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FORMULATION DEVELOPMENT AND EVALUATION OF ANTIOXIDANT POTENTIAL OF HESPERIDIN NANOCRYSTALS

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ABSTRACT

Objective: The objective of this study was to prepare and investigate hesperidin nanocrystal and investigate its antioxidant potential. **Materials and Methods:** Nanosuspensions of hesperidin were prepared by NANOEDGE™ method using optimized combination of HPMC E5 and Poloxamer 188 as stabilizers. The nanosuspension was spray dried to obtain solid dry nanocrytals of hesperidin and formulated into capsules and evaluated. **Results:** The nanosuspensions of hesperidin were successfully prepared. The concentrations of different stabilizers influenced the particle size of nanosuspensions. Spray drying of nanosuspension resulted in free flowing nanocrystals. Spray dried nanocrystal were needle shaped. The X-ray powdered diffraction and differential scanning calorimetry results indicated that there was no change in the crystalline nature of hesperidin. Hesperidin nanocrystals increased the saturation solubility to an extent of 5-times. Capsules incorporating hesperidin nanocrystal dissolved 5 times more as compared to pure hesperidin capsules. *In-vitro* antioxidant studies carried out on hesperdin nanocrystals showed that it has antioxidant potential as comparable to pure hesperdin. *In-vivo* studies on carbon tetrachloride induced liver toxicity studies on rat confirmed significant antioxidant potential of hesperidin nanocrystals at a dose of 50 mg/kg compared to pure hesperidin. **Conclusions:** Dissolution studies contended the ability of dried hesperidin nanocrystals to enhance dissolution rate of the drug compared to its crude form. Furthermore, dried hesperidin nanocrystals could significantly improve and retain the antioxidant potential of hesperidin.

KEYWORDS: Hesperidin, NANOEDGE™, nanocrystals, liver toxicity, *in vivo* antioxidant.

1. INTRODUCTION

The human body is a complex system of natural enzymatic and non-enzymatic antioxidant defenses which neutralizes the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including many types of cancers, cardiovascular diseases, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease and hepatotoxicity, ulcerative colitis, aging and atherosclerosis.[1]

Hesperidin is a flavanone glycoside found in citrus fruits (family *Rutaceae*). Its name is derived from the word "hesperidium", for fruit produced by citrus trees. The flavonoid hesperidin comprises of the flavanone hesperitin and disaccharide rutinose. Sweet oranges (*Citrus sinensis*) and tangelos are the richest dietary sources of hesperidin.

Hesperidin has low solubility in water. It is much more soluble in water than its aglycone hesperetin. It is chemically 2(S)-7-[[6-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one, is a yellow to brown crystalline powder having melting point at 260^{o}C. This compound was initially referred as 'Vitamin P' as it could decrease capillary permeability and fragility.[2] Literature suggests that hesperidin prevents microvascular leakage by virtue of its vasoprotective action through the inhibition of the enzyme hyaluronidase which is reported to regulate the permeability of capillary walls and supporting tissues.[3] Hesperidin, in combination with a flavone glycoside called diosmin, eg. Daflon 500 is used widely in Europe for the treatment of venous insufficiency and hemorrhoids. Besides their effect on vascular permeability and ocular blood flow, hesperidin demonstrates strong antioxidant properties by quenching oxidative radical chain reactions.[4] Hesperidin also exhibits significant anti-inflammatory, hypolipidemic, anti-hypertensive and diuretic activity.[5,6,7,8] It also shows potential anticancer activity mediated through the suppression of cell proliferation.[9,10]

Currently hesperidin is used as a dietary supplement for improving blood flow and for its vasoprotective properties, and is available as an oral dosage form. Ameer *et al.* reported that following oral administration, hesperidin is absorbed across the gastrointestinal tract, but cumulative urinary recovery indicates low bioavailability (<25%).[11] Several factors limit

oral bioavailability of hesperidin, including poor water solubility and precipitation in an acidic environment.[12] Attempt has been made to improve the solubility of hesperdin by its chemical derivatization, complexation with cyclodextrin, micronization offers a technology to increase dissolution, however it does not change the saturation solubility.[13,14,15] Therefore, further steps to reduce particle dimension to nanometer size range has emerged to be a powerful formulation approach that can increase the dissolution rate and the saturation solubility.[26]

Drug nanocrystals can be prepared by bottom up techniques (precipitation methods) or top down techniques (size reduction by milling or high pressure homogenization) and combination technologies like NANOEDGE™ (Baxter International Inc.) which involves precipitation followed by a homogenization, SmartCrystal and Precipitation-lyophilization-homogenization (PLH) technology.[18,26,27]

In this study, the feasibility of production of capsules of hesperdin nanocrystal was investigated, using NANOEDGE™ Technology with an aim to have formulations with increased dissolution velocity, oral absorption and better antioxidant potential.

2. MATERIAL AND METHOD

2.1. Material

Hesperidin was purchased from Otto Inc, Mumbai. The stabilizer Poloxamer 188 from BASF (India), Hydroxypropylmethylcellulose E5 from LOBA Chemie Pvt.Ltd. All the other chemicals used were of analytical grade.

2.2. Method

2.2.1. Preparation of hesperidin nanosuspensions and optimization of preparation process.

Nanosuspensions with different stabilizers were produced using combination technique involving evaporative precipitation augmented by high pressure homogenizer (Avestin EmulsiFlex C5) with a batch size of 50 mL. Stabilizers were added to purified water maintained at a cold temperature using ice bath and stirred using mechanical stirrer at the speed of 800 rpm. Simultaneously Hesperdin was solubilised into 0.5% Dimethyl sulphoxide to form a clear solution. Drug solution was maintained at 60^{o}C and added to cold stabilizer solution under mechanical stirring dropwise. Suspension was kept on stirring for 1 hour. Then suspension was passed through High pressure homogenizer at 15,000 psi for 15 cycles.

Batches were prepared using 1:2 ratio of Hesperidin: HPMC E5 (Batch X) and also using different ratios of poloxamer 188 and HPMC E5 as shown in the figure 1.

Ingredients	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch X
Hesperidin	50mg	50mg	50mg	50mg	50 mg	50 mg	50mg	50mg	50mg	50mg
HPMC E5 IP	100mg	50mg	50mg	150mg	150mg	75mg	75mg	_	_	100mg
POLOXAMER 188 IP	_	_	_	_	_	25mg	25mg	150mg	50mg	_
Tween 80 IP	1%	_	1%	_	1%	_	1%	_	_	_
DMSO IP	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%
Distilled Water	50ml	50ml	50ml	50 ml	50ml	50ml	50ml	50ml	50ml	50ml

Figure 1: Trial Batches using NANOEDGE™ Method

2.2.2. Particle size analysis

The mean particle size and polydispersity index of the prepared nanosuspension was obtained by using Malvern particle size analyzer Nano-S2000.

2.2.3. Spray drying of nanosuspension

Solid dispersion of Hesperidin nanocrystal was prepared using Spray Drying method. The optimized formulation of nanosuspension was spray dried to obtain solid and stable powder. The solid nanocrystals was prepared using Labultima spray drier (LU-222 advanced) parameter was set regarding to temperature inlet (190°C), outlet (85°C), pump speed (1.5ml/min), Atomization Pressure (0.8 to 1 kg/cm).

2.2.4. Characterization of spray dried powder

2.2.4.1. Crystalline status evaluation

a. Differential Scanning Calorimetry (DSC)

DSC thermograms were recorded using differential scanning calorimeter (PerkinElmer Inc., Shelton, CT, USA). About 1-3 mg of samples were sealed in flat bottomed aluminium cubicles and heated at a scan rate of 10^{o}C/min from 30 to 300^{o}C.

b. Powder X-ray diffractometry (P-XRD)

X-ray diffractograms of hesperidin, mixture of (drug and excipients) and formulation were recorded on a Philips Analytical X'pert Pro MPD with copper anode using a voltage of 40 kV and a current of 35 mA. The diffractograms were recorded in 2θ angle range between 10^{o} and 90^{o} at a scanning rate of 18/min.

2.2.4.2. Scanning electron Microscopy

Surface morphology of hesperidin nanocrystal was observed using scanning electron microscope (SEM). Spray dried nanocrystal powder was spread as a thin layer on aluminium foil loaded on to two way carbon film coated copper grid for SEM. The pictures were taken at excitation voltage of 20kV using scanning electron (Microscope Quanta 200).

2.2.4.3. FTIR studies of hesperidin nanocrystal

FTIR analysis of pure hesperidin mixture of hesperidin poloxamer 188 and HPMC E5 and hesperidin nanocrystals were performed and spectrum was obtained using FT-IR (Perkin Elmer 200 series). All spectras were recorded within a range of 4500-400 cm^{-1}.

2.2.4.4. Re-dispersability and Particle size of Nanocrystals

The re-dispersability of hesperidin nanocrystals was determined by shaking vials by hand and until all of the sediment had been uniformly dispersed in the aqueous phase and the particle sizes was determined by Malvern particle size analyzer.

2.2.4.5. Saturation Solubility

Solubility studies of hesperdin nanocrystal and pure hesperidin was done in water and various buffers i.e. pH 1.2, 6.8 and 7.4 phosphate buffer. Spray dried powder was added in excess to 10 ml of mentioned buffers till no further drug was soluble in it. Then solutions were kept for shaking in orbital shaker for 48 hours at 37^{o}C. After 48 hours solutions were filtered using Whatmann filter paper and absorbance was taken using UV/Visible Spectrophotometer.

2.2.4.6. Assay

Spray dried product of nanosuspension formulation is considered to have 100% drug content. 50 mg nanocrystal powder was dissolved in 100ml 0.1N sodium hydroxide solution and analyzed at 283 nm using UV spectrophotometer. The drug content was calculated using calibration curve of drug in 0.1N sodium hydroxide solution.

2.2.5. Preparation of the Capsule and Drug Release

hesperidin nanocrystal capsules was formulated . Hesperidin nanocrystals were admixed to the excipients by geometric mixing. The mixed powder was filled into hard gelatin capsule of size 00 using simple filling capsule equipment for lab scale. The final product of the capsules was collected and immediately transferred into dry plastic containers and tightly sealed.

Table 1: The formulation of hesperidin nanocrystal capsules

Ingredients	Weight per capsule
Hesperidin Nanocrystal	493.68mg (weight equivivalent to 250 mg)
Talc	1%
Magnesium Stearate	0.05%

Dissolution rate studies of pure hesperidin and hesperidin nanocrystals were performed in USP dissolution testing apparatus (type I) with rotating basket at 100rpm using 900ml of pH 1.2 buffer and pH 6.8 phosphate buffer as dissolution medium. The temperature was maintained at 37±0.5^{o}C throughout the experiment. 5ml of samples were withdrawn at various time intervals and same volume of dissolution medium was replaced for maintaining the constant volume of dissolution medium. Samples were filtered through whatmann filter and analyzed for drug concentration using UV spectroscopy.

2.2.6. *In vitro* antioxidant studies

I. Reducing power method

1 ml of various concentrations (20, 40, 60, 80, 100 µg/ml) of hesperdin nanocrystals were mixed with potassium ferricyanide (2.5 ml, 1%), 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml water, 0.5 ml Ferric chloride (0.1%) and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The results were compared by using pure hesperidin and ascorbic acid (same concentrationas that hesperidin nanocrystals) as standards using the same procedure.[16]

II. Hydroxyl radical scavenging assay

The scavenging capacity for hydroxyl radical was measured according to following method. Stock solutions of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM), H_2O_2 (10mM), deoxyribose (10mM) were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $Fecl_3$, 0.1 ml H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of the stock solution of different concentration (10,20,30,40,,50,60 mg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM,pH-7.4), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37˚C for 1 hr. A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% Trichloroacetic acid and 1.0 ml of 0.5% thiobarbituric acid to develop the pink chromogen measured at 532 nm. The same

procedure was carried out using curcumin as standard (2, 4, 6, 8, 10 µg/ml). The hydroxyl radical scavenging activity of the hesperidin nanocrystal was reported as % inhibition of deoxyribose degradation is calculated by using Eq.

Percentage inhibition = (1– absorbance of test/absorbance of control) × 100.

The IC_{50} value of hesperidin nanocrystal was determined and compared with that of pure hesperidin and curcumin.[17]

III. Thiobarbituric acid method

1 ml of different concentrations (20,40,60,80,100 µg/ml) of the hesperidin nanocrystal were mixed with 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% of thiobarbituric acid.

The mixture was placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000 rpm for 20 min. The same procedure was carried out using curcumin as standard (20, 40, 60, 80, 100 µg/ml).The absorbance activity of the supernatant was measured at 552 nm and recorded after it has reached its maximum.The IC_{50} value of ethyl acetate rich extract was determined and compared with that of pure hesperidin and curcumin using the same equation as mentioned in hydroxyl radical scavenging assay.[1]

2.2.7. *In Vivo* Antioxidant Study

2.2.7.1. Animals

Male Wistar albino rats (*Rattus norvegicus*) used for the present study were procured from Bharat Serum and Vaccines Ltd, Thane kept in SVKM's animal house facility were allowed to acclimatize for 7 days prior to commencement of experiment. The experiments were performed based on animal ethics guidelines of Institutional Animals Ethics Committee (Approval Number: CPCSEA/IAEC/BNCP/P-37/2014).

2.2.7.2. Experimental design

After seven days of acclimatization, the rats were divided into four groups (n=6). Treatment was done for 8 days as follows.

Group I**:** Normal control (0.9% normal saline; 1 ml/kg i.p.)

Group II**:** CCl_4 control (CCl_4: liquid paraffin (1:2); 1ml/kg i.p.)

Group III**:** CCl_4 + Pure Hesperidin (50 mg/kg/day; p.o).

Group IV**:** CCl_4 + Hesperdin nanocrystal (50mg/kg/day; p.o).

Group V**:** CCl_4 + Hesperdin nanocrystal (40mg/kg/day; p.o).

Group II-V received CCl_4 in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h.

After 24 h of the last dose, all the animals were sacrificed and liver tissues were collected for the evaluation of antioxidant status through parameter viz., catalase assay, lipid peroxidation assay and histopathological studies.

2.2.7.3. Estimation of lipid peroxidation (LPO)

Tissue sample preparation for LPO assay: 1 g of liver tissue was collected from each experimental rat, washed in normal saline and soaked in filter paper. The tissues were then homogenized in 10 ml of 0.15 M tris buffer (pH-7.4) and centrifuged at 3000 g at 4° C for 30 min. The supernatant was collected was taken for lipid peroxidation.

Procedure

Lipid peroxidation (LPO) was assayed according to the method of Okhawa et al. To 1ml of tissue homogenate, 1ml of normal saline (0.9% w/v) and 2.0 ml of 10%TCA were added and mixed well. The mixture (3000 g) was then centrifuged at room temperature for 10 min to separate proteins. 2 ml of supernatant was taken, 0.5ml 1.0% TBA was added to it followed by heating at 95° C for 60 min. to generate the pink colored MDA. OD of the samples was measured at 532 nm using UV spectroscopy. The levels of lipid peroxides were expressed as nM of MDA/mg wet tissue using extinction co-efficient of 0.155 M^{-1} cm^{-1}.[17,29]

2.2.7.4. Catalase Assay

Tissue sample preparation for catalase assay: 900 mg of liver tissue was collected from each experimental rat, washed in normal saline soaked in filter paper. The tissues were then homogenized in 3.0 ml M/150 phosphate buffer (pH-7.0) and centrifuged at 3000 g at 4° C for 1 hr. The supernatant collected was taken for the assay.[17]

Assay Procedure

Catalase activity was assayed by the method of Luck. Briefly, assay mixture of 25 µl of sample and 3ml of phosphate buffer for blank solution and 3 ml phosphate buffer with hydrogen pereoxide for test in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/gm of protein using molar extinction coefficient of 0.071.[28]

2.2.7.5. Histopathological studies

After animals were sacrificed and liver was excised, washed immediately with saline and then fixed in 10% buffered formalin. Tissues were embedded in paraffin, sectioned at 5 µm

and stained with hematoxylin and eosin (H&E). The sections were examined under light microscope.

2.2.8. Statistical analysis

The statistical evaluation was performed using the Graphpad InStat for 32 bit Windows version. Groups were compared to assess the statistical significance using one way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post-hoc test. The data is represented as mean ± SEM values and n = 6 per group

3. Result and Discussions

3.1. Preparation of nanosuspension and particle size analysis

Different batches of hesperidin nanosuspension were prepared. The particle size was confirmed by Zeta sizer. The average particle size for all the batches is given in Table 2.

Table 2: Particle size and PDI results of trial batches.

Batches	Batch X	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7
Z-Average (d-nm)	2223	2710	404.1	2502	406.5	2286	**377.8**	499.3
PDI	1	1	0.656	1	0.435	1	**0.052**	0.816

3.2. Spray Drying

Spray drying process was accomplished to obtain dried hesperidin nanocrystals. Spray drying imparted better flow property to nanocrystals and completely dried nanocrystal.

3.3. Characterization of spray dried nanocrystal

3.3.1. Crystalline status evaluation

a. Differential Scanning Calorimetry (DSC)

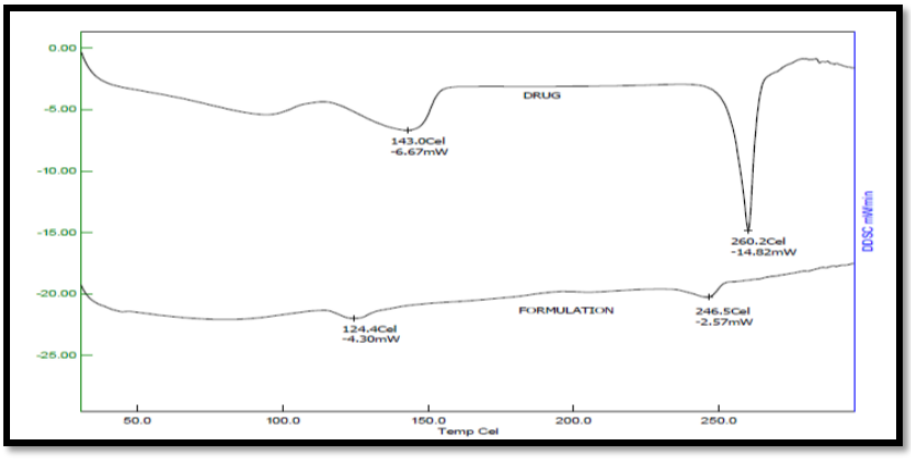


Figure 2: Overlay of DCS graphs of drug and formulations

DSC thermogram showed of drug showed peaks at 260.2°C and 143°C and that of hesperidin nanocrystals was observed at 246.5°C and 124.4°C.

b. Powder X-ray diffractometry (P-XRD)

X-ray diffractograms of Hesperidin, mixture of (drug and excipients) and formulation were recorded and there graphs are as follow.

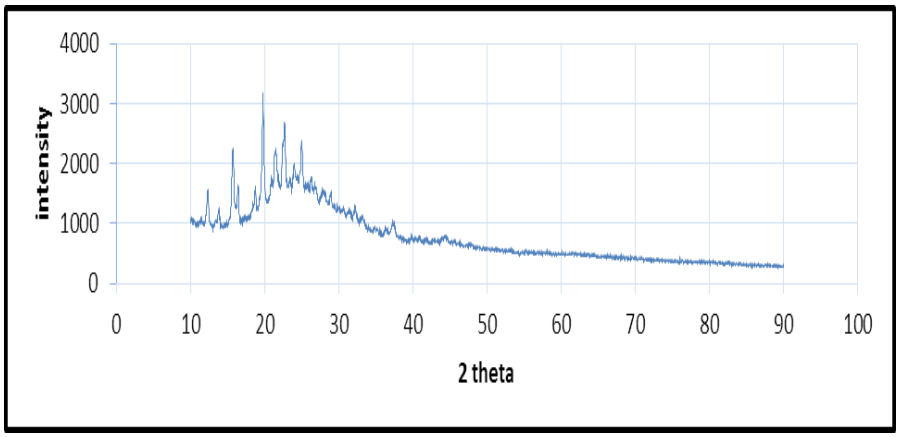


Figure 3: XRD data of pure hesperidin

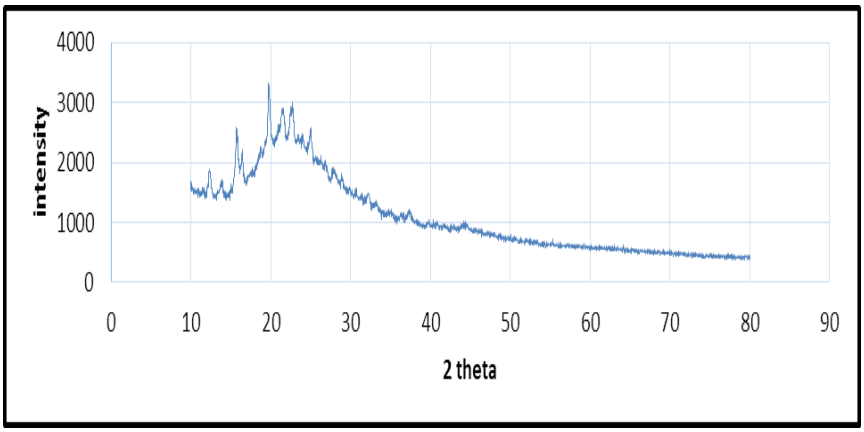


Figure 4: XRD data of pure hesperidin with poloxamer 188 and HPMC E5

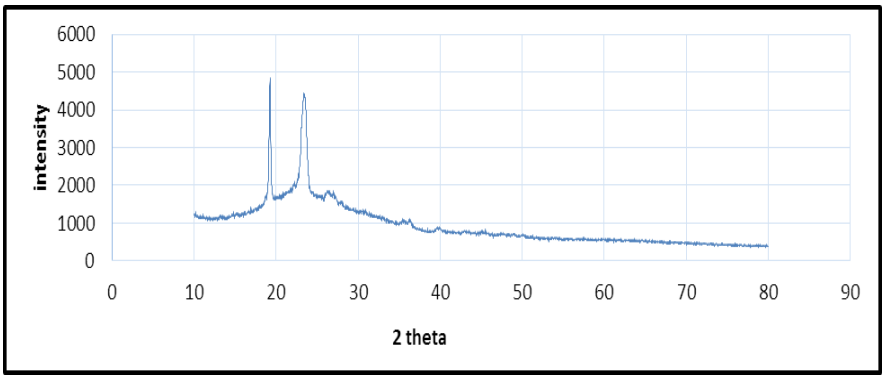


Figure 5: XRD data of hesperidin nanocrystals

XRD data of pure hesperidin and hesperidin nanocrystals have been shown in above figures 3-5.

3.3.2. FTIR of Nanocrystals of Hesperidin

FTIR of Hesperidin Nanocrystals was done. Following image shows overlay of hesperidin and mixture of hesperidin +HPMC E5+ Poloxamer 188 and hesperidin nanocrystal.

Black- Hesperidin

Blue- Nanocrystal

Green- Mixture of Hesperidin + HPMC E5 + Poloxamer 188

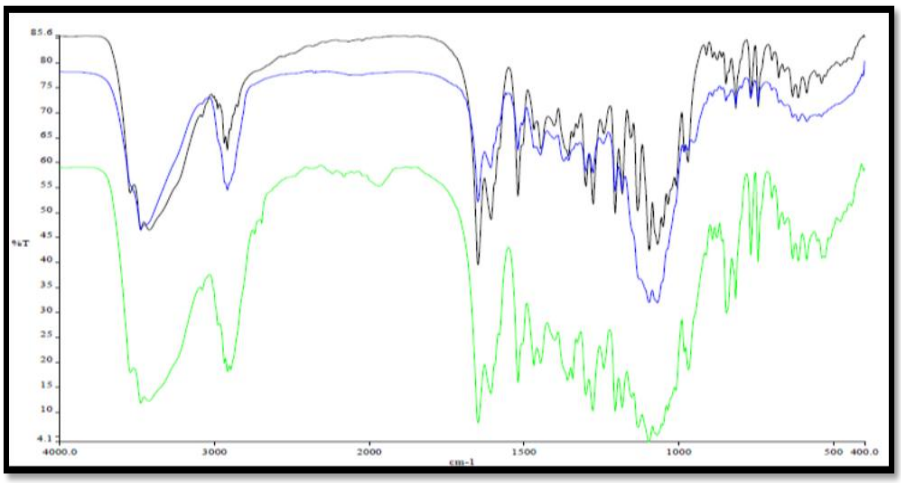


Figure 6: Overlay of FTIR graphs of hesperidin, mixture of hesperidin +HPMC E5+ Poloxamer 188 and nanocrystals

3.3.3. Redispersability and particle size

The Z- average of re-dispersed formulation was found to be 294.7nm and PDI was 0.407.

3.3.4. Saturation Solubility

The saturation solubility of the particles is important as it affects the rate of release of drug into dissolution medium hence, the therapeutic efficiency and bioavailability of the pharmaceutical product. The results observed have been reported in table no.

Table 3: Saturation solubility of hesperidin nanocrystal and pure hesperidin

Media	Solubility of Nanocrystal (µg/ml)	Solubility of Drug(µg/ml)
Distilled Water	111.95	21.09
1.2pH buffer	88.6	18.75

6.8 pH buffer	85.81	16.48
7.4 pH buffer	99.53	17.73

3.3.5. Assay

The UV calibration curve of hesperidin in 0.1N NaOH solution follows the Beer- Lambert's law in the range of 10-60 µg/mL. Percent Content of hesperidin in spray dried hesperidin nanocrystal was calculated using the above equation and was found to be 98.74±0.49%.

3.4. Dissolution rate

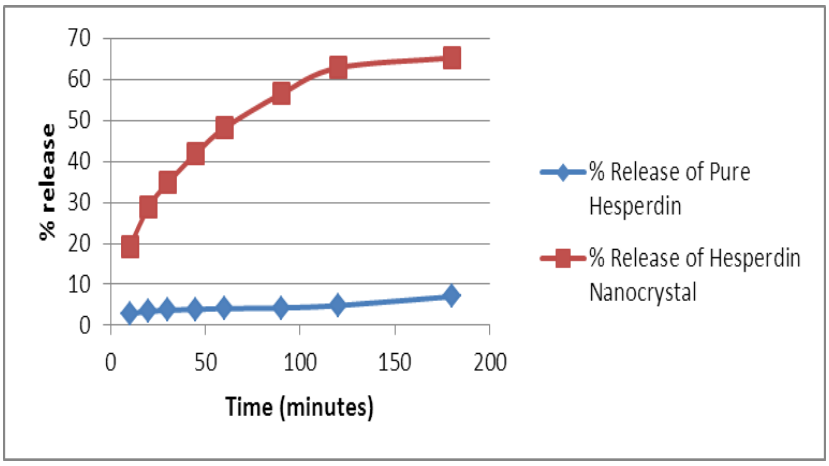


Figure 7: Comparison of dissolution profile of hesperidin and hesperidin nanocrystals in pH 1.2 buffer

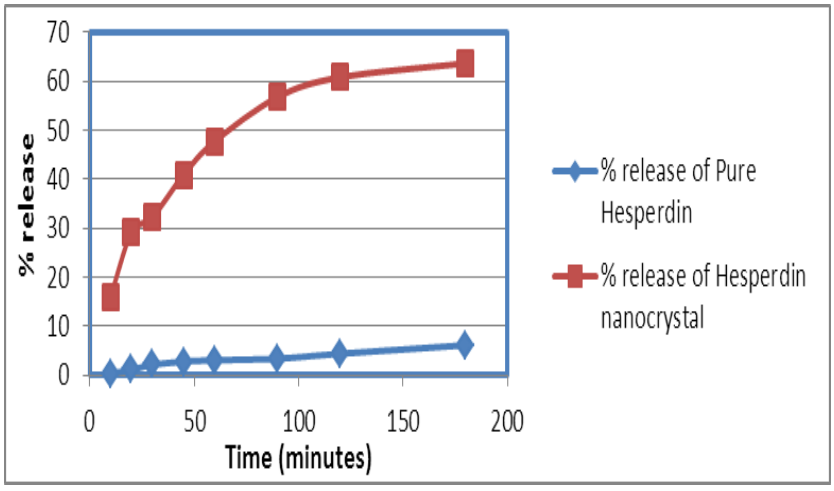


Figure 8: Comparison of dissolution profile of hesperidin and hesperidin nanocrystals in 6.8 buffer.

Dissolution velocity evaluation showed superior dissolution profile of hesperidin nanocrystals. The comparative dissolution characteristics of Nanocrystal capsules with pure drug capsules in pH 1.2 buffer and phosphate buffer pH 6.8 are shown in Figure 7 and 8.

3.5. *In vitro* antioxidant studies

I. Reducing power method

There was a dose (20-100 µg/ml) dependent increase in the, reducing power of hesperidin nanocrystal from 0.326 to 0.72 whereas that of pure hesperdin and ascorbic acid increased from 0.328 to 0.717 and 0.48 to 1.63 respectively.

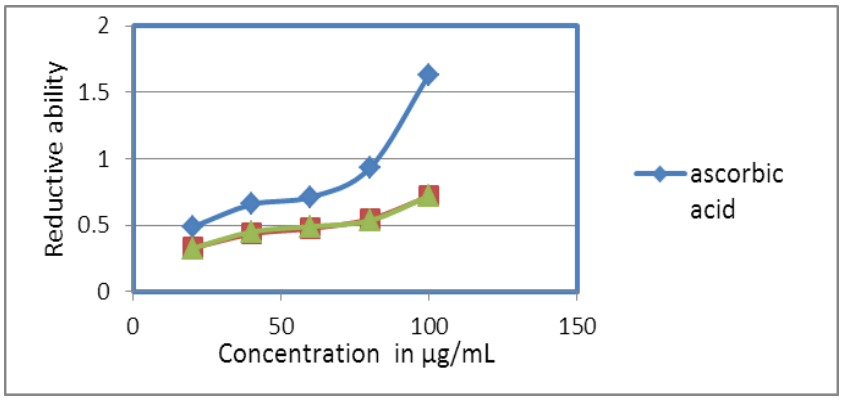


Figure 9: Comparison of reducing power of hesperidin, hesperidin nanocrystal and ascorbic acid

II. Hydroxy radical scavenging assay

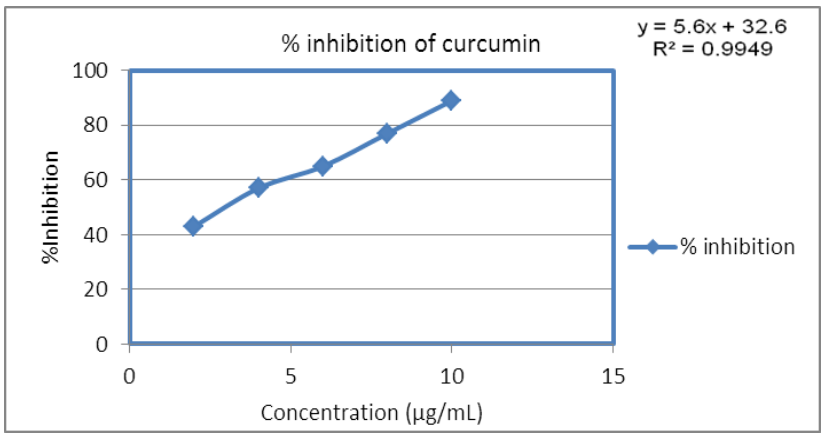


Figure 10: Graph for % inhibition curcurmin in HRAC

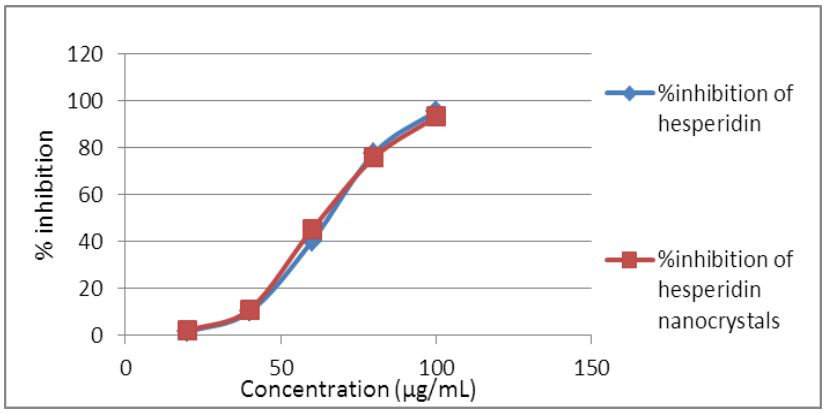


Figure 11: Comparison of % inhibition of pure hesperidin and hesperidin nanocrystal in HRAC

IC50 value for hesperidin nanocrystal and hesperidin was found to be: 62.41±1.22 µg/ml and 64.64 ±1.173 µg/ml respectively. IC_{50} of curcurmin was found to be 3.02±0.29µg/ml.

III. Thiobarbituric acid method

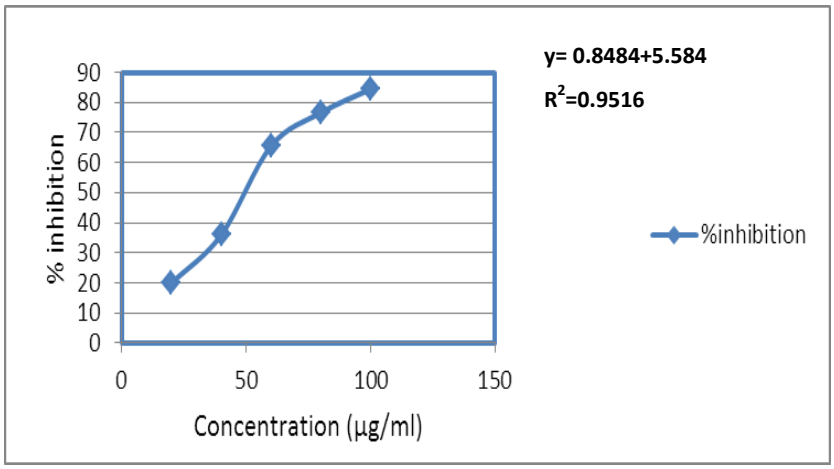


Figure 12: Graph showing % inhibiton of curcurmin in TBA

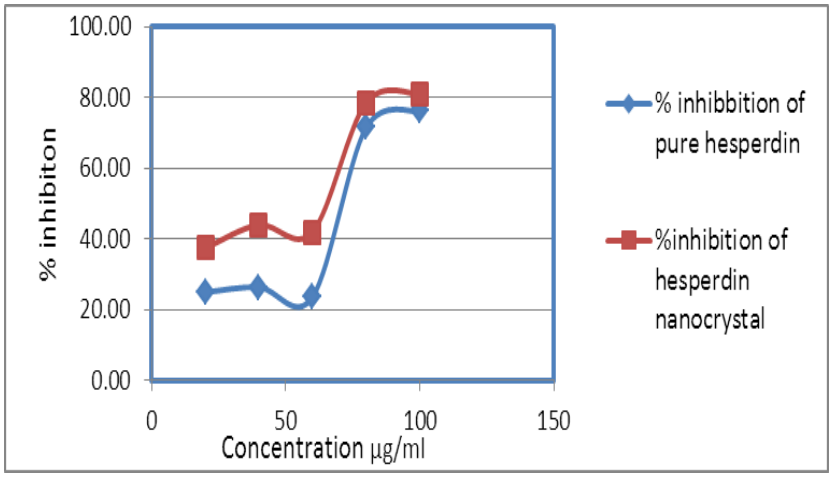


Figure 13: Comparison of % inhibition of pure hesperidin and hesperidin nanocrystal in TBA IC50 value for Hesperidin nanocrystal was found to be: 49.66±1.8 µg/ml whereas that of pure hesperidin was found to be 67.3±0.99 µg/ml.

3.6. *In-vivo* antioxidant studies

In-vivo antioxidant studies were carried out on male wistar rats to study the efficacy of scavenging action of hesperidin nanocrystal formulation oxidative stress was induced on the rat liver by carbon tetrachloride treatment.

3.6.1. Catalase Activity

Table 4: Effect of Hesperidin nanocrystal CAT levels of CCl_4 intoxicated rats.

Sr. No.	Treatment groups	H_2O_2 consumed (µM/min/gm)
1	Control	100.686 ± 0.9075
2	CCL4 Induced	32.07 ± 1.243
3	Standard	63.106 ± 0.5445
4	HN dose 1 (50mg/kg)	71.07 ± 0.8426
5	HN dose 2 (40mg/kg)	58.2 ± 0.6367

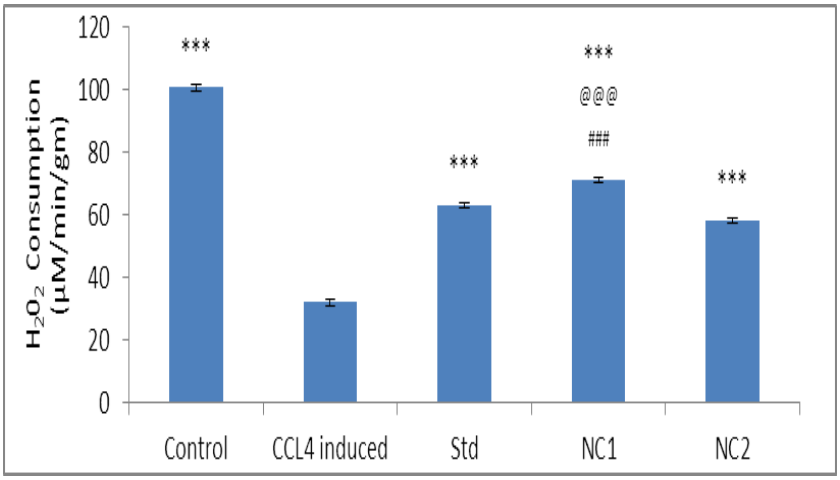


Figure 14: Effect of Hesperidin nanocrystal Catalase levels of CCl_4 intoxicated rats

Catalase Activity: Values are mean ± S.E.M.; n=6 in each group. Drug treatment was done for 8 days. Significance is denoted by ***P<0.001 as compared to the CCl_4 induced group; $^{@@@}$P<0.001 as compared to the standard treated group; $^{###}$P<0.001 as compared to the NC2 treated group; where the significance was performed by Oneway ANOVA followed by Tukey's honest significant difference post-hoc test.

3.6.2. Lipid Peroxidation

Table 5: Effect of Hesperidin nanocrystal Lipid peroxidation levels of CCl_4 intoxicated rats.

Sr. No.	Treatment groups	Lipid Peroxidation [MDA content (in nano mole/gram)]
1	Control	17.143± 0.4256
2	CCL_4 Induced	112.733 ± 1.213
3	Standard	40.55 ±0.5102
4	HN1 (50mg/kg)	33.186 ±1.016
5	HN2 (40mg/kg)	45.013±0.7019

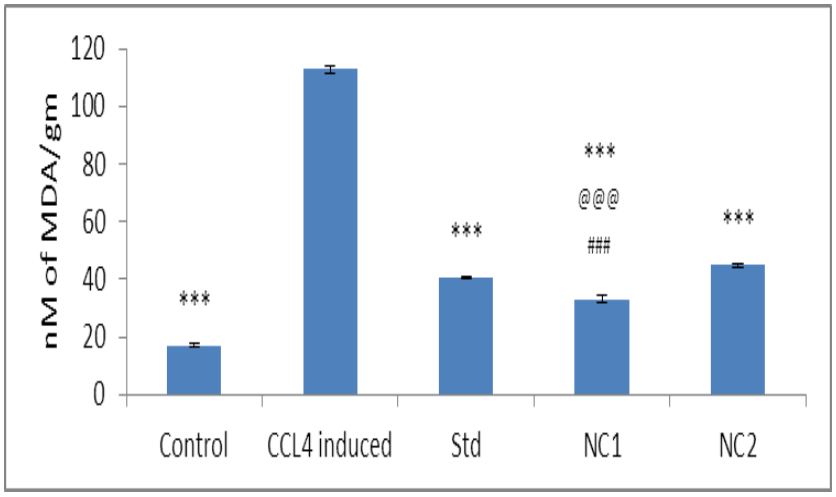
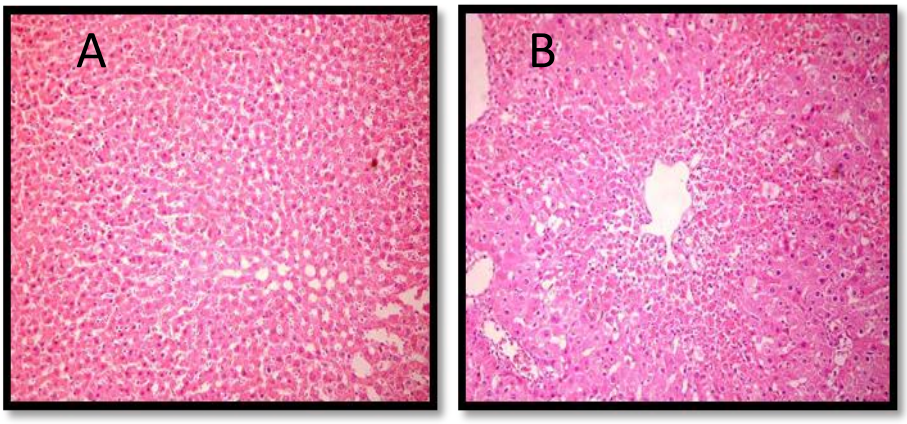


Figure 15: Effect of Hesperidin nanocrystal Lipid peroxidation levels of CCl_4 intoxicated rats

Lipid Peroxidation: Values are mean±S.E.M.; n=6 in each group. Drug treatment was done for 8 days. Significance is denoted by ***P<0.001 as compared to the CCl_4 induced group; $^{@@@}$P<0.001 as compared to the standard treated group; $^{###}$P<0.001 as compared to the NC2 treated group; where the significance was performed by Oneway ANOVA followed by Tukey's honest significant difference post-hoc test.

3.6.2.3. Histopathological studies

Carbon tetrachloride (CCl_4) is a highly toxic chemical agent, the most famous drug used to induce liver damage experimentally. Histopathological sectioning of the liver tissues indicated that, CCl_4 induced fibrosis, cirrhosis and hepatocarcinoma.[23,24] The toxic effect of CCl_4 is attributed to trichloromethyl radical produced during oxidative stress.[22] The number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer cells are significantly increased after liver injury induced by hepatotoxins such as CCl_4 .[21]



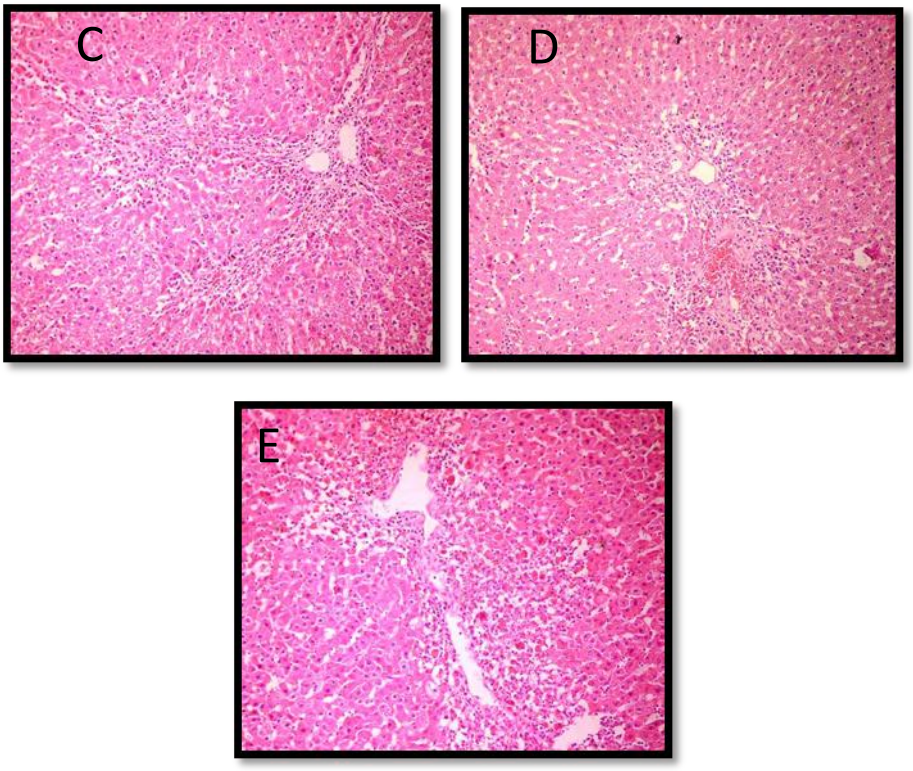


Figure 16: Histopathology Slides (A) Normal Control (B) Negative control (C) Standard (D) HN1 (50mg/kg) (E) HN2 (40mg/kg)

Table 6: Observations of histopathological studies of liver

Group	Observations
Normal control	Mild degree diffuse granular degeneration
Negative control	Sections showed centrilobular necrosis of moderate to marked severity with hepatocytic depopulation and mild degree mononuclear cell infiltration, mildly multifocal individual cell macrovesicular degeneration was seen.
Standard	The centrilobular areas showed mild severity with hepatocytic depopulation and mild degree mononuclear cell infiltration, minimally multifocal individual cell macrovesicular degeneration was seen.
HN1 (50mg/kg)	Sections showed centrilobular necrosis of minimal degree hepatocytic depopulation and mild degree mononuclear cell infiltration
HN2 (40 mg/kg)	Sections showed centrilobular necrosis of moderate severity with hepatocytic depopulation and mild degree mononuclear cell infiltration, minimally multifocal individual cell macrovesicular degeneration was seen.

The ascending order of hepatoprotective effect was as

Negative control< HN 2< Standard < HN 1 < Normal control

4. DISCUSSIONS

The key to preparing nanosuspensions by NANOEDGE™ is to create conditions that favor very rapid particle formation and little particle size growth. Combination of HPMC E5 and Poloxamer 188 was chosen as choice of stabilizers for Hesperidin nanocrystals based on

particle size and PDI results. Combinations of stabilizers are added to nanosuspension formulations to reduce the free energy of the system by decreasing interfacial tension and to prevent nanoparticle aggregation by electrostatic stabilization.[18] Mechanical stirring has been used to reduce the particle size of the newly formed particles and suppressing the agglomeration of fine particles. High pressure homogenization is used to further reduce the particle size by shear forces and cavitation forces.[20] The second energy-addition step avoids further crystal growth and aggregation after precipitation and converts the amorphous and semi unstable crystalline form arising from precipitation process to the crystalline form.[31]

DSC thermogram showed that the presence of different stabilizers in the hesperidin nanocrystal formulation had no influence on the crystalline state. According to these thermograms, the melting points of the nanocrystals did not change significantly. The melting points occurred in the range of 245^{o}C. The presence of stabilizer could explain the slight shift in those melting points. Water was also evaporated from dried nanocrystals and from hydrated form of hesperidin during heating process, as clearly shown by the peaks at around 124^{o}C.[30] However, all peaks in the thermogram indicate the same crystaline state of the hesperidin nanocrystals and the raw material. This showed that the dried hesperidin nanocrystals were not converted to an amorphous state and remained in crystalline form. XRD results confirmed that Nanocrystals which were formed were in crystalline state. The crystalline state of hesperidin was not influenced by the drying process. Both the drug and hesperidin nanocrystals lie in the same fingerprint region confirming hesperidin existed in crystal form.From FTIR graph it can be concluded that drug peaks observed in final formulation (hesperidin nanocrytsal) matches with peaks of pure drug. Thus confirming neither the drug degrades nor excipients interact with the drug during the formulation of nanocrystal.

Dried powders of nanocrystals should re-disperse into nanometer-sized particles when placing the dosage form in water and in alternate water-based environments (e.g. gastric fluid or simulated gastric fluid, etc.).[30] On visual observation spray dried Hesperidin nanocrystal completely re-dispersed in water. Particle size of hesperidin nanocrystals in nanosuspension was 377.8 nm and spray dried product was 294.7 nm. Thus the spray drying process could be responsible for further reduction in particle size of nanocrystals.[27]

From the above results it has been observed that the solubility of hesperidin was found to increase considerably in its nanocrystal form when compared to the pure hesperidin. There was almost 5 times increase in hesperidin solubility in distilled water. This increase in the solubility has been related to reduction of particle size resulting in increased saturation solubility.[25]

The formulation of hesperidin nanocrystal into capsule was successful. The rate and extent of drug dissolution from capsules incorporating hesperidin nanocrystal was significantly higher i.e. 65.211% and 63.631% as compared to pure hesperidin capsules i.e. 7.033% and 6.15% in pH 1.2 buffer and pH 6.8 buffer respectively at the end of 180 minutes. The increase in dissolution velocity may be attributed to its smaller particle size and increased surface area. The simultaneous increase in saturation solubility and decrease in diffusion distance also leads to increase in dissolution velocity in addition to the surface effect.[30] This formulation resulted in a significant increase in solubility and dissolution velocity as compared to that of pure hesperdin. This was due to increase in surface area due to reduction in particle size to nano-meter range.

In vitro antioxidant studies showed that hesperidin nanocrystal shows comparable antioxidant activity to pure hesperidin. There was no decrease in antioxidant potential on formulation of hesperidin to nanocrystals. In vivo antioxidant studies showed that the level of Catalase enzyme increased and Lipid peroxidation decreased in the normal rats. The inhibition of the generation of free radical is important in the protection against CCl_4-induced liver lesion. Treatment with hesperidin nanocrystals (50mg/Kg and 40mg/Kg) and pure hesperidin (50mg/Kg) significantly reversed these changes. Hesperidin nanocrystals (50 mg/Kg) showed significant antioxidant activity as compared to other treatment groups indicating enhanced biochemical defenses to scavenge the overproduced free radicals. Histopathological studies showed liver of CCl_4-intoxicated rats treated with pure Hesperidin and hesperidin nanocrystals at a dose of 50mg/kg exhibited clear hepatic recovery characterized by a complete regeneration of hepatocytes.

5. CONCLUSION

The current work adopted development and in vitro evaluation of dried hesperidin nanocrystals. Drying could be successfully done via spray drying technique. The nanocrystals could be tailored for the drug with 98.74% drug loading, and nanometric range. Spray dried hesperidin nanosuspension showed good redispersibility upon reconstitution retaining the

nano metric size range of hesperidin nanosuspension. Dissolution studies contended the ability of dried hesperidin nanocrystals to enhance dissolution rate of the drug compared to its crude form. Furthermore, dried hesperidin nanocrystals could significantly improve and retain the antioxidant potential of hesperidin.

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