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RESEARCH ARTICLE

Quercetin Nanocrystal Formulation: *In Vitro* Anti-tumor Activity against Dalton Lymphoma Cells

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ABSTRACT

In this present work quercetin nanocrystal (QNC) has been formulated and investigated its anti-tumor activity against Dalton's lymphoma cells (DLA) in an *in vitro* model. Since quercetin has poor water solubility, it has been formulated into nanocrystal in order to improve the solubility as well as dissolution rate of the drug. Quercetin nanocrystal (QNC) was formulated using anti-solvent precipitation method followed by lyophilization process. The QNC was subjected to particle size, zeta-potential, FTIR, solubility study, X-ray diffraction study, in-vitro dissolution study and stability study. Further QNC was subjected to *in vitro* anti-oxidant study using DPPH method and *in vitro* anti-tumor study using Dalton's lymphoma cells. The results showed that Particle size of the QNC was found to be within the range of ~600-700nm. The zeta potential values of QNC were obtained as (2.59mV). FTIR study showed that there is no interaction materials used in the preparation of QNC. QNC could increase the saturation solubility as well as dissolution rate. X-ray diffraction pattern confirmed that QNC were found in the crystalline state. Stability study showed that at low temperature the stability of QNC could be maintained for six months. QNC showed significant antioxidant activity using *in vitro* DPPH method. QNC exhibited dose-dependent anti-tumor activity with DLA cells. Therefore, this study concluded that formulated QNC exhibited potent antioxidant activity as well as anti-tumor activity.

Keywords: Quercetin; Nanocrystal; DPPH; DLA cells

INTRODUCTION

Cancer is a primary cause of death worldwide. Lung, stomach, colon and breast cancer cause the most cancer deaths each year. Tobacco use, alcohol use, unhealthy diet and chronic infections from hepatitis B and hepatitis C virus are leading risk factors for cancer. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030. Treatment of cancer, which usually includes chemotherapy, radiation, and surgery, has numerous adverse side effects and may in itself lead to death. Radiation treatments of cancer have led to increased risks of other types of cancer, sterility, etc. Surgery may cause long-term changes in health status that may also lead to death. Therefore, development of anti-cancer drugs with better efficacy is gaining momentum [1,2,3]. Numbers of drug candidates are identified in drug discovery programs, but most of them are fairly poorly soluble. This challenges in pharma research to develop novel approaches to achieve a high solubility, stability and bioavailability of the drugs. Among these novel formulations, lipid-based nanocarriers are important carriers to develop novel drug formulations [4].Today, many drugs are exhibit such a low solubility that micronization does not lead to improve the solubility and dissolution property of the drug. But, micronization to nanonization that means producing drug nanocrystal has gained much interest to improve the solubility of the drug. Nanocrystals may be able to reduce the dose to be administered and provide a sustained drug release and increase patient compliance. A particle size reduction down to the nanometres rage can increase the drug solubility. Quercetin, a major representative of the flavonol subclass, has received considerable attention. Quercetin has been reported for anti-oxidant activity as well as prevention of cancer, atherosclerosis, and chronic inflammation. But the clinical application of quercetin is limited due to its poor solubility and dissolution rate [5, 6]. This inspired us to develop quercetin nanocrystal (QNC) formulation, in order to improve the solubility and dissolution rate of the drug and investigated its anti-tumor activity against Dalton's lymphoma cells (DLA) in an *in vitro* model.

MATERIALS AND METHODS:

Materials

Quercetin, DMSO, DPPH (22,diphenyl-1-picryl hydrazyl), 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide), Potassium Bromide Ascorbic acid, polyethylene glycol, sodium hydroxide, and potassium dihydrogen phosphate were obtained from Sigma-Aldrich, USA.

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Formulation of Quercetin Nanocrystals (QNC) by anti-solvent precipitation method

Qercetin nanocrystals were prepared by anti-solvent precipitation method augmented by sonication. In this method, 1mg/mL quercetin was dissolved in 1 mL of DMSO solution (Solvent) and it was injected in to 10mL of pH - 4 water (Anti-solvent) at room temperature. The nanocrystals were obtained by suddenly adding an anti-solvent into drug dissolved solvent under intense sonication (by an FS20 sonication bath from Fisher Scintific Co). The final product was filtered. Drug crystals in the nanosuspension were obtained by quick evaporation of the solvent and anti-solvent, followed by vacuum drying and then nanocrystals were freeze-dried [7].

Characterization of Quercetin Nanocrystal Formulation

Particle size distribution and Zeta potential analysis of QNC using photon correlation spectroscopy

The average hydrodynamic diameter and poly-dispersity index (PDI) of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using Zetasizer Nano ZS90 (Malvern Instruments limited, UK), 1ml of QNC sample dispersion was placed in disposable cuvettes for particle size measurements. Each experiment was conducted in triplicate. The electrophoretic mobility (zeta potential) measurements were made using the Malvern Zetasizer (Nano ZS90, Malvern Instruments) at 25ºC.

Scanning electron microscope (SEM) analysis of QNC

The surface morphology of the prepared QNC was determined for by using scanning electron microscopy (JEOL model JSM-6390LV). A drop of QNC was placed on a carbon film coated copper grid for SEM. Studies were performed at 80kv using JEOL model JSM-6390LV, Japan equipped with selected area electron diffraction pattern (SAED) .The copper grip was fixed in to sample holder and placed in a vacuum chamber of the scanning electron microscope and observed under low vacuum and SEM images were recorded.

FTIR studies of QNC

The Fourier transform infrared spectroscopy (FT-IR) spectrums were studied to detect any sign of interaction between quercetin and excipients used in the preparation of nanocrystal. FTIR analysis of pure quercetin and quercetin nanocrystals were performed and spectrum was obtained using FT-IR (Shimadzu 8300 Japan). All these samples were grounded and mixed thoroughly with potassium bromide, at 1:5(sample: potassium bromide) weight ratio. The spectrum obtained was in between the wave number of (4500-500 cm-1).

Solubility studies of QNC

Solubility is the property of solid, liquid or gaseous chemical substances called solute to dissolve in a solid, liquid, or gaseous solvent to form a homogeneous solution of the solute in the solvent. The solubility of substances basically depends on the physical and chemical properties of solute and solvent as well as on temperature, pressure and pH of the solution. The extent of the solubility of a substance in a specific solvent is measured as the saturation concentration, where adding more solute does not increase the concentration of the solution and begin to precipitate the excess amount of solute solubility studies were performed with a shaker (orbital shaker incubator GiNei, Bangalore). The saturation solubility was determined by excess quercetin was added in 20ml DI water and stored with shaking at 37±0.5^{o}C. After 24 hour suspensions were filtered and analyzed spectrometrically at 370nm using (Shimadzu UV-1700 spectrophotometer). Experiments were carried out in triplicate, and solubility data were averaged [8].

X-ray powder diffraction studies of QNC

X-ray powder diffractometry is one of the most powerful and established technique for material structural analysis, capable of providing information about the structure of a material (crystal) at the atomic level. In which the crystalline atoms cause a beam of incident X-rays to diffract in many specific directions. By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a three dimensional picture of the density of electrons within the crystal. From this electron density, the mean positions of the atoms in the crystal can be determined as well as their chemical bonds. Since many materials can form crystals such as salts, metals, minerals, semiconductors, as well as various in organic, organic and biological molecule's X-ray crystallography has been fundamental in the development of many scientific fields. X ray diffraction is used to study the atomic and molecular structure of crystalline substances such as drugs and excipients. X-ray diffraction patter (diffractogram) can be used to confirm the crystalline nature of the sample. Therefore, this information is used to verify whether the substances are crystalline or amorphous. PXRD (diffractograms) of the lyophilized powder of quercetin nanocrystals were recorded using (Bruker AXS D8 Advance diffractometer) [9].

Stability studies of QNC: The first stability can relate to the materials themselves are they stable or chemically break down. It could relate to their orientation, the materials may not chemically degrade, but physically alter their arrangement over time or under different

conditions (temperature, light, humidity, pH, etc.). If the nanoparticles are serving as drug carriers it could relate to the stability of the drug within or attached or associated with carrier, or its ability to remain with the carrier under different conditions. The dispersed quercetin nanocrystals were stored at 4 °C and room temperature. Particle size and zeta potential, and poly-dispersity index of the samples were measured at 0, 1, 2, 3 and 6 months [10].

***In vitro* dissolution studies of QNC**

The *in vitro* dissolution of the quercetin nanocrystals samples as well as the pure Quercetin was determined using the paddle method (Electrolab tablet Dissolution tester USP apparatus II), in 900 mL of DI water. The paddle rotation was set at 100 rpm. The temperature was maintained at 37 ± 0.5°C. The original Quercetin and Quercetin nanocrystals containing an equivalent 10 mg of Quercetin were tested for their dissolution. The dissolved solution samples of 3 ml were collected at 15, 30, 45, 60, 90,120 and 180 min of dissolution time. The dissolution test for each sample was performed in triplicate and the dissolution data was averaged. The concentration of drug was determined spectrometrically at 370 nm [11].

***In vitro* Antioxidant Activity of QNC**

The antioxidant activity was measured by the DPPH assay.2 ml of DPPH (0.3 mM in methanol) was incubated (in darkness) with 0.5 ml of original Quercetin , Formulated nanocrystals and ascorbic acid at concentrations of 250, 500 and 1000 µg/ml at 37±0.5°C. After the incubation period of 30 min the absorbance was measured at 517nm using (Shimadzu UV-1700 spectrophotometer) [(20).] The percentage inhibition of the Experimental samples was evaluated comparing the absorbance values of control and test samples. Free radical scavenging activity of all the formulations were determined my DPPH assay method and compound with ascorbic acid used as the standard [12].

Percentage of scavenging activity was calculated by using the following formula:

% of inhibition = [A(control) – A(sample)/A (control)] x100

A (control) - Absorbance of DPPH alone

A (sample) - Absorbance of DPPH with different concentrations of samples and ascorbic acid

In vitro* cyto-toxicity study of QNC against Dalton Lymphoma Cell line: *MTT assay

The MTT 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) dye reduction assay was conducted to diagnose the cyto-toxic activity of quercetin and formulated Quercetin nanocrystals. The assay depends on the reduction of MTT by mitochondrial dehyrogenase, an enzyme present in the mitochondria of viable cells, to a blue formazan product. Briefly 1 mL of 1 × 106 DLA cells was seeded in RPMI1640 medium supplemented with 10% fetal bovine serum, streptomycin (100 µg/mL) and penicillin (100 units/mL) and incubated in a 5% of carbon dioxide incubator with 0.1% DMSO(vehicle) with various doses of (250, 500 and 1000µg/ml) QNC for 48 h. Pure quercetin was used as standard. After incubation, MTT solution (1.2 mg/mL) was added to each well, and the cells were incubated for an additional 4 h. The MTT formazan product was dissolved in DMSO, and the optical density was measured at 570 nm using an ELISA plate reader (Biorad, Model 680, Japan) [12].

Statistical analysis

Student t-tests were performed for comparing the statistical difference among *In vitro* methods. The p-values that were 0.05 or less, the difference was considered significant.

RESULT AND DISCUSSION

Quercetin nanocrystals (QNC)

Quercetin nanocrystals were prepared by anti-solvent precipitation method. In this method, high super-saturation is needed and can be achieved by choosing a proper combination of good solvent and an anti-solvent. Therefore, this study indicated that anti-solvent precipitation method is cost effective and could be suitable for large scale production.

Characterization of QNC

Particle Size distribution and Zeta Potential analysis of QNC

The particle size distribution of formulated QNC Showed range from ~ 600 to 700 (d.nm) and PdI was found to be 0.390. The zeta potential values of formulated Quercetin nanocrystals were obtained as 2.59mV (Figure 1).Therefor this study indicated that the formulated quercetin was in nanoscale range and sufficiently stable. The zeta potential is caused by the net electrical charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. The determination of the zeta potential of a nanocrystal is essential as it gives an idea about the physical stability of the nanocrystal formulation.

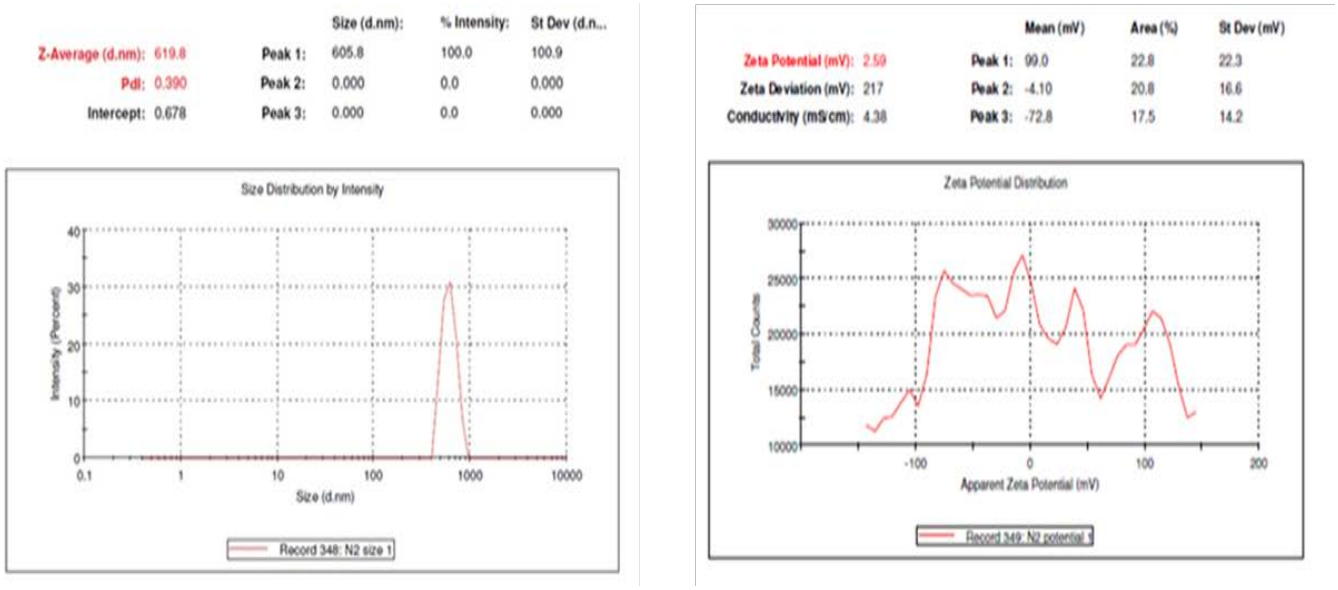

Figure 1: Particle size distribution & zeta potential analysis of QNC

SEM analysis of Quercetin Nanocrystal

In this study the particle size of commercial Quercetin and formulated quercetin nanocrystals were characterized by SEM analysis. The result showed that quercetin have relatively large particle about (~34µm). The particle size of formulated of quercetin nanocrystals was found to be ~430nm (Figure 2).

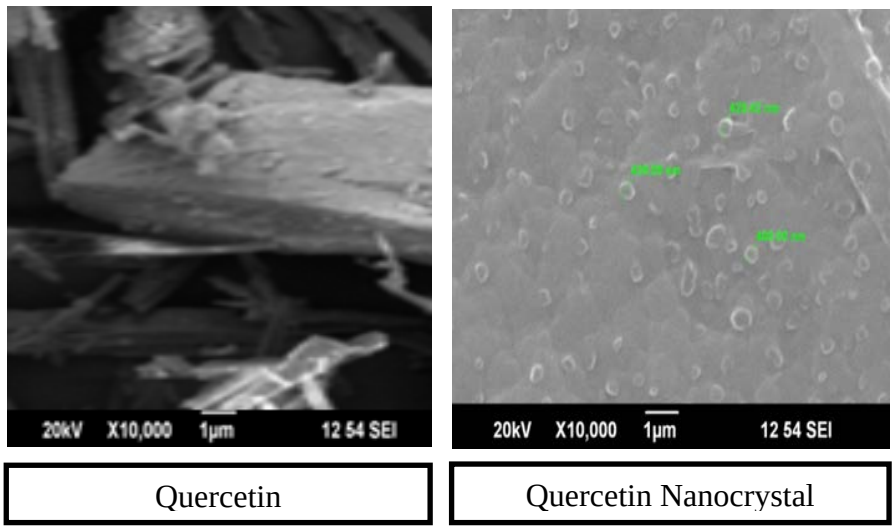

Figure 2: SEM analysis of Quercetin & Quercetin Nanocrystal

FTIR study of QNC

The FTIR of pure Quercetin and the prepared Quercetin nanocrystals were analyzed for the comparison. The FT-IR showed (Figure 3) one of the unique characteristic peaks of pure Quercetin was observed at 3283.94 (OH-streching vibration). In formulated QNC indicated that stretching vibration peak at 3456, showed presence of OH group. FTIR peak between 3416-3290 showed presence of OH stretching. The stretching peak between the range of 1663-1609 denoted that presence of (CCO,Ar-OH) stretching. The pure Quercetin and formulated QNC showed stretching peak at 1661.75and the formulated nanocrystals showed peak at 1641.49 respectively due to presence of (CCO, Ar-OH). Streching vibrations peaks between 1517-1312 represent that presence of (C=C). The pure Quercetin showed peaks at 1319.37,1357.94,1461.14,1513.22, as well as formulated QNC showed peaks at 1319.37, 1407.13 due to presence of (C=C). The Fourier transform infrared spectroscopy (FT-IR) spectrum was studied and found that there is no interaction in materials used during the preparation of QNC.

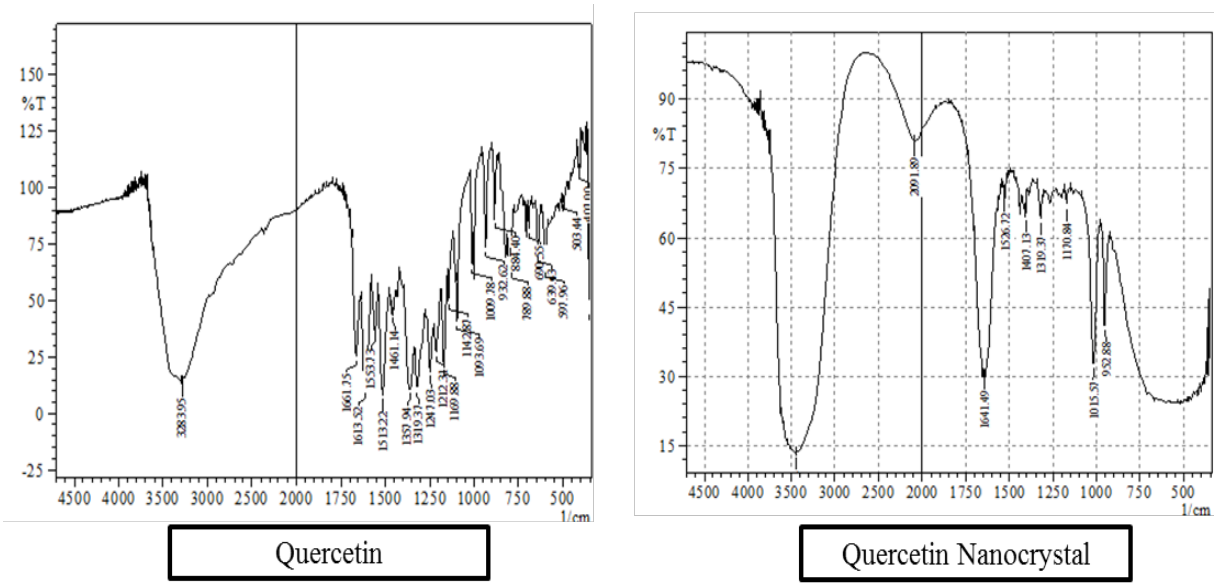

Figure 3: FTIR study of Quercetin and QNC

Solubility studies of QNC

In this study, solubility of quercetin was found to be 2.5±0.03 µg/ml and the solubility of formulated quercetin nanocrystals was found to be 13.50±1.32 µg/ml (Figure 4)**.** This study indicated that formulation of QNC by anti-solvent precipitation method enhanced the saturation solubility of quercetin approximately 5 times (13. 50±1.32µg/ml) than pure quercetin (2.5±0.03 µg/ml).

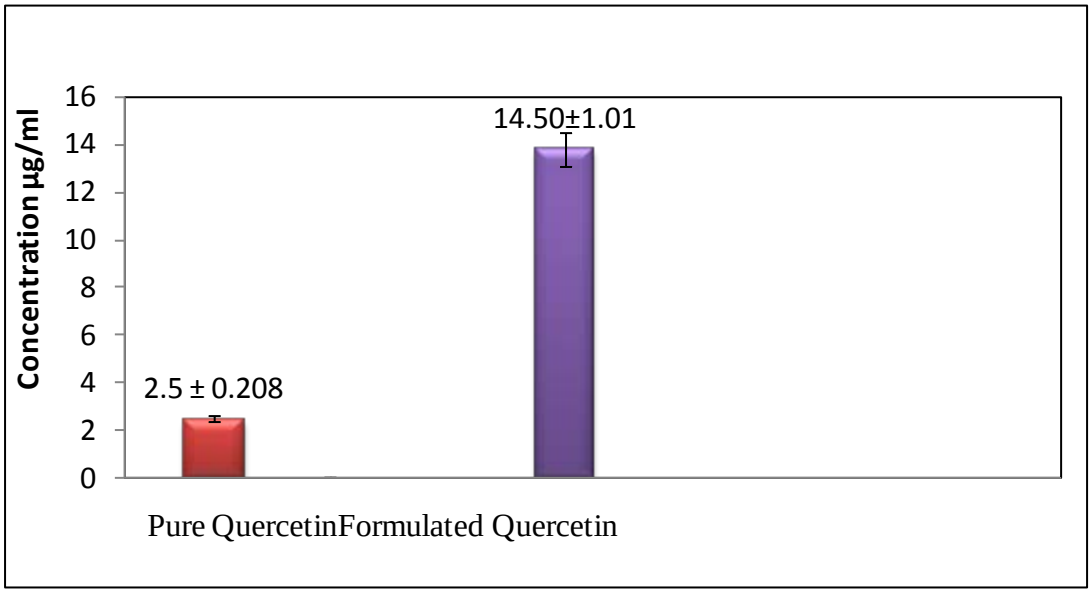

Figure 4: Solubility study of Quercetin Nanocrystal

X-ray powder diffraction study of QNC

The X-ray diffraction pattern of the original Quercetin displayed the presences of numerous distinct peaks at 2Ɵ of 5.54^0, 10.220, 11.820, 14.180, 17.240,22, 160and 27.48^0, which suggested that high crystalline form of the drug (Figure 5). The lyophilized Quercetin nanocrystals also showed the similar peaks but with slightly different peak intensities. Nanocrystallinity is known to cause such effect in the X-ray powder diffractograms. Hence, X-ray diffraction pattern confirmed that prepared quercetin nanocrystals were found in the crystalline state.

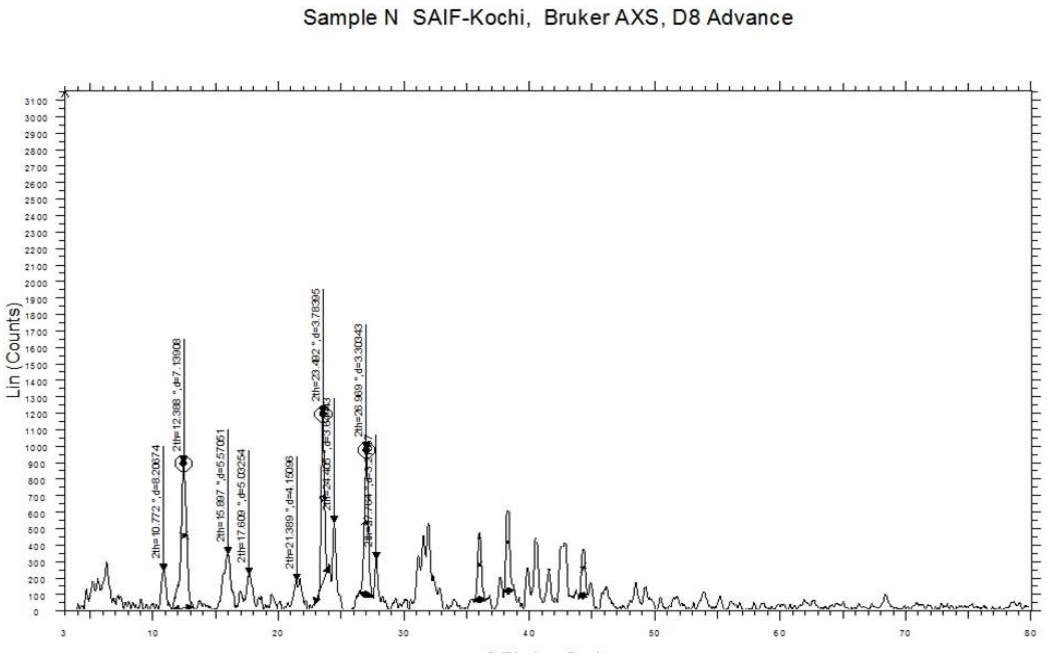

Figure 5:X-ray powder diffraction spectrum of QNC

Stability studies of QNC

Dispersed Quercetin nanocrystals were stored at 4 ◦C and room temperature respectively. Particle size, polydispersity index (PdI), zeta potential, and quecetin nanocrystals were monitored over six months (Table 1). Stored at the lower temperature, the particle size showed no significant change while the PdI was increased over the period of six months. Conversely, when stored at the room temperature, both size and PdI changed significantly. Therefore, storage temperature clearly affected the stability of the nanocrystals; at low temperature, the stability of nanocrystals can at least be maintained for six months. .

Table 1: Stability study of Quercetin Nanocrystals

Duration (Month)	Particle size(nm)		PdI		Zeta potential(mV)[b]	
	4^{o} C	RT	4^{o} C	RT	4^{o} C	RT
0	400±32.6	401±32.6	0.139±0.04	0.140±0.04	2.89±1.02	2.90±0.99
1	419±33.4	422±33.8	0.390±0.05	0.401±0.05	3.65±0.98	3.67±1.00
2	429±32.8	436±38.2	0.553±0.03	0.568±0.07	3.73±1.03	4.01±1.01
3	430±35.8	464±39.0	0.562±0.06	0.617±0.09	5.06±1.16	7.12±1.02
6	432±37.3	723±180	1.000±0.09	3.167±1.02	9.01±1.36	11.00±1.23

In vitro dissolution study of QNC

The dissolution profiles of pure quercetin and formulated quercetin nanocrystals were studied. The results showed that QNC exhibited dissolution profiles about 77.7% within 180 minutes as compared to pure Quercetin (dissolution profile 16.2%) within the same time (Figure 6). In this study the dissolution of pure quercetin showed a very poor dissolution rate. The greatest increase in the dissolution rate is exhibited by QNC. Therefore, the Quercetin nanocrystals have more dissolution compared to the pure Quercetin.

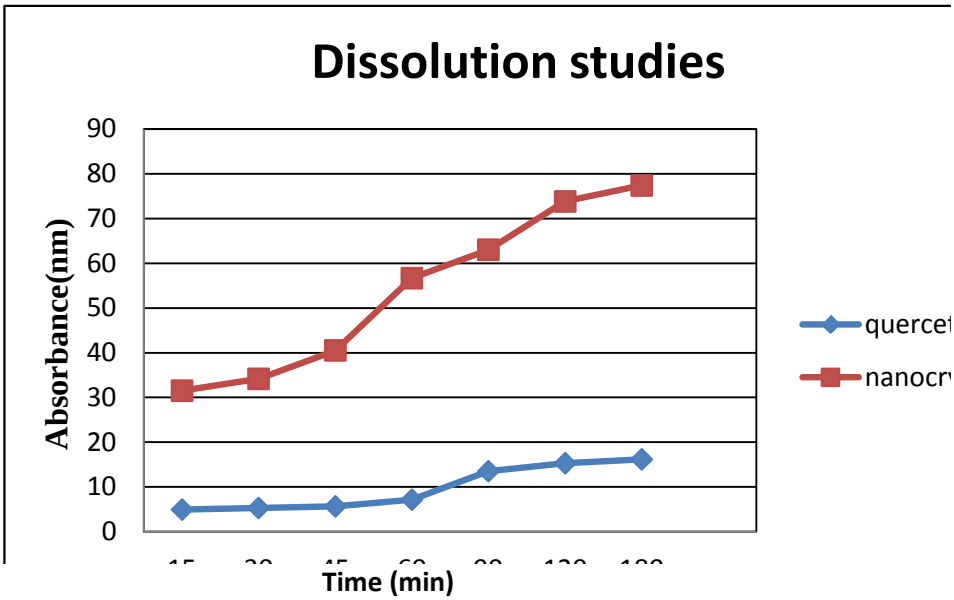

Figure 6: *In vitro* dissolution study of QNC

***In vitro* Antioxidant Study of QNC**

The observed scavenging effect of Quercetin on the DPPH radicals decreased in the following order: the standard ascorbic acid > prepared Quercetin nanocrystals > original Quercetin, which was at 90.41%, 73.32 and 70.36%the concentration of 250 mg/mL, respectively (Figure 7). Surendra et al reported that DPPH is a free radical and stable at room temperature, which produces a deep violet solution in organic solvents. It is reduced in the presence of Quercetin molecules, giving rise to uncoloured solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidant properties of Quercetin. Quercetin shows an anti-oxidative effect which is mainly due to its phenolic hydroxyl groups. These phenolic hydroxyl groups are able to donate the hydrogen to reduce the free radicals to prevent oxidation of lipids, proteins, and DNA. Antioxidant activity of Quercetin Nanocrystals was calculated by using the formula:

% of inhibition= [(Control OD -Test OD)/Control OD] ×100 (1)

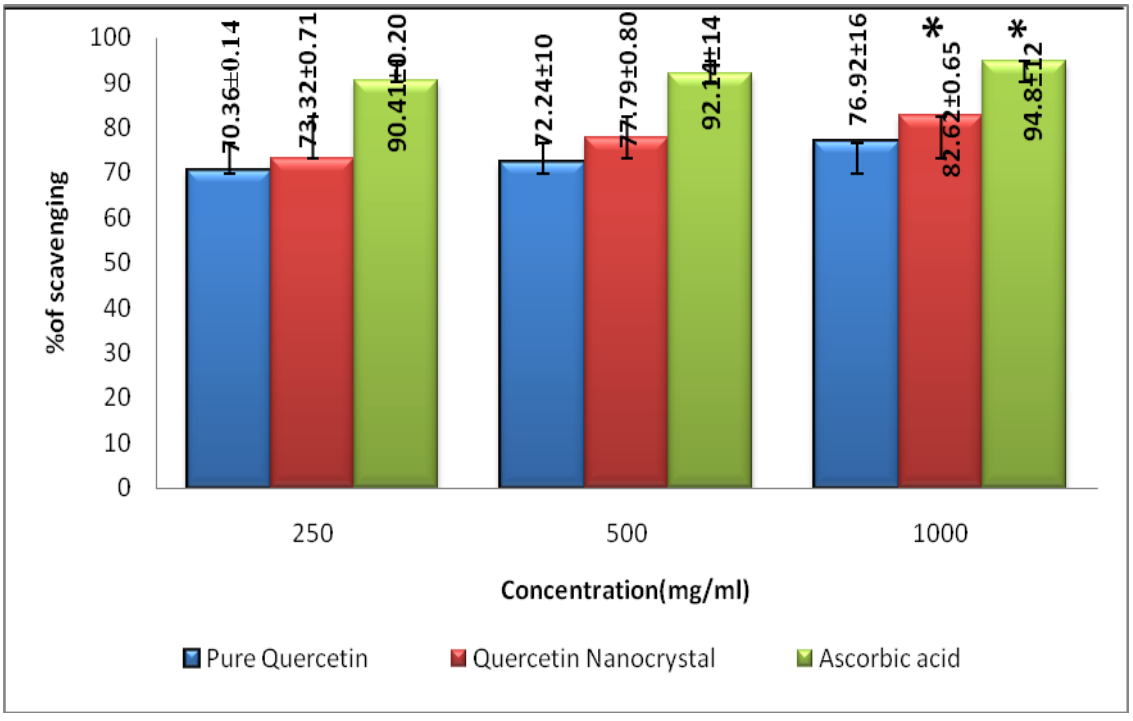

Figure 7: *In vitro* Antioxidant Study of QNC

In vitro cytotoxicity study of QNC against Dalton Lymphoma Cell line

The maximal % of inhibition after 48 h exposure was obtained with 1000 µg/ml of formulated QNC with a percentage of inhibition nearly (91.90%). Using the DLA cells and it was treated for a period of48 h was selected because the control cells were still in the exponential growth phase at that time. In this study, the results showed that QNC exhibited dose-dependent anti-tumor activity with DLA cells. The percentage of inhibition will be more in 1000X dilution for all sample (N1,N2,N3).when we are analyzing the report it is clear that percentage of inhibition was more for Quercetin nanocrystals than normal Quercetin.

Table 1*: In vitro* **cytotoxicity study of QNC against DL cells**

% of inhibition			Pure Quercetin
N1	N2	N3	
96.90±0.12 %*	90.63±0.12 %*	86.90±0.12 %*	38.64±0.12 %

Control 99.97%; N1= 1000 µg/ml; N2=500 µg/ml; N3=250 µg/ml

CONCLUSION

In the present study, the Nanocrystal technique has been specifically used to increase the solubility and dissolution rate of poor water soluble quercetin. Anti-solvent precipitation method was used to prepare QNC in order to improve the quercetin solubility and dissolution property. Sonication process proved to be important for reducing particle size and producing nanosized uniform crystal. Particle size distribution of formulated QNC showed range from 600 to 700 (d.nm) and zeta potential values were obtained as (2.59mV) using anti-solvent precipitation method. Therefore, this study indicated that anti -solvent precipitation method is cost effective and simple preparation method. SEM analysis of formulated quercetin nanocrystals showed particle size around 432nm. FTIR study showed that there is no interaction materials used in the preparation of QNC. Solubility study indicated that QNC enhanced the solubility of the quercetin produced by the precipitation method. X-ray diffraction pattern confirmed that prepared quercetin nanocrystals were found in the crystalline state. Stability study showed that at low temperature the stability of nanocrystals can be maintained for six months. In vitro dissolution study showed that greatest increase in the dissolution rate by QNC. In addition, QNC exhibited potential antioxidant using DPPH method and anti-tumor activity against Dalton Lymphoma cells at dose dependent manner. This study concluded that QNC showing potential antioxidant as well as anti-tumor activity.

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