO* STUDY

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ABSTRACT

The curcumin is naturally occurring antimicrobial, anticancer, poorly water soluble drug. The nano-emulsion based gel containing curcumin was prepared with view of its enhanced antibacterial activity and drug release kinetics using membrane dialysis method. The solubility study was useful to optimize emulsion system. Oleic acid was optimized as an oil phase while Span 20 and PEG 400 were optimized as surfactants and co-surfactant respectively. The surfactants and co-surfactants are mixed in different weight ratios (1:1, 1:2, 1:3, 1:4, 4:1, 3:1 and 2:1 respectively) to optimize proportion of surfactants, co-surfactant and oil phase. The pseudo ternary phase diagrams were developed by using different weight ratios of oleic acid (oil phase) and Smix (mixture of surfactant and co-surfactant) mixed in different weight ratios ranging from 1:9 to 9:1. The optimized nano-emulsions were selected to form nano-emulgel. The agarose were used as a gel forming agent. The emulsion region of pseudo ternary phase diagrams was considered to optimize emulsion. The formulated optimum emulsion was used to load curcumin. The probe sonicator was used to maintain nanometer size ofself-assemble micelle. The nano-emulgel formulations of curcumin were used to study its potent antibacterial activity. The drug release study was done by using membrane dialysis method. The Nano-gel form is optimized for transdermal administration of drug.

Keywords: Curcumin, Nano-emulsion, Nano-emulsion gel, Micelles and Drug release study.

INTRODUCTION

Curcumin is naturally occurring polyphenolic compound use as a active pharmaceutical ingredient in the world. For the topical drug delivery, poor bioavailability of drug is the major cause of high cost of therapy and decreases its potential. In order to overcome such limitations nano-emulgel formulation is use. This technology helps for penetration of drug through intercellular pathway.[1] Metal nanoparticles also show catalytic activities during various reactions.[2]

The nano-emulgel (NEG) formulation with homogeneous distribution and near about same micelle size, provides some advantages for drug delivery of pharmaceutical agents. NEG has ability to dissolve lipophilic drugs, increases penetration in intercellular pathway and extend the release of lipophilic and hydrophilic drugs. Moreover, it creates complete dispersion into skin as well as better skin hydration in cosmetic products.[3] (The transdermal route of administration).The curcumin is aromatic polyphenolic compound with potent antimicrobial as well as anticancer activity. It is also use in skin infections such as fungal and bacterial infections. The objective of the present study is enhancement in ability of curcumin to penetrate membrane for better drug delivery through nano-emulgel

formulation. The solid lipid nanoparticles were successfully used to carry curcumin.[4] The nanotechnology of metals like gold shows therapeutic effects.[5,6,7] Nano emulsions of curcumin possess high bioavailability and has enough stability in gel form.[8,9,10] In the last decades, nanocarriers have been extensively used as drug carriers to enhance therapeutic effect of drugs. The structural elements encoded in each nanocarrier, which rule the self-assembly, provide efficient methods for building new materials with predictable structure and function.[11]The self-assembling in nanotubes exhibit easily tunable morphology, size and surface characteristics. The self-assembled nano-emulsions were investigated experimentally and theoretically.[12]Thecurcumin is hydrophobic aromatic polyphenolic compound with potent anticancer activity towards different type of cancer cells.[13,14,15] Therapeutic efficiency of curcumin can be enhanced by increasing its bioavailability by protection from degradation as well as from metabolism. The polymer with hydroxyl group shows end to end interaction by hydrogen bonding.[16]Nano-emulsions are useful tool for the proper drug delivery and mostly for drugs having low water solubility.[17,18] The Scanning electron microscopy (SEM) of nano-emulsions indicates that the different size and shape of micelles.[19,20,21]

MATERIAL AND METHODS

The present study was focus on the development of curcumin loaded nano-emulgel formulation. The evaluation of physiochemical properties of CNEG has been done. During the curcuminencapsulation curcumin was dissolved in appropriate solvent like acetone. The curcumin was trapped in cross-linked polymeric network of agarose polymer. There is strong intermolecular hydrogen bonding exist between curcumin and polymer. The curcumin was extracted from rhizome of curcuma longa plant using soxhlet apparatus and purified by column chromatography.[22] The purity of extracted curcumin was confirmed by H^1 NMR. The H^1NMR which was done by sophisticated analytical instrumentation facility (SAIF) Punjab University Chandigarh, India. The oleic acid (cis-9-Octadecenoic acid, Elainic acid, CAS Number 112-80-1), Agarose polymer (low EEO, CAS 9012-36-6), Span 20 (CAS Number 1338-39-2 MDL Number MFCD00005365), PEG 200 (Polyethylene glycol 200 CAS Number 25322-68-3, EC Number 500-038-2), PEG 400 (Polyethylene glycol 400 for synthesis. CAS 25322-68-3) were purchased from Sigma-Aldrich Co., Bengaluru, India. All chemicals used were of analytical grade.

Solubility study

The solubility of curcumin was determined in various recipients, Span 20 as a surfactant and PEG 400 as Co-surfactant. The excess quantity of curcumin (0.25 gm) was mixed with 20 ml of solvents separately in stopped bottles for 6 h at 25 °C and stir at 200 rpm using magnetic stirrer. The solvent drug mixture was then centrifuged at 3500 rpm (Remi Industries Ltd. Mumbai, India 400053 model no 412 LAG) for 20 min then left to reach equilibrium for 48 h. The supernatant was filtered through 0.45 mm Millipore membrane filter and diluted with methyl alcohol. The amount of curcumin dissolve was quantitatively determined by using spectrophotometer (UV-Visible spectrophotometer Shimadzu Corporation Tokyo, Japan.) at λ max 425 nm using methyl alcohol as a blank as shown in figure 01.

Criteria for oil phase selection

The appropriate emulsion system for lipophilic drugs is oil in water type (o/w). The lipophilic drug is soluble in o/w emulsion system. The water in oil system (w/o) system is more preferred the hydrophilic drugs. The loading of drug in nano-emulsion formulation is depends on solubility of drug in different formulation components. The study shows that there must be low volume of surfactants in formulation in order to improve drug delivery. In present study oil is selected on the basis of solubility of drug in oil phase. The selection of oil phase is more critical as well as sensitive in case of nano-emulsion formulation. The curcumin shows solubility 8.2 mg/ml for oleic acid. Hence oleic acid was selected as a good solvent (oil phase) for nano-emulsion formulation.

Criteria for selection of surfactants and co-surfactants

The concentration of surfactants in emulsion must be low as possible because surfactants cause toxic effects. Large amount of surfactants may produce irritation of skin in topical drug delivery. The non-ionic surfactants are comparatively less toxic than ionic surfactants. The critical micelle concentration (CMC) for nonionic surfactants is also low. The Hydrophilic-lipophilic balance (HLB) value also considerable to lower the necessary energy required to form stable emulsion. The HLB value must be greater than 10. In the present study, on the basis of solubility and less toxicity Span 20 was selected as a surfactant

while PEG 400 use as a co-surfactant. The co-surfactant and surfactant mixture (Smix) are formed in its appropriate concentration in order to form stable nano-emulsion.

Preparation of curcumin-nano-emulsion formulae

The nano-emulsion formulation of curcumin was done on the basis of selection of solvent, surfactant system and pseudo ternary phase diagram.

The pseudo ternary phase diagrams(PTPD)

The development of pseudo ternary phase diagrams was done by using solubility study[23]. The pseudo ternary phase diagrams were use to optimize the nano-emulsion zone as well as to determine the appropriate concentration ratios of components of nano-emulsions. The weight ratios (surfactant to co-surfactant) for the pseudo ternary phase diagrams PTPD-01, PTPD-02, PTPD-03, PTPD-04, PTPD-05, PTPD-06, and PTPD-07 are 1:1, 1:2, 1:3, 1:4; 2:1, 3:1and 4:1 respectively. The selected emulsions were used for nano-emulsion formation. For each pseudo ternary phase diagram Smix and oil were combined in different weight ratios ranging from 1:9 to 9:1 in separate sample glass cells. The titration with aqueous phase was carried out slowly drop by drop for each sample at each weight ratio of Smix and oil phase until turbid solution obtained .[24]The pseudo ternary phase diagrams were developed by using excel software tool. The shaded region in the diagram indicates the nano-emulsion region. The diagram with largest emulsion region (shaded area) of PTPD-01, PTPD-02, PTPD-03, PTPD-04 areas are 77.10%, 72.50%, 67.33% and 66.33% respectively as compared to the shaded regions of PTPD-05, PTPD-06 and PTPD-07 are 65.80%, 64.10%, and 59.5% respectively as shown in figure 02.

Preparation of nano-emulsions

On the basis of nano-emulsion region in pseudo ternary phase diagrams, optimization of emulsion system was done. The appropriate ratio of emulsion contains minimum concentration of Smix. The different compositions of nano-emulsion system as discussed in table 01. The codes obtained NE1, NE2, NE3, NE4, NE5, NE6, NE7 and NE8 are the nano-emulsion system from (NE1 to NE8) selected due to low oil to water ratio on the basis of pseudo ternary phase diagrams as shown in figure 03.

Loading of curcumin

Twenty five mg of curcumin was weighed and dissolve in optimized Smix at their determined ratios and mixture was stirred using high-speed homogenizer at 4000 rpm (Rajendra electric. India.Ltd. homoginic type RQ-127A) for one hour. The oil phase in the form of oleic acid was then added drop wise in to the mixture containing curcumin and both was mixed using high-speed homogenizer followed by drop wise addition of specified weight of water (as shown in Table 01) at 4000 rpm for two hours. The resultant emulsion was kept in ice bath and subjected for ultrasonication using probe sonicator (Sonics & Materials INC made in U.S.A.) at 300W for 45 minutes. The nano-emulsion was obtained new codes CNE1, CNE2, CNE3, CNE4, CNE5, CNE6, CNE7, and CNE8.

Preparation of curcumin loaded nano-emulsion gel (CNEG)

The nanocarriers in the form of agarose polymers and Smix exhibit potent antimicrobial activity to curcumin by self-assembly. The agarose polymer is use as a gel forming polymer as curcumin loaded nano-emulsion is of o/w type. The Triethanolamine (2 ml) was use as a neutralizing agent and it was added to 2.5 ml 20% agarose solution in PEG 400. The agarose gel with neutralizing agent was added in 50 ml appropriate curcumin loaded nano-emulsion. The mixture was stir using high speed homogenizer at 5000 rpm at 50 °C temperature for one hour. The resultant emulsion was kept in ice bath and subjected for ultrasonication using probe sonicator at 300W for 25 minutes. The present study shows that when agarose polymer concentration close to 1% for that case nano-emulgel show adequate performance during drug release study. Consequently the agarose polymer was act as gel forming polymer, was prepared as 1% w/w at 1% concentration of polymer, a gel behavior was exhibited by the aqueous agarose system and the corresponding nano-emulsion having better consistency. The hydrogen bonding exists between curcumin and agarose indicated in figure 04.

Determination of encapsulation efficiency (%EE)

The determination of encapsulation efficiency of the developed NG is the measure of the how much amount of curcumin entrapped in micelle. The encapsulation efficiency (EE) was calculated using following equation[25].

$$(\%EE) = \frac{Mass\ of\ curcumin\ in\ nanoemulgel}{Total mass\ of\ curcumin} X100$$

The encapsulation efficiency was determined by measuring the amount of curcumin that was remained in the nano-emulgel after nano-emulgel were filtered (0.45µm), centrifuged at 4000 rpm (Centrifuge 412 LAG, Remi industries Ltd. Mumbai, India) and subjected for membrane dialysis (16 h). The final concentration of curcumin in nano-emulgel was determined by measuring absorbance at 425 nm using UV/Visible spectrophotometer. The physical appearance as shown in figure 06 for free curcumin and curcumin loaded nano emulsion gel.The % loading indicated in figure 07.

RESULT AND DISCUSSION

^{1}H NMR of curcumin

The ^{1}H NMR spectra of curcumin indicates that the purity of extracted and column purified curcumin was excellent. The ^{1}HNMR (400.1324 MHz, [D_S]DMSO, 25 °C, TMS): δ= 5.96 (s, 1H; CH), 16.36 (bs, 1H; Ar-OH), 6.84-6.82 (d, *J*= 8Hz, 2H; CH), 7.55-7.51 (d, *J*= 16Hz, 2H; CH), 7.09-7.07 (dd, *J*= 8Hz, 2H; Ar-H), 6.84-6.82 (d, *J*= 8Hz, 2H; Ar-H), 7.22 (s, 2H; Ar-H), 3.88 (s, 6H; O-CH_3), 6.65 (s, 2H; Ar-H).

FTIR of curcumin

The identity of isolated curcumin was further confirmed by FTIR spectra (FTIR Shimadzu corporation Kyoto Japan CORP) of curcumin showed by stretching frequency of –OH group at 3429.43 cm^{-1}. The peak at 2941.44 cm^{-1} indicates the C-H stretching and 1514.12 cm^{-1} showing C=C symmetric aromatic ring stretching. The peak at 1571.99 cm^{-1} indicates the C=O, while C-O peak was obtained at 1282.95 cm^{-1}.

Characterization of CNEG

The size of curcumin loaded nanocarriers was obtained by FE-SEM and particle size analysis. The curcumin loaded nanocarriers was obtained by self-assembly of micelle.

Scanning electron microscopic analysis of CNEG

FE-SEM images of curcumin loaded nano-emulgel were investigates the surface morphology as well as size of micelles in CNEG as show in figure 08. The “road shape” micelles mostly observed in sample that was subjected for the FE-SEM analysis. The nanogel shows different size of micelles. The present study shows that the distribution of size is observed near 244 nm. The FE-SEM analysis shows the image of micelles in the nanometer size. The round shape micelles were assembling in rod like structure. The average droplet size was measured is about 14 nm. The surface morphology of nano-emulgel was observed at 10 µm indicates that the structure of gel appears like a network of fibers The present study indicated that the FE-SEM images CNEG was appears like a network incorporated with micelle of gel formulation as shown in figure 08.

Particle size analysis

The particle size of micelle was investigated by using Malvern particle size analyzer. The particle size distribution of curcumin loaded gel of microsphere is totally depends on reaction conditions such as reaction time and Smix. The average size of microsphere is measured and plotted in particle size analyzer statistics. However, it is evidently seen that the average size of nano-emulsion was found to be near about 244.2 nm as shown in figure 09 and 10.

Zeta potential measurement

The potential of Nano-emulgel formulation was measured by using an electrophoretic light scattering technique using a Malvern Zetasizer (Malvern Instruments, Ltd., UK) at wavelength 633 nm by applying 1v electric field. The nano-emulgel formulation dispersed in double distilled water (1% solution in water). The zeta potential of emulsion droplets value was found to be 14.2 mV as shown in figure 11.

Energy-dispersive X-ray spectroscopy (EDX)

Energy-dispersive X-ray spectroscopy was carried out for the element detection. The element interpretation indicates that the sample must contains element having atomic number 6 (Carbon) 77.06 % , sample was also shows presence of element having atomic number 8 (Oxygen) 18.53 % ,The remaining portion of the extra peak indicates the amount of Nitrogen 4.41% as shown in figure 12.

Thermodynamic and physical studies

Heating - Cooling cycle

The CNEG was subjected for six heating cooling cycles between 5 °C to 46 °C for 48 hours. CNEG sample were found stable. The stable sample was subjected for further study. In the present study it was found that the nano-emulsions systems NGCU5, NGCU7, NGCU8, were not found thermally stable while remaining are found thermally stable as shown in table 03.

Freeze thaw cycle

Nano-emulsions were subjected to check whether it withstand at lower temperature or not. The nano-emulsions were kept in deep freezer (Remi deep freezer, Remi industries Ltd. Mumbai, India model No. 396 LAG) for (-

20 °C) for 24 hours. 4 cycles were repeated to insure the nano-emulsion again regains its original form within 10 min. In the present study it was found that the nano-emulsion system NGCU8 was not found stable in Freeze thaw cycle while remaining was found stableas shown in table 03.

Centrifugation

After freeze thaw cycle nano-emulsions were subjected to centrifugation (Remi centrifuge Model No. 412-LAG, Remi industries Ltd. India) in which nano-emulsions undergo centrifugation for 30 min at 4000 rpm in centrifuge. The nano-emulsion formulations which are stable after centrifugation did not show phase separation. In the present study it was found that the nano-emulsion system NGCU6 was not found stable after centrifugation at 4000 rpm while remaining was found stableas shown in table 03.

Refractive index and pH

The transparency of nano-emulsion can be determined by refractive index (RI) measurement (SSU Abbe Refractometer) The RI of CNEG was somewhat more than that of water (1.33) as depicted in table 03. It indicates that prepared nano-emulsion was o/w type. The acidic nature of drug indicates by pH measurement (HANNA Instruments HI 2215 pH/ORP meter) as shown in table 03.

Viscosity

The viscosity study of nano-emulgel shows that the viscosity of nano-emulgel increases with increase in oil contents. In the present study the formulae of nano-emulsion with increase in concentration of oil contents was measured as shown in table 03. The maximum viscosity value measured for the formulae CNEG1 and NGCU2 (26.18 cp ± 6.5, 24.46 cp ±9.6 respectively) due to maximum content of Span 20 in the Smix (Brookfield engineering laboratories viscometer made in U.S.A. Model No D220).Thermodynamic and physical stability of nano emulsion gel indicated in table 3.

Drug release study

The release kinetics is investigated by using dialysis membrane technique.[26,27,28] The membrane dialysis was performed using solution containing of ethanol and water (1:1 proportion) as a dissolution medium for CNEG which kept in membrane (Dialysis membrain – 150 LA 401-5MT). The pH of CNEG was somewhat increase by adding 0.1N NaOH solution till pH comes to 6 from 3.5. It was observed that the drug release ability increases with pH. At the pH above 5 the stability of micelle decreases. The drug release kinetics investigated by sampling out 1 ml of dissolution medium after every 2 hours for 40 hours and the absorbance was measured at 425 nm using UV-Visible spectrophotometer. Drug release kinetic study was investigated by UV-Visible spectrophotometer. The graph was plotted absorption verses wavelength of incident light using spectrophotometer (UV-Visible spectrophotometer Shimadzu carporation Kyoto. Japan) in the present study it was found that the curcumin releases somewhat faster than the CNEG as shownin figure 14 and 15 indicates comparative study of % release of curcumin verses time for free curcumin and curcuminnano-emulgel.

In Vitro study (Antimicrobial activity)

The antimicrobial activity of CNEG was investigated against *S.aureus, B.subtilis, E-coli, and S. typhi*obtained from the Department of Biotechnology M.J.C. Jalgaon, India. Nutrient agar was used to culture the bacteria. The well diffusion method was used for *in-vitro* study. The agar well diffusion method is widely used to determine antimicrobial activity.[23,24,25] to prepare inoculums, turbidity of bacterial culture was adjust according to McFarland turbidity standard by using saline. 15 ml of Muller Hinton agar medium was poured in to previously sterilize and labeled petri plates. The petri plates are swabbed with 100 µlinocula of each test bacterium and kept for 20 minutes for proper absorption. The well of diameter size 6 mm diameter bored in to seeded agar plates and these were loaded with concentration of 100 µl/ml of each compound constituted in dimethylsulphoxide (DMSO). The plates were incubated for 24h at 37 °C under aerobic conditions. After incubation, confluent bacterial growth was observed. The diameter of inhibited zones was measured in mm where DMSO served as a control. The same procedure was followed for the curcumin as a reference standard. The antibacterial activity of CNEG was assessed. Nano-emulgel CNEG has high significant antibacterial activity than curcumin as shown in figure 16 and table 04.

CONCLUSION

In the summary, we have succeeded in preparing curcumin loaded potent nano-emulgel carriers obtained by self-assembly of agarose polymer and we have succeeded to measure drug release kinetic study by membrane dialysis method. The present study indicates that optimum nano-emulgel systems of curcumin were from oleic acid (oil phase), Span 20 (surfactant), PEG 400 (Co-surfactant)

and agarose polymer (gel formulating agent). The nano-emulgel formulation passing through thermodynamic stability studies like pH, RI, viscosity measurements was carried out, while droplet size measured my FE SEM and particle size analyzer. The Zeta potential was found to be 14.5 mV. The *in-vitro* (antimicrobial study) of optimized CNEG formulation shows that nano-emulgel exhibited the high antibacterial effect as compared with curcumin. The drug release kinetic study of CNEG formulation was done by using membrane dialysis method. The drug release study indicates that the nano-emulgel formulation exhibit ability to penetrate. The ability to penetrate is mostly helpful for transdermal administration.

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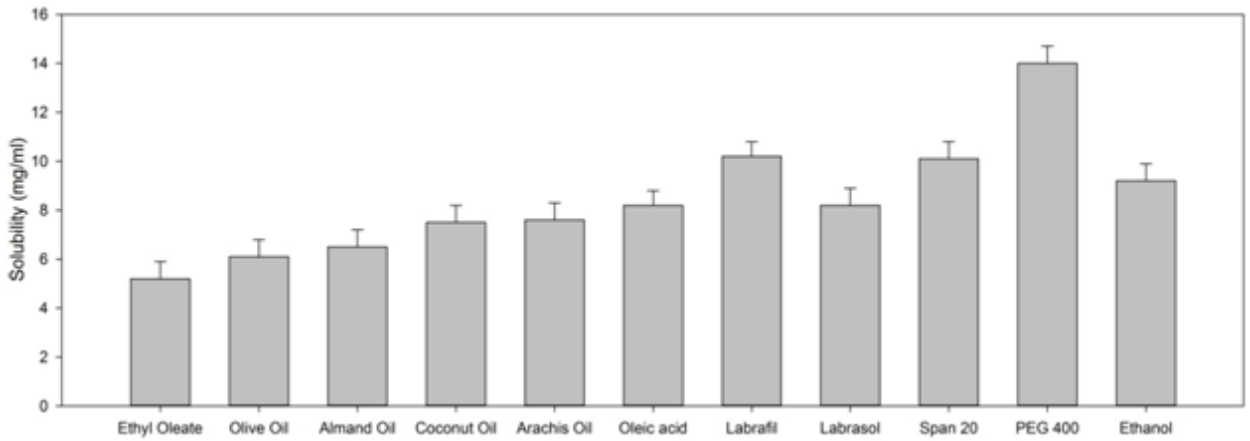


Fig. 1: Solubility of curcumin in water in different surfactants and different co-surfactants

Table 1: The different ratios of oil and Smix in nano-emulgel formation

Pseudo ternary phase diagrams	nano-emulsion	Smix ratio	Oil (%W/W)	Smix (%W/W)	Water (%W/W)
PTPD-01	NE1	1:1	10	35	55
PTPD-01	NE2	1:1	15	25	60
PTPD-02	NE3	1:2	10	35	55
PTPD-02	NE4	1:2	15	25	60
PTPD-03	NE5	1:3	10	35	55
PTPD-03	NE6	1:3	15	25	60
PTPD-04	NE7	1:4	10	35	55
PTPD-04	NE8	1:4	15	25	60

