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Quercetin Nanosuspension: *In-vitro* Anti-tumor Activity against Dalton Lymphoma Cells

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ABSTRACT

In this present work quercetin nanosuspension (QNS) has been formulated and investigated its anti-tumor activity against Dalton's lymphoma cells (DLA) in an *in vitro* model. Since quercetin is insoluble in water, it has been formulated into nanosuspension in order to improve the solubility as well as dissolution rate of the drug. Quercetin nanosuspension (QNS) was formulated using homogenization method followed by lyophilization process. The QNS was subjected to particle size, zeta-potential, FTIR, solubility study, *in-vitro* dissolution study and stability study. Further QNS was subjected to anti-oxidant study and anti-tumor study using Dalton's lymphoma cells. The results showed that Particle size of the QNS was found to be within the range of ~160-200nm. The zeta potential values of QNS were obtained as (3.69mV). Solubility of QNS was found to be 15.41±0.6683µg/ml. QNS could increase the dissolution rate as well as the saturation solubility. QNS showed significant antioxidant activity compared with that of standard ascorbic acid. QNS exhibited dose-dependent anti-tumor activity with DLA cells. This study concluded that formulated QNS exhibited potent antioxidant activity as well as anti-tumor activity.

Keywords: Nanosuspension; MTT assay; DLA cells.

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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]. Few techniques such as precipitation methods, milling methods and homogenization methods are developed to produce drug nanosuspensions. Among these methods, high pressure homogenization is the simplest method and has been successfully employed in large-scale production. Above all, a new thought of nanosuspensions (NS) are proposed. The advantages of nanosuspension are (a) improved drug dispersibility and drug solubilization, increased therapeutic efficacy and reduced toxicity. In addition, NS could be suitable for large scale production[5, 6]. 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[7, 8]. This inspired us to develop quercetin nanosuspensions (QNS), in order to improve the solubility and dissolution rate of drug and investigated its anti-tumor activity against Dalton's lymphoma cells (DLA) in an *in vitro* model.

MATERIALS AND METHODS

Quercetin, 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.

Formulation of Quercetin Nanosuspension (QNS) by Homogenization Method

QuercetinNanosuspension (QNS) was prepared by homogenization method. Briefly quercetin powder was dispersed in an aqueous surfactant solution containing 0.2% w/v polyethylene glycol

and 0.1%w/v ascorbic acid. The suspension was pre-milled and mixed by magnetic stirrer for 15 minutes. Then it undergoes homogenization for 30 minutes. Then QNS was subjected to lyophilization process.[9]

Characterization of Quercetin Nanosuspension (QNS)

Particle size distribution

The average hydrodynamic diameter and polydispersity index (PDI) of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using Zetasizer Nano ZS90 (Malvern Instