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DESIGN AND INVITRO EVALUATION OF A NEW HERBAL NANO-MICROPARTICULATE SYSTEM: THE NEXT PROMISING INHERITED DISORDER ALTERNATIVES IN SICKLE CELL ANEMIA TREATMENT

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ABSTRACT

Sickle cell disease can result in a range of acute and chronic outcomes, some of which have the potential to be life-threatening. A vaso-occlusive crisis occurs when sickle-shaped red blood cells block capillaries, leading to reduced blood flow to an organ. This causes ischemia, discomfort, and often organ damage. The prescription drugs now on the market include Hydroxyurea (sold under the brand names Droxia, Hydrea, and Siklos) and L-glutamine oral powder (marketed as Endari). However, it is important to note that these medications might cause significant negative effects. As a substitute, curcumin nanoparticles are developed and investigated for their ability to prevent sickling. The herbal plant material was obtained from the authorized vendor, then dried and finely ground. The produced coarse powder was subjected to a 24-hour extraction process, and the resulting extract was condensed and described. The docking studies were conducted for the elements of the extract. The active components were separated from the extract using column chromatography. The extract was transformed into Nano-particles and analyzed. Various compositions of F1-F5 were produced. The evaluation of the extract's ability to prevent sickling, as well as the determination of the most effective formulation, was conducted, yielding satisfactory results. The formulations were developed and analyzed using SEM (Scanning Electron Microscopy) to examine their structure. Additionally, the Drug Encapsulation Efficiency, Percent yield, and In-vitro release experiments were conducted to assess their performance.

Keywords: Sickle cell anaemia, Herbal, Nano-particles, Anti-sickling, Phytochemical Screening, Treatment, Curcuma longa.

INTRODUCTION

Sickle haemoglobin (HbS) is a structural variation of normal adult haemoglobin (HbA) generated by a mutation in the HBB gene that results in the substitution of valine for glutamic acid at position 6 of the globulin sub-unit (S) of the haemoglobin molecule. [1,2] Sickle cell disease (SCD) refers to any situation in which the generation of HbS has pathophysiological repercussions. The most frequent variant (>70% of SCD globally) [2] is caused by homozygous inheritance of the S-mutation and is known as 'SCD SS' sickle cell anaemia' (SCA)[3]. SCD, on the other hand, can be caused by the inheritance of S in conjunction with a wide range of other HBB mutations [4], the two most common being a second structural -globulin variant C (SCD SC) [5,6] and one of the many thalassemia mutations that lead to reduced production of normal -globulin (SCD S/-thalassemia). [7,8] There are several drugs on the market, including Hydroxyurea (Droxia, Hydrea, and Siklos) and oral Lglutaminepowder (Endari), both of which have serious side effects. As an alternative, curcuminnanoparticles are created and their anti-sickling properties are examined.

ISOLATION OF CURCUMA LONGA

The dried root (rhizome) of Curcuma Longa was procured from the Yucca enterprises, Mumbaiand identified by the HarshadM.Pandit, PhD. (Botany) and air-dried in a cool place, mechanically powdered with the help of hand mill and stored in an airtight container. In a Soxhlet extractor, First cotton is placed at the bottom and again filtering disc is placed on that about 0.6kg of powdered material was placed and again filtering disc was placed and refluxed successively with 1000ml of solvent methanol for 48 hours. The extract obtained here was condensed with the help of vacuum rotatory evaporator and later the extract obtained here was further condensed in hot water bathas shown in fig. 1. [9-14].

Figure 1 : Isolation of Curcuma longa

PHYTOCHEMICAL SCREENING OF HERBAL EXTRACT

The plant extracts obtained was subjected to different phytochemical tests to identify the plant constituents by using standard following methods.

Preparation of Test Solution:

The test solution was prepared by taking 1 gm of the extract in 20ml of methanol.

PHYTOCHEMICAL CHARACTERIZATION STUDIES FOR CURCUMA LONGA [15-29]

1. **Test for Alkaloids**

a. ***Mayer' s test :***
In a few millilitres of plant sample extract, add two drops of Mayer reagent along the side of the test tube. The appearance of a white milky precipitate indicates the presence of alkaloids.

b. ***Wagner's test :***
A few drops of Wagner reagent was added to a few millilitres of plant extract on the side of the test tube. The reddish brown precipitate confirmed that the test was positive.

2. **Test for Amino acids**
The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whitman No. 1 filter paper, and the filtrate was tested for amino acids.

a. ***Ninhydrin test :***
Two drops of Ninhydrin solution (10 mg Ninhydrin in 200 ml acetone) were added to 2 ml aqueous filtrate. The appearance of purple indicates the presence of amino acids.

3. **Test for Carbohydrates**

a. ***Molisch' s test :***
Add two drops of α-Naphthol in alcohol solution to 2ml of plant sample extract. Shake the mixture well, and slowly add a few drops of concentrated sulfuric acid along the side of the test tube. The purple ring indicates the presence of carbohydrates.

b. ***Benedict' s test:***
Add 0.5 ml of Benedict's reagent to 0.5 ml of filtrate. The mixture was heated on a boiling water bath for 2 minutes. The coloured precipitate indicates the presence of sugar.

4. **Test for Fixed oils and Fats**

a. ***Spot test :***
Press a small amount of extract between two filter papers. Oil stains on the paper indicate the presence of non-volatile oil.

b. ***Saponification test :***
A few drops of 0.5N potassium hydroxide alcohol solution and one drop of Phenolphthalein are added to a small amount of extract. The mixture was heated on a

water bath for 2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

5. Test for Glycosides

For 50 mg of the extract, hydrolyze with concentrated hydrochloric acid on a water bath for 2 hours, filter and subject the hydrolyzed product to the following test.

a. Borntrager's test :

To 2ml of the filtered hydrolyses, 3ml of chloroform was added and shaken, the chloroform layer was separated, and 10% ammonia solution was then added after. Pink indicates the presence of glycosides.

b. Legal's test :

Dissolve 50 mg of the extract in pyridine, add sodium nitroprusside solution, and basify with 10% NaOH. The presence of glycosides is indicated in pink.

6. Test for Phenolic compounds and Tannin

a. Ferric Chloride test :

The extract (50 mg) was dissolved in 5ml of distilled water. Add to these few drops of neutral 5% ferric chloride solution. Dark green indicates the presence of Phenolic compounds.

7. Test for phytosterols

a. Lieberman-Burchard's test :

The extract (50 mg) was dissolved in 2ml of acetic anhydride. To do this, slowly add 1 or 2 drops of concentrated sulfuric acid along the side of the test tube. A series of colour changes indicate the presence of plant sterols.

8. Test for Proteins

The extract (100 mg) was dissolved in 10ml of distilled water and filtered through Whitman No. 1 filter paper, and the filtrate was tested for protein.

a. Millon's test :

Add a few drops of Millon's reagent to 2ml of filtrate. The white precipitate indicates the presence of protein.

9. Test for Saponins

The extract (50 mg) was diluted to 20ml with distilled water. The suspension was shaken in a graduated cylinder for 15 minutes. A 2cm foam layer indicates the presence of Saponins.

10. Test for gum and Mucilage

The extract (100 mg) was dissolved in 10ml of distilled water, and added to the 2ml of absolute alcohol under constant stirring. White or cloudy precipitation indicates the presence of Gums and Mucilages.

11. Test for volatile oil

In order to estimate the volatile oil, 50mg of powdered material (crude medicine) was taken and subjected to hydro-distillation. The distillate is collected in the graduated

tube of the module, and the water content is automatically separated from the volatile oil.

DETERMINATION OF TOTAL PHENOLIC CONTENT

A mixture of extract, distilled water and Folin-ciocalteu reagent was prepared and kept this preparation 3min a side. Then 1ml of Na_2CO_3 20% (W/V). Incubated for 1hr and observed Calorimetry at 725nm. The following equation is used to determine the total Phenolic content.

Y=1.7097ln(x) +5.2062, Where,X=Absorbance, Y=equivalent of Gallic acid (mg/ml)

DETERMINATION OF TOTAL FLAVONOID CONTENT:

The mixture of extract and $AlCl_3$ was stirred for 3min. Incubated for 1hr and observed in calorimetry. The following equation is used to determine total Flavonoid Content.

Y=0.5001 ln(x) + 3.442, Where, X=Absorbance, Y=Equivalent of Quercetin (mg/g)

DETERMINATION OF TOTAL ANTHOCYANINS:

The mixture of extract, vanillin-methanol and HCL stirred for 3min and incubated for 1hour and observed under calorimetry. The following equation is used to determine total Anthocyanins Content.

Y=0.0728 ln(x) + 0.0171, Where, X=AbsorbanceY=Equivalent of Catachein (mg/g)

ASSESSMENT OF THE ANTI-SICKLING ACTIVITY OF CURCUMA LONGA EXTRACT:

Blood samples:

The blood samples should be collected from the sickle cell patients. Once in the laboratory, they were kept in the refrigerator and the tests was performed 24 hours later to ensure that these samples were actually taken from sickle cell patients.

ISOLATION OF PURE COMPOUNDS FROM THE HERBAL EXTRACT:

- Column chromatography technique is used for the isolation of active compounds from the herbal extracts. There are two types

1. Dry approach: In the dry method, the column is first filled with dry powdered silica. The mobile
phase is then flushed through it with a suitable solvent until all of the silica is moist and settles. From
this point forward, the column must remain moist with solvent.

2. Wet method:A slurry of silica and solvent is made first and then put over the column using a funnel. More solvent must be added until the silica is dissolved.

Curcumin: [32]

- A technique for extracting and purifying choline from curcumin longa extract is developed in this study. The mobile phase in the column chromatographic procedure

is a combination of chloroform and methanol (40:60), while the stationary phase is silica gel.

- Differential scanning calorimetry will be used to characterize each fraction extracted during the chromatographic process (DSC),
- Infrared spectroscopy using the Fourier transform (FTIR).

Preparation of the column:

The column was prepared according to the dry method. The silica gel of 600g was taken for the 30ml of the extract. First glass wool was placed at the bottom and later silica gel 60 was poured into it and

above that the sample was placed and the mixture of the continuous phase was poured on to above it and continuous phase was added slowly whenever required.

Characterization of the isolated pure component:[30-33]

- **DSC:** Differential scanning calorimetry (DSC) is a thermo-analytical technique that measures the difference in heat required to raise the temperature of a sample and a reference as a function of temperature. Throughout the experiment, both the sample and the reference are kept atapproximately the same temperature. The temperature programme for a DSC study is oftenconstructed so that the sample holder temperature increases linearly as a function of time. Thereference sample should have a well-defined heat capacity over the temperature range beingscanned.
- DSC spectra of extracts were recorded using STA 7300 (HITACHI, Japan) operating with TA 700 software. Cell constant and temperature axis were calibrated using Alumina. Here the samples were heated at 100 C min-1 in the range of 300 C - 3500C under nitrogen flow rate with pin holed aluminium pans. The DSC graphs were shown in fig 4.
- **FTIR:** Fourier Transform Infrared (FTIR) spectroscopy is a technique used in industry and academia to study the structure of individual molecules and the composition of molecular mixtures. To probe a material, FTIR spectroscopy employs modulated mid-infrared radiation.

  Infrared light is absorbed at precise frequencies that are closely connected to the vibrational bond energies of the atoms in the molecule. The bond can absorb energy when the bond energy of the vibration and the energy of mid-infrared light are comparable. Because various bonds in a molecule vibrate at different energies, they absorb IR light at different wavelengths. The location (frequency) and strength of each distinct absorption bands contribute to the overall spectrum, forming the molecule's unique fingerprint. [34,35]
- FTIR spectra of extracts were recorded on a Perkin Elmer, IR Spectrophotometer model: Spectrum RXI, using KBr disc as reference. The FTIR graphs were shown in fig 5.

Calibration curve of Curcumin: [36]

10mg of curcumin was dissolved in 10ml of methanol which is referred as standard. From the above solution 1ml was transferred into a 50ml V.F and 49ml of SLS buffer was added

referred as stock. Dilutions of (0.5, 1, 1.5, 2, and 2.5) were prepared. Carried in triplicate and measured the absorbance at 421nm using UV-visible Spectrophotometer .

PREPARATION OF CURCUMIN NANOPARTICLES:

For the preparation of nanoparticles, the selection of the appropriate method is based on the drug to be loaded and physico-chemical properties of the polymer. The primary preparation methods of nanoparticles includes:

Ionic-gelatin method:

The ionic gelatin process will be used to create the herbal nanoparticles. With magnetic stirring, tri poly-phosphate will be added to this solution once the separated herbal active component extract (5%) has been completely dissolved. It will then be agitated for another 2 hours before being centrifuged for 10 minutes at 2000 rpm. The supernatant is discarded, and the pellet is re-suspended in phosphate buffer saline (PBS). Centrifugation at 2000 rpm for 10 minutes will collect the nanoparticles, which will then be rinsed with distilled water. Stirring shall be maintained for 30 minutes after the Opalescent hue was detected, and the pellet produced will be rinsed three times with distilled water. The nanoparticles will be lyophilized and kept 40°C until further usage shown in table 1.

Table 1: Formulae for the Nanoparticles formulation of isolated active component

Name of the ingredient (inpercentages)	F1	F2	F3	F4	F5
Curcumin	5	5	5	5	5
Chitosan	0.25	0.5	1.0	1.5	2.0
Acetic acid	1.6	1.6	1.6	1.6	1.6
TPP	0.5	0.5	0.5	0.5	0.5

CHARACTERIZATION OF CURCUMIN NANOPARTICLES:

1. **Morphological studies by scanning- electron microscopy: [36]**

   Scanning electron microscopy will be used to examine the Nano-particles morphology. In the first stage, 100l of the herbal Nano-particles formulation will be placed to a 10mm glass slide and dried overnight in a vacuum desiccator at room temperature till SEM examination is done. For the examination of nanoparticles, they will be mounted on appropriate support and coated with gold in a higher vacuum evaporator using a gold sputter module. At 15kv, observations will be made at various magnifications.

   2. Drug encapsulation efficiency:

   The ultra-centrifugation technique will be used to assess the drug encapsulation efficiency of

   herbal nanoparticles. In brief, additional Nano-particles constituents will be removed from the herbal Nanoparticles using ultra-centrifugation at 12,000 rpm for 30 minutes. The pellets will be re-dissolved in distilled water, and the supernatant will be scanned with a UV-visible Spectrophotometer in this parameter. The efficiency of drug encapsulation was calculated utilizing the relationship in this equation.

% Drug encapsulation efficiency= Experimental drug content/ Theoretical drug content X100.

3. Production yield of Nano-particles:

The yield of nanoparticles will be calculated by comparing the total weight of the nanoparticles generated to the combined weight of the co-polymer and drug.

% Yield calculation = Amount of drug / Amount of drug + polymer X100.

4. In-vitro release study of drug release: [37-39]

The in-vitro release of produced herbal nanoparticles in phosphate buffer saline (PBS) (PH 7.4) at 37°C will be examined. Herbal nanoparticles will be dialyzed for 1 hour against 50 ml of PBS with continual shaking in a dialysis bag. Aliquot will be removed on a regular basis. The discarded sample volume in PBS will be replaced with a new volume of PBS. The amount of medication released will be calculated by measuring absorbance with a UV-visible Spectrophotometer.

5. Emmel's test: [41-50]

The concentration of extract was prepared notably 1mg/ml using saline solution as a solvent (0.9% Nacl).From above solution we make a microscopic slide preparation where a drop of SCA blood was placed to which a drop of $Na_2S_2O_5$,2% was added to create a hypoxic condition then a drop of extract was added to that preparation which was mixed carefully to avoid air bubbles. Another slide was prepared without extract under same conditions and serve as a negative control. The edges of the slide were super cooled with paraffin to maintain the hypoxic condition. The results were seen after 24hours and carried out in triplicate

RESULTS AND DISCUSSION:

Phytochemical screening of herbal extract:

From the phytochemical screening, it was found that in Curcuma longa carbohydrates, alkaloids, flavonoid's, tannin's, proteins, phenol's were present and glycosides, lipids, ketones, cholesterol were absent as shown in table 2. cooled with paraffin to maintain the hypoxic condition.The results were seen after 24hours and carried out in triplicate.

Table: 2 Phytochemical Screening test

Sl No	Name of Test	Curcuma longa
1	Molisch's test	+VE
2	Seliwinoff's test	+VE
3	Osazone test	+VE
4	Dragendroff's test	+VE
5	Wagner test	+VE
6	Hagers test	+VE
7	Legal's test	-VE
8	Baljet's test	-VE
9	Froth formation test	-VE

10	Shinoda test	+VE
11	Ferric chloride test	+VE
12	Chlorogenic test	+VE
13	Warming test	+VE
14	Hydrolysis test	+VE
15	Saponification test	**-VE**
16	Test for pentose	**-VE**
17	Liebermann-Burchard test	**-VE**
18	Phenolic test	+VE

Table: 3 Phenolic, Flavonoid, Anthocyanins content of Curcuma longa

S.No	**Plant name**	**Total polyphenolic content mg/EGA**	**Flavonoid content mg/EQ**	**Anthocyanins content mg/EC**
1	Curcuma longa	4.928	3.503	0.0565

Emmel's test:

For the Emmel's test it was found that the herbal plant extracts Curcuma longa, has the anti-sickling property as shown in the fig 2,3.

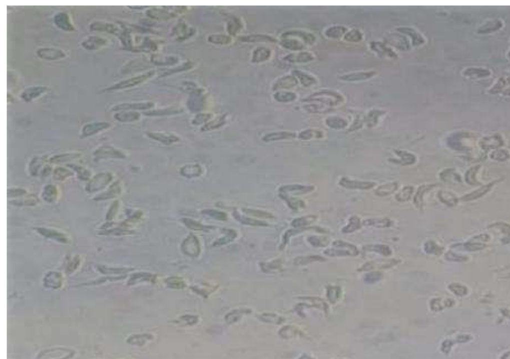

Figure: 2 Control slides of RBC of Sickle cell anaemia patient

Anti-sickling property of the plant Curcuma longa:

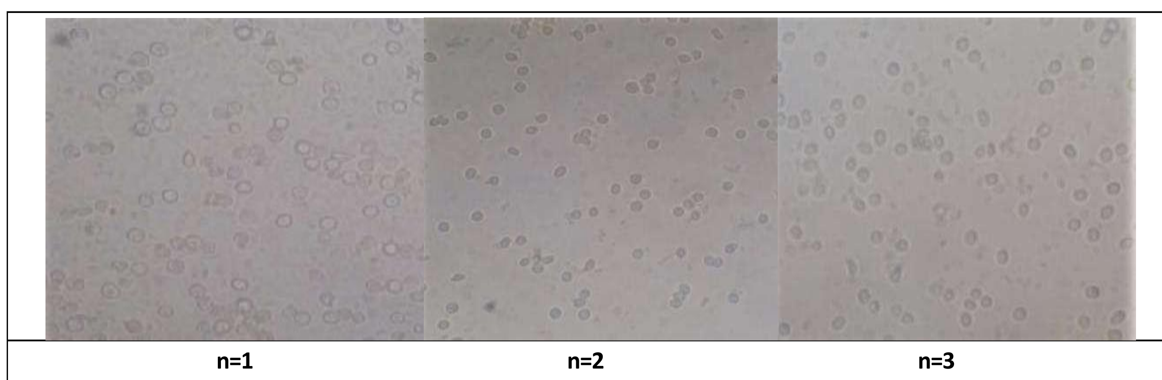

Figure: 3 RBC after treatment with Curcuma longa extract (Emmel's test)

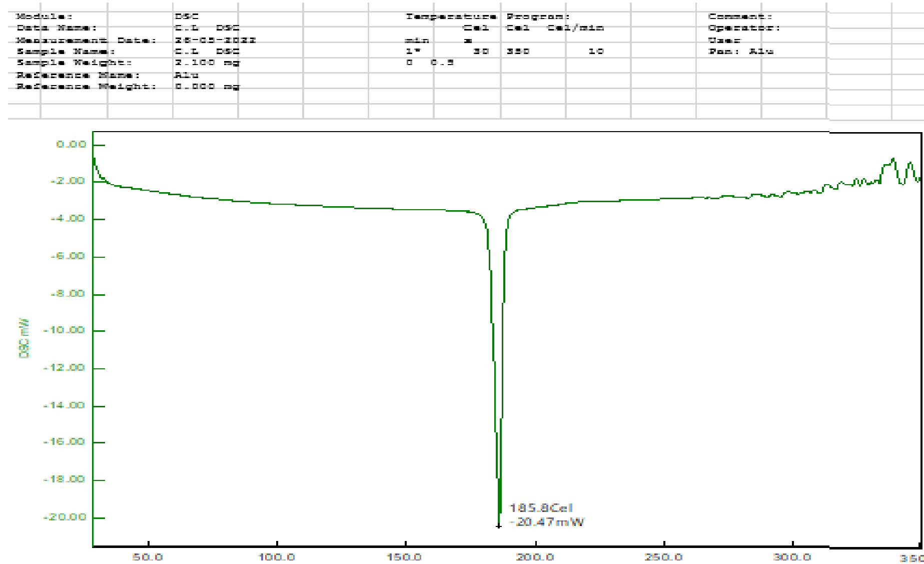

Figure: 4 DSC graph of pure component from Curcuma longa

The DSC thermograms of extract exhibited endothermic peak at 185.8 of isolated component of Curcuma longa. Where the melting point was close to the component in extract which is Curcumin (183-186oC) so, the components isolated from the extract may be this respectively.

FTIR- Fourier transmission infrared spectroscopy:

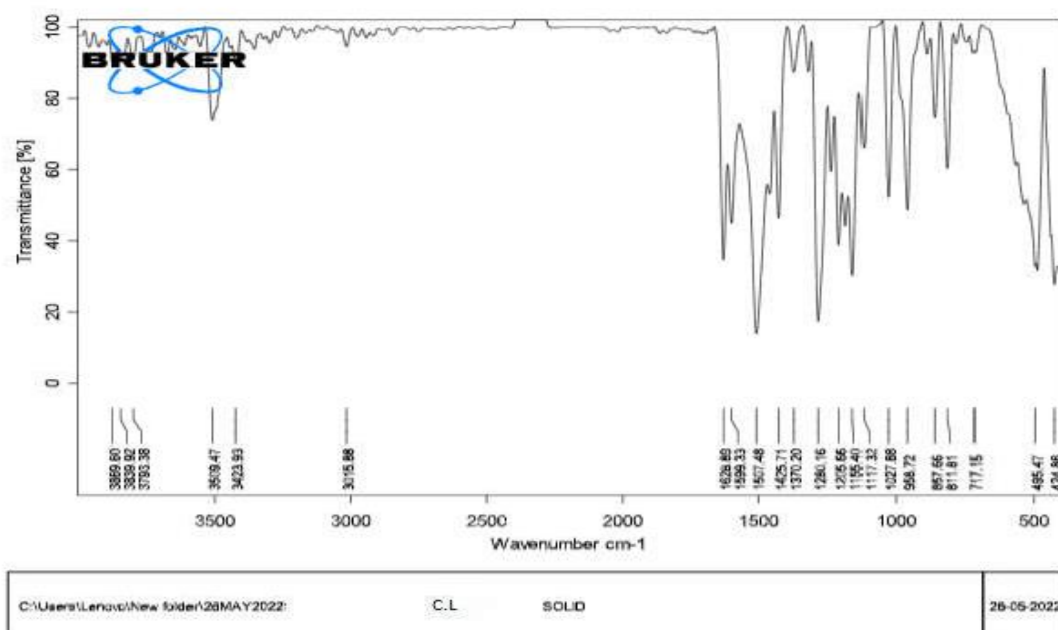

Figure: 5 FTIR graph of component from Curcuma longa

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FTIR analysis of Curcuma longa:

Table: 4 FTIR analysis of Curcuma longa

GROUP	WAVENUMBER
O-H	3509.47 cm^{-1}
C=C	1507.48 cm^{-1}
C=O	1628.89 cm^{-1}
C=C	1425.71 cm^{-1}
C=O	1280.16 cm^{-1}

From the FTIR spectra wavenumber of pure component from Curcuma longa were found to be O- H: 3509.47cm^{-1}, C=C: 1507.48cm^{-1}, C=O: 1628.89cm^{-1}, C=C: 1425.71cm^{-1}, C=O: 1280.16cm^{-1} which wasassumed to be Curcumin.

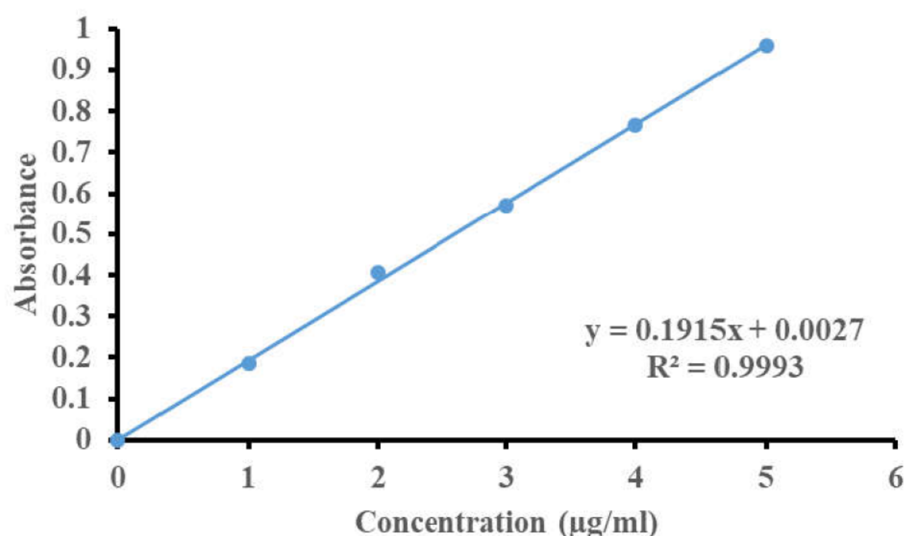

Figure: 6 Calibration curve of curcumin

From the Calibration curve of the pure components obtained from the Curcuma longa was found to be linear and obeyed Beer-Lambert's law.

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Scanning Electron Microscopy studies

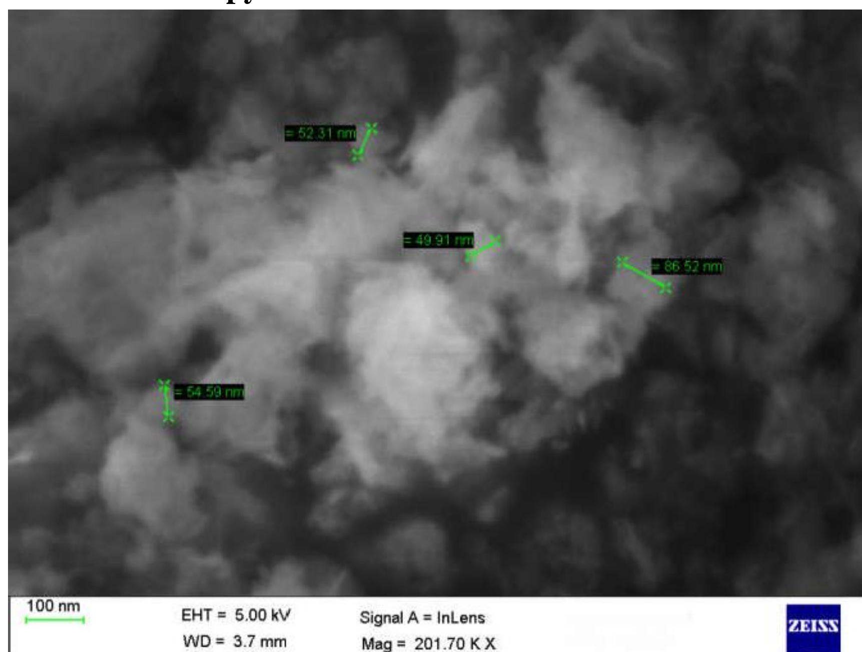

Figure: 7 SEM of Curcumin nanoparticles of (F5)

The SEM images of the formulation was given in the fig 7. The nanoparticles in Curcumin F5 were in the range of 49.91-86.52nm. Hence, the nanoparticles formed were in the range of required size.

Drug encapsulation efficiency:

Drug encapsulation efficiency is calculated by experimental drug content divided by theoretical drug content and multiplied by hundred. It gives the entrapment efficiency of the drug with the polymer.

Table: 5 Percent Drug encapsulation efficiency, n=3, mean±S.D

Name	% Drug Encapsulation efficiency
Curcumin	51.0±1.49

The drug encapsulation efficiency of the F5 nanoparticles of Curcumin was found to be 51±1.49.

Production yield of isolated curcumin Nano-particles:

Table: 6 Production yield of isolated component nanoparticles, n=3, mean±S.D

Isolated Component	F1	F2	F3	F4	F5
Curcumin	8.49±1.52	9.47±1.54	18.34±1.41	51.89±1.41	90.40±0.48

Production yield ofisolated active constituent nanoparticles of F5 is more when compared to other formulationsCurcumin is 90.40±0.48 which is most desirable.

In-vitro release studies

Cumulative percent release of curcumin:

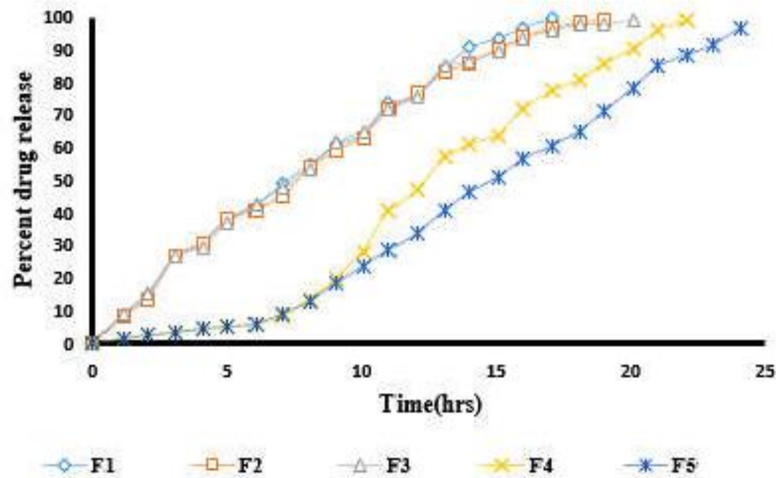

Figure: 8 Percent drug release vs Time percent release plots of curcumin (F1-F5) from nanoparticles

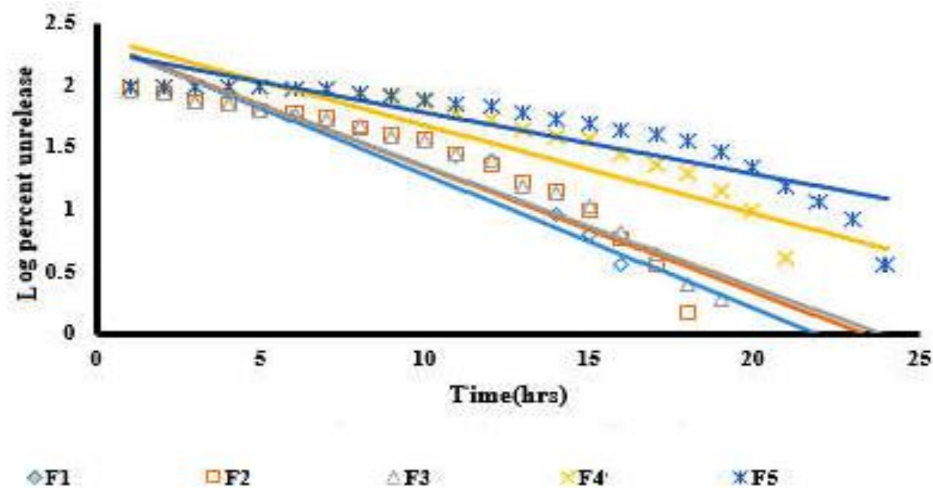

Figure: 9 Log percent unreleased vs Time percent release plots of curcumin (F1-F5) from nanoparticles

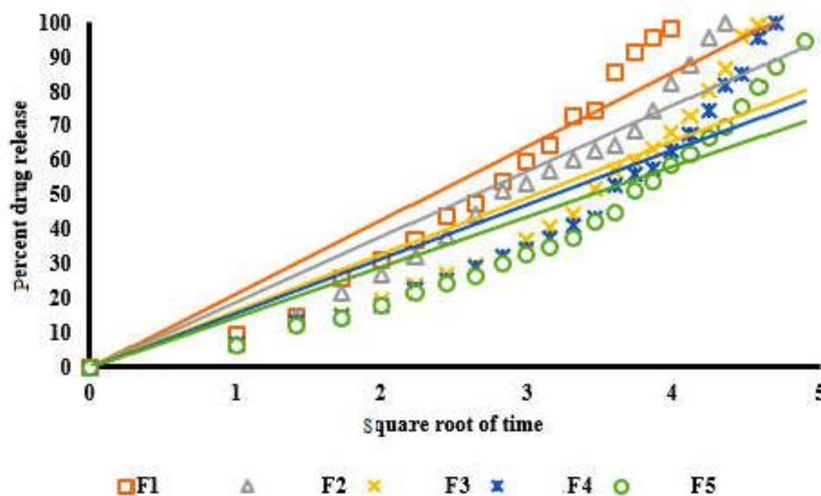

Figure: 10 Percent drug release vs Square root of time percent release plots of curcumin (F1-F5) from nanoparticles

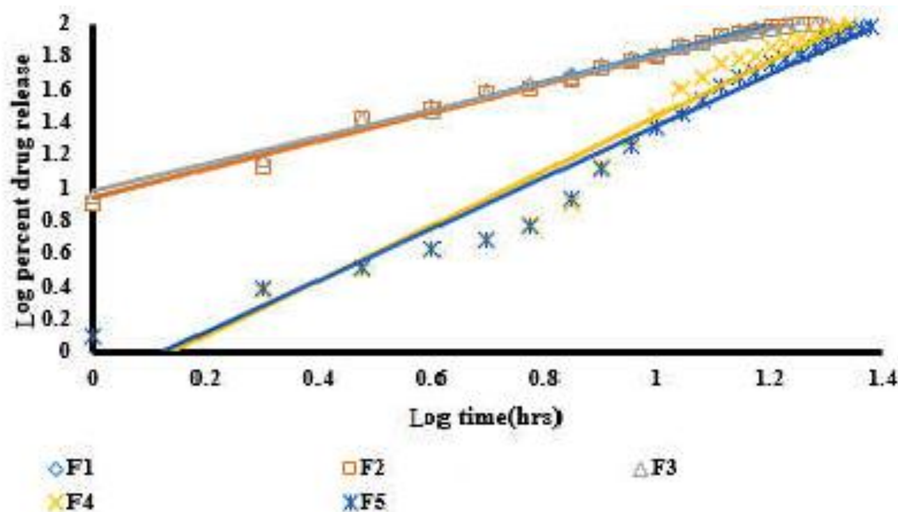

Figure: 11 Log percent drug release vs Log time percent release plots of curcumin (F1-F5) nanoparticles

Release characteristics of isolated component nanoparticles:

Table: 7 Regression(r) values for the isolated component nanoparticles

ISOLATED COMPOUND	FORMULATION	ZERO ORDER	FIRST ORDER	HIGUCHI	PEPPAS EQUATION
Curcumin	F1	0.995	0.813	0.978	0.923
	F2	0.989	0.896	0.982	0.926
	F3	0.984	0.637	0.985	0.919
	F4	0.981	0.685	0.987	0.915
	F5	0.983	0.914	0.911	0.982

Table: 8 'n' value in Peppas equation

ISOLATED COMPOUND	FORMULATION	'n' IN PEPPAS EQUATION
Curcumin	F1	0.9342
	F2	0.9686
	F3	0.9967
	F4	0.9843
	F5	0.9827

In-vitro release studies was done and it was found that the F5 has shown more controlled release when compared to other formulations in isolated active constituent Curcumin release was 99.35±0.33 for 24hrs. All the formulations followed Zero order kinetics and 'n' values indicated non- fickian diffusion.

Emmel's Test for the isolated active constituent nanoparticles:

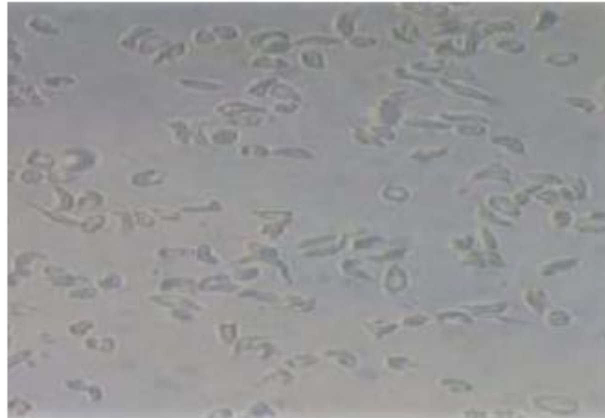

Figure: 12 Control blood of sickle cell patient

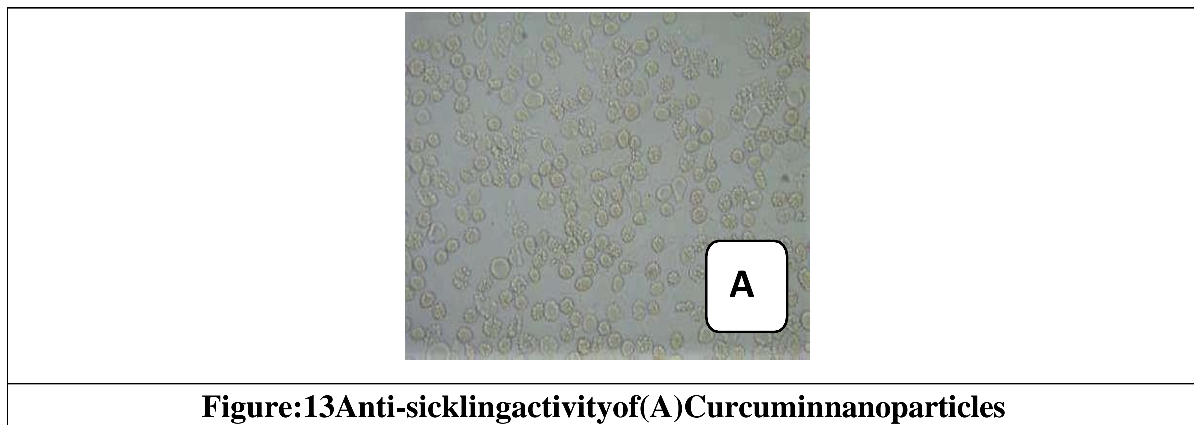

Figure:13Anti-sicklingactivityof(A)Curcuminnanoparticles

The Emmel's test also carried out with the isolated active component (Curcumin) nanoparticles the resultswere satisfactory reviving the RBC shape and has anti-sickling activity for the nanoparticles.

CONCLUSION:

Therefore, Curcuma longa has anti-sickling property and the active constituents (Curcumin) can be formulated into nanoparticles for the effective and economical treatment of sickle cell anaemia with lessside effects and is beneficial for tribal people as the dosage forms formulated are more economical.

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