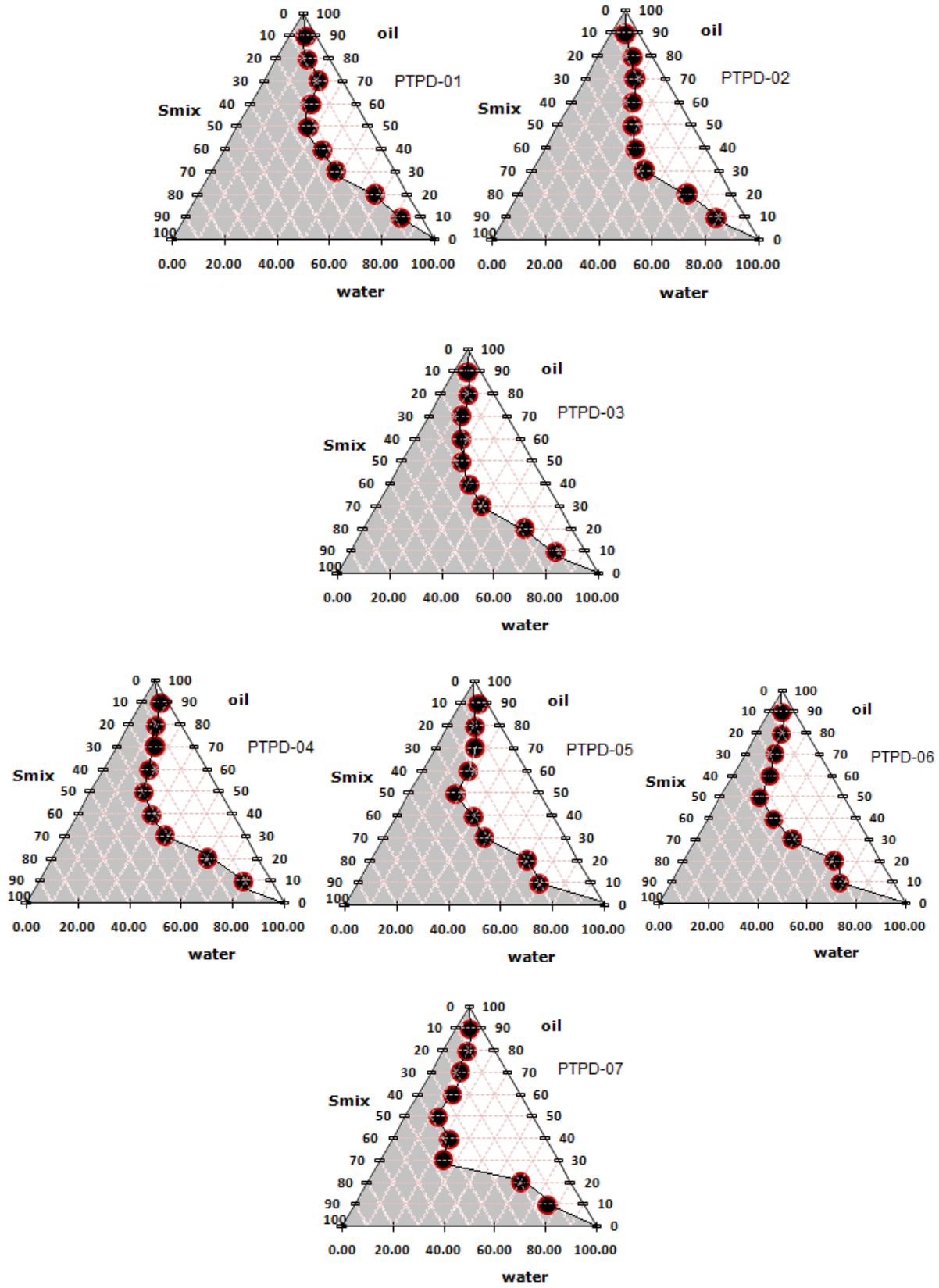


Fig. 2: The pseudo ternary phase diagrams

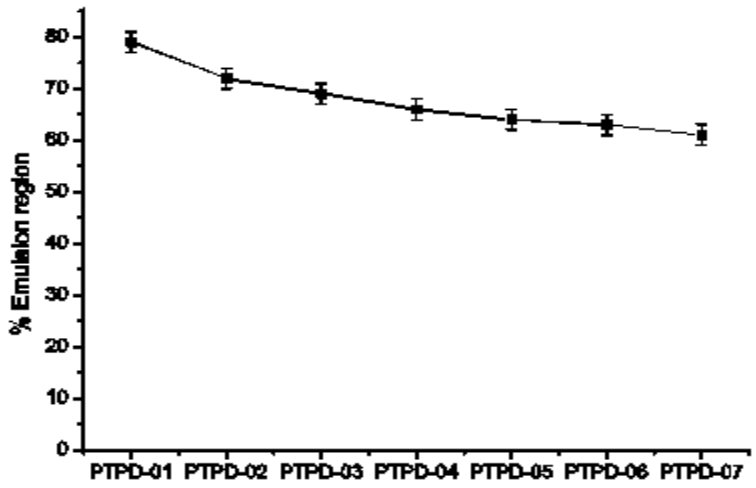


Fig. 3: Optimization of emulsion system on the basis of % emulsion region

Table 3: Thermodynamic and physical stability measurements

Curcumin loaded Nano-emulgel	Viscosity[a]	pH[a]	RI[a]	Centrifugation	Freeze thaw cycle	Heating-cooling Cycle
CNEG1	26.18 ±6.5	3.41 ± 0.2	1.38 ± 0.006	✓	✓	✓
CNEG2	24.46 ±9.6	3.42 ± 0.2	1.39 ± 0.002	✓	✓	✓
CNEG3	21.33 ±7.4	3.48 ± 0.1	1.39 ± 0.002	✓	✓	✓
CNEG4	18.27 ±6.8	3.419 ± 0.2	1.38 ± 0.007	✓	✓	✓
CNEG5	16.47 ±7.2	3.49 ± 0.4	1.38 ± 0.007	✓	✓	X
CNEG6	13.86 ±5.2	3.53 9 ± 0.3	1.38 ± 0.006	X	✓	✓
CNEG7	14.17 ±3.6	3.56 ± 0.4	1.38 ± 0.004	✓	✓	X
CNEG8	11.38 ±5.8	3.55 ± 0.1	1.38 ± 0.006	✓	X	X

a: Values were expressed in mean ±SD, (n=3),
✓:Symbols were represent that the sample was passed in the test,
X: Symbols were represent that the sample was failed in the test.

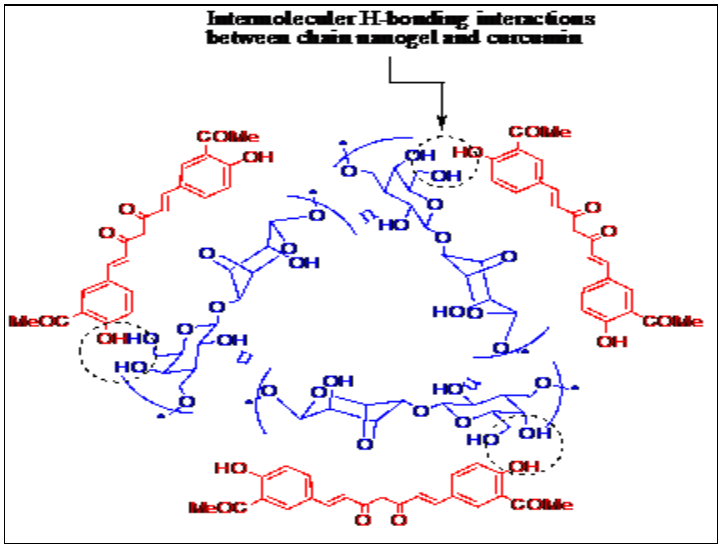


Fig. 4: The curcumin loaded agarose polymer in micelle showing intermolecular hydrogen bonding

Table 04: Potent antibacterial effect of CNEG over control (curcumin)

Organism	Inhibition diameter (mm)	
	curcumin	CNEG
S. typhi	22	28
E.coli	24	29
S.aureus	22	27
B.subtilis	17	26

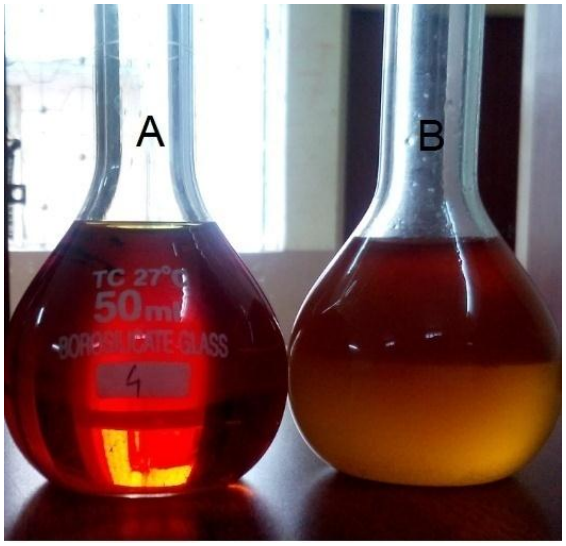


Fig. 5: free curcumin (A), curcumin loaded nano-emulgel (B)

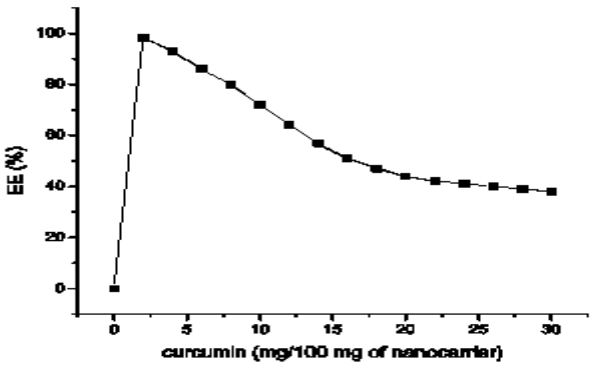


Fig. 6: Determination of % Encapsulation Efficiency

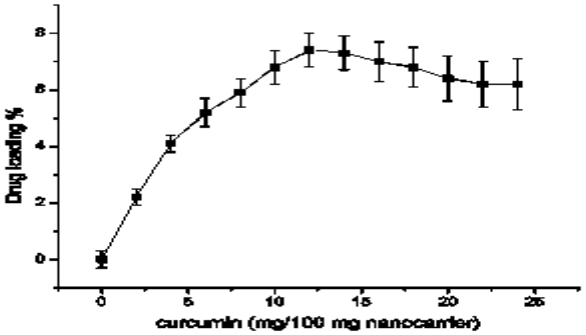
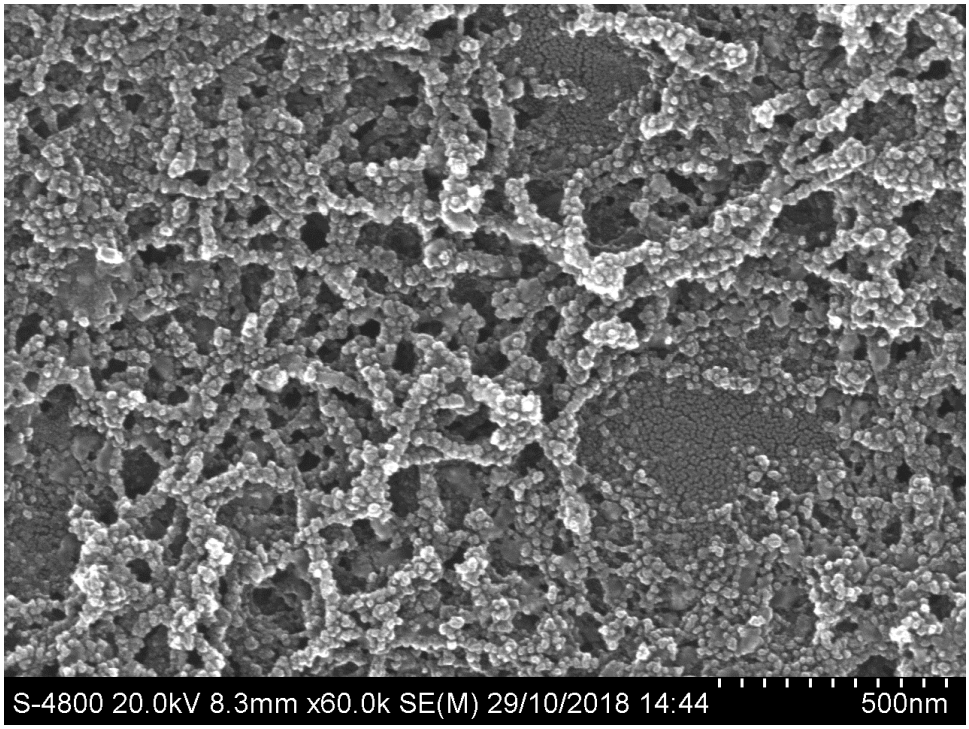
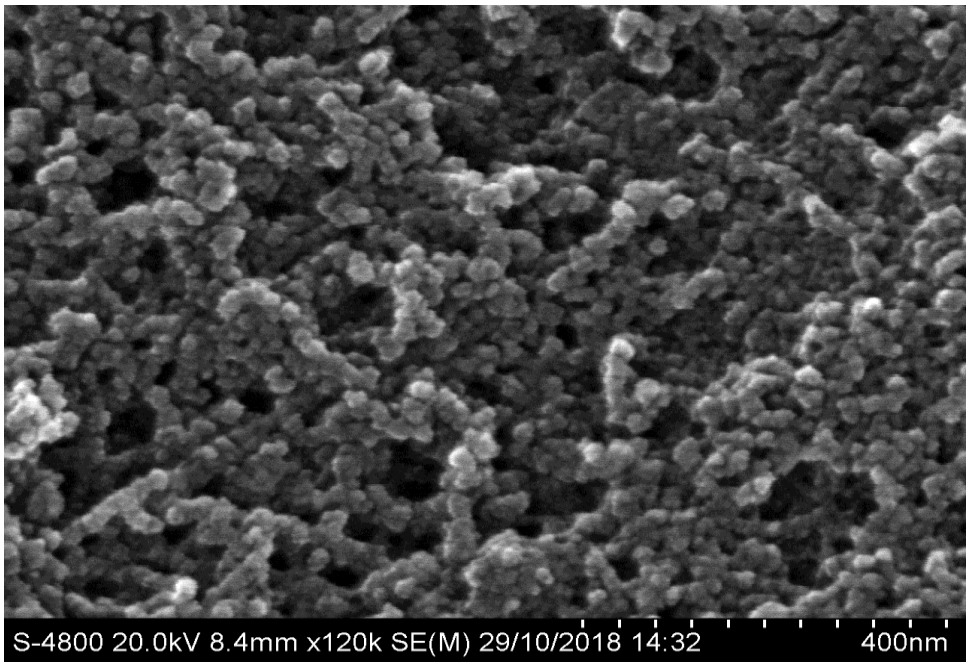
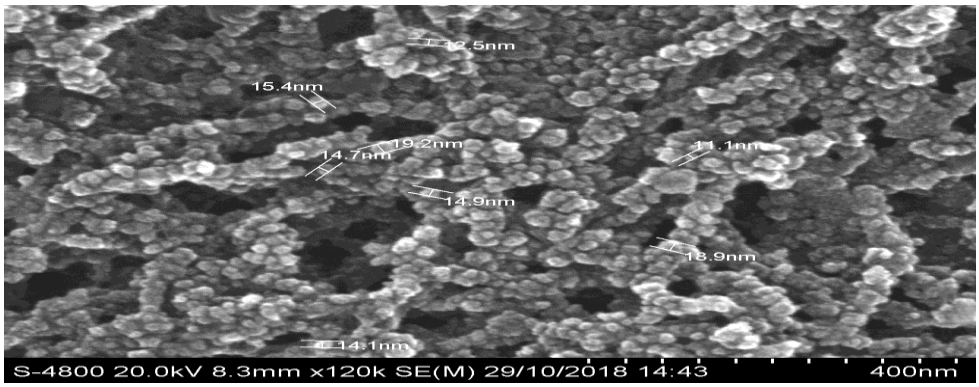


Fig. 7: Determination of % loading







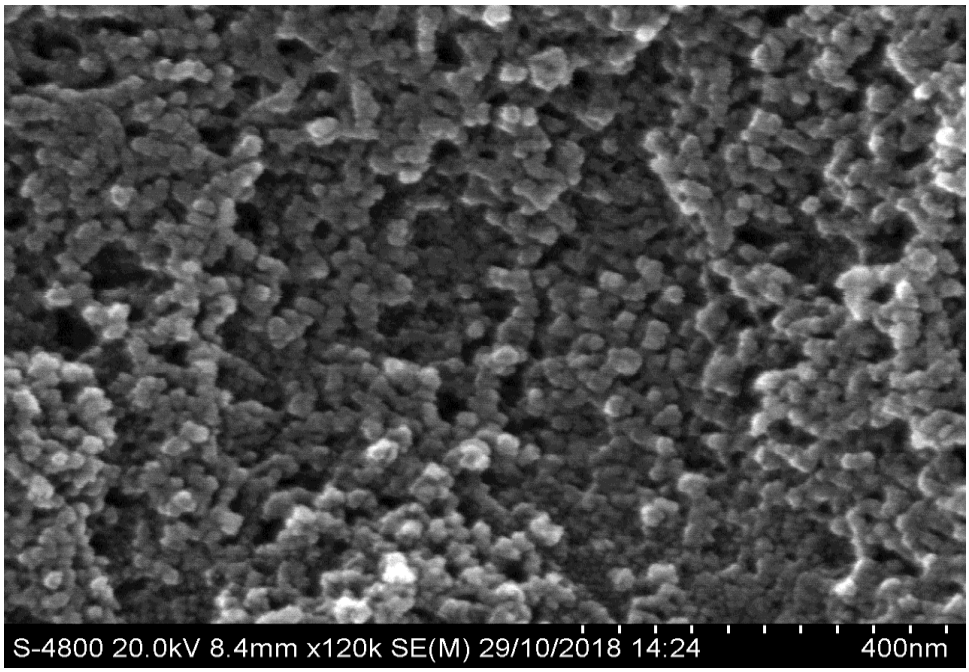


Fig. 8: FE-SEM Images of CNEG showing morphology and size

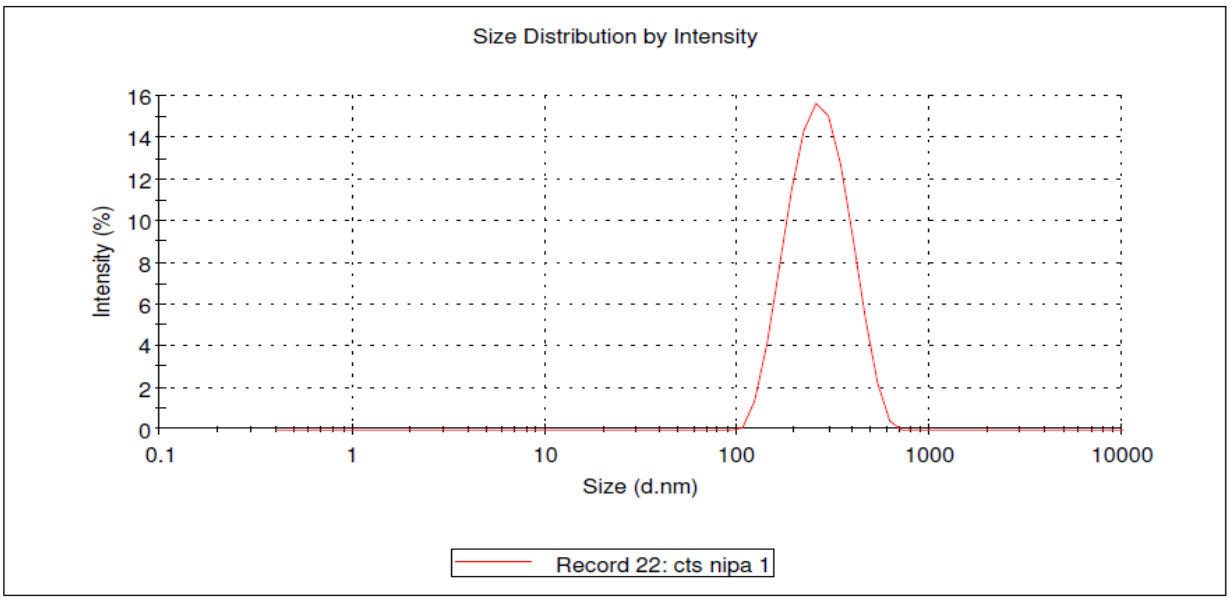


Fig. 9: The particle size distribution by intensity

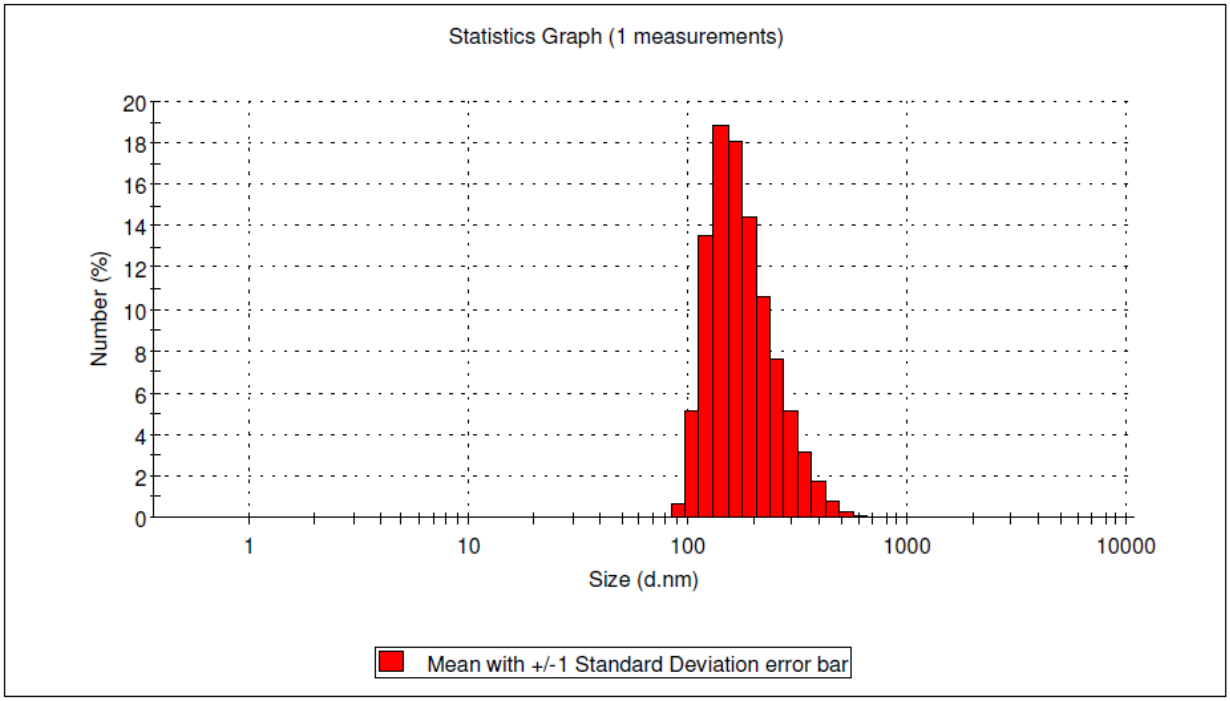


Fig. 10: The particle size distribution statistics

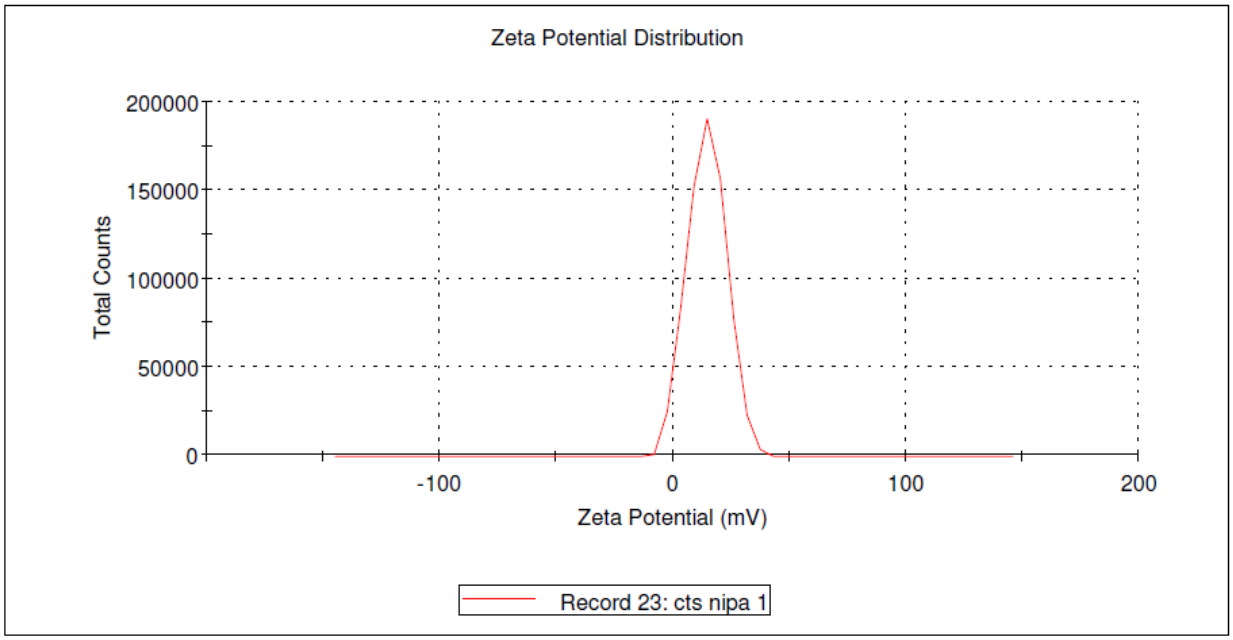


Fig. 11: The zeta potential distribution

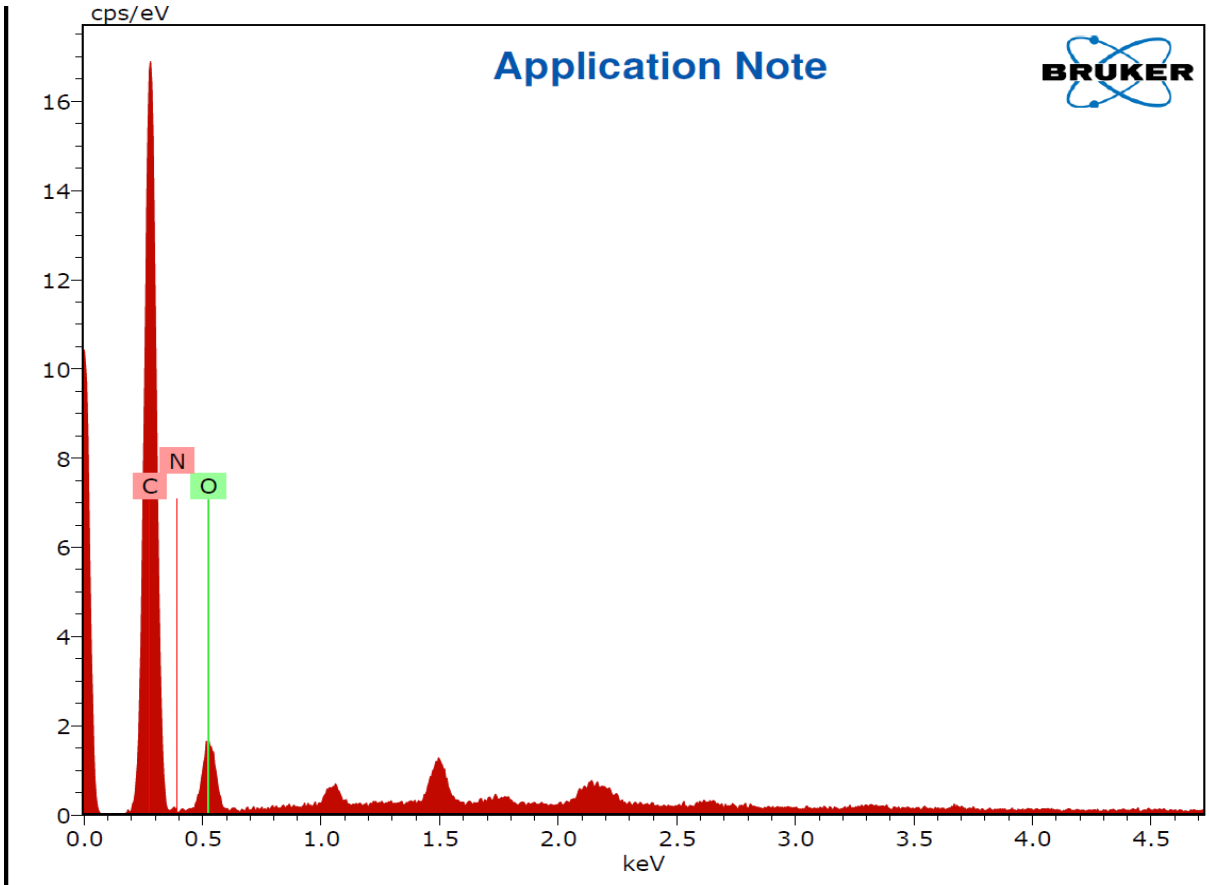


Fig. 12: Energy-dispersive X-ray spectroscopy result

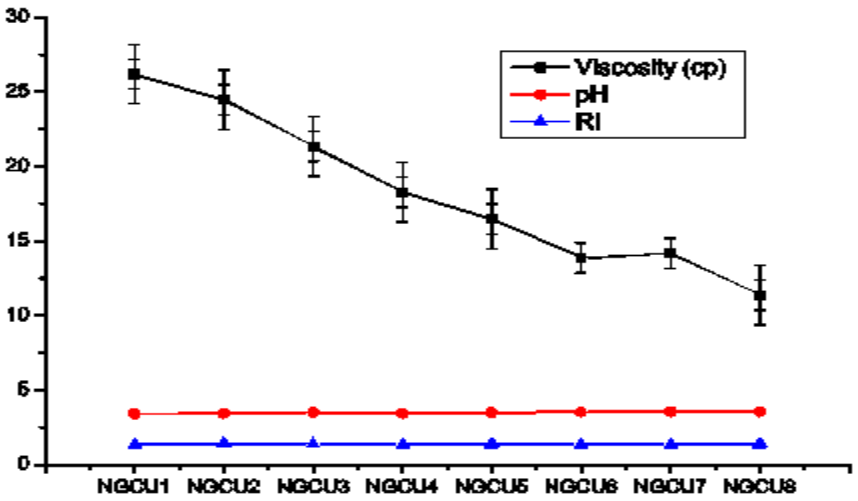


Fig. 13: Thermodynamic and Physical properties studies on CNEG

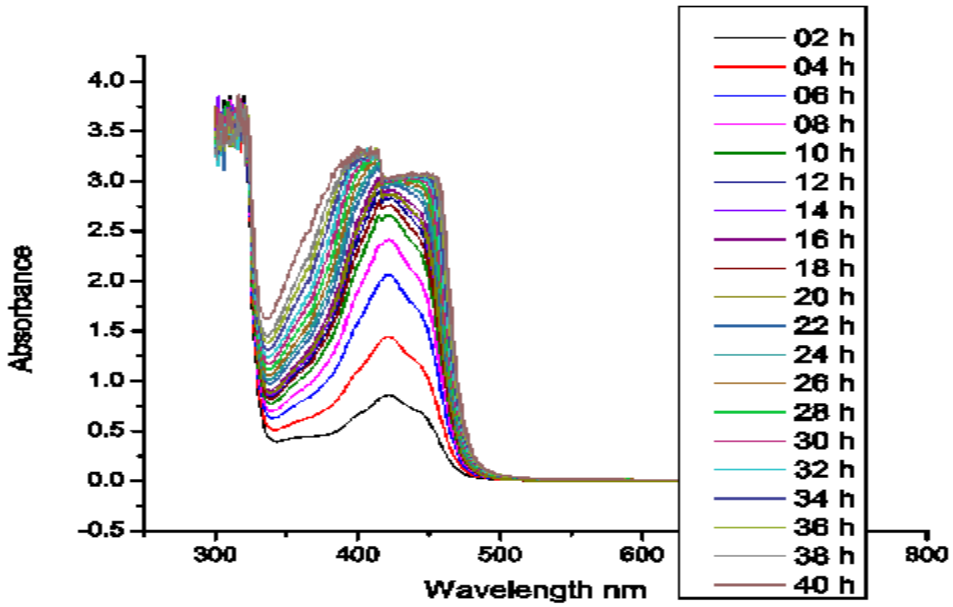


Fig.14: Measurement of UV-Visible absorbance in order to measure how much drug was released

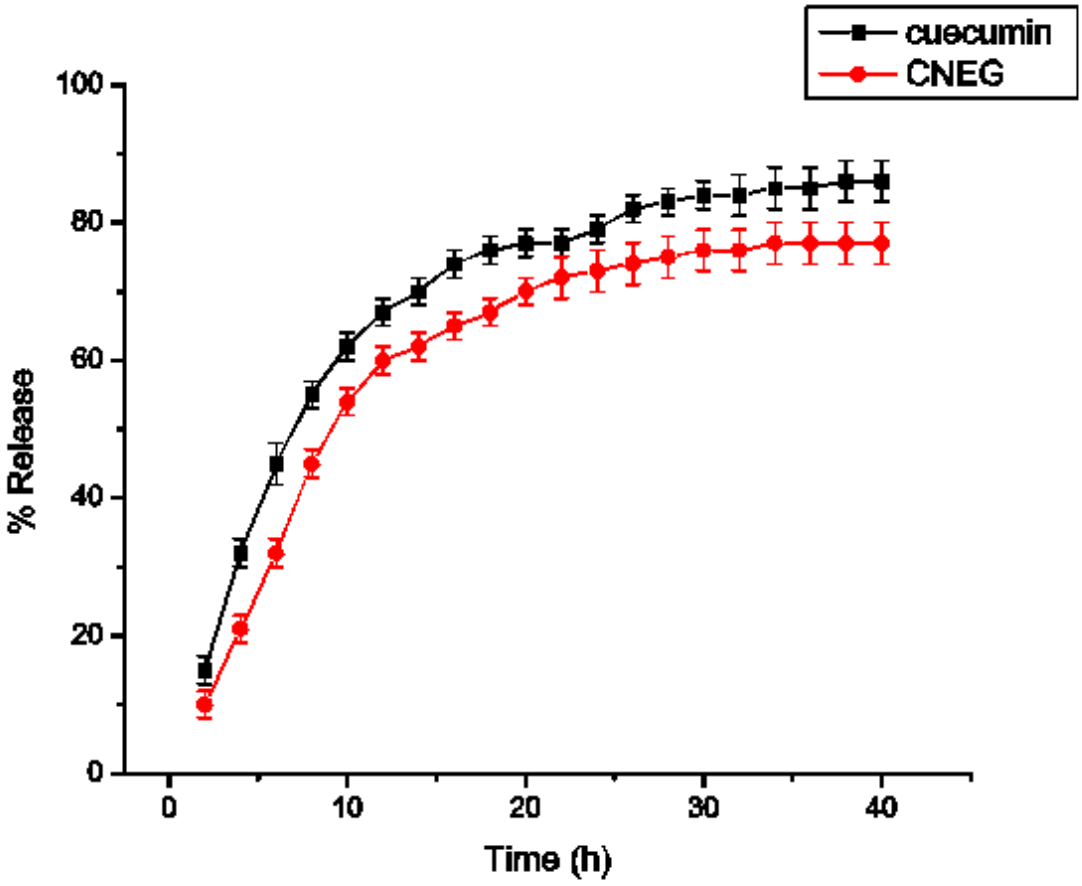


Fig. 15: Drug release kinetic study

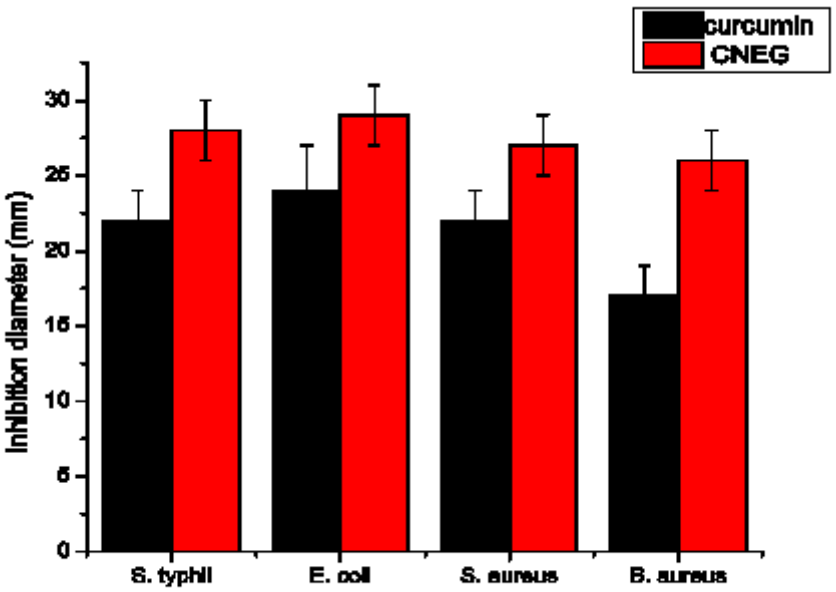


Fig.16: Potent antibacterial effect of CNEG over curcumin

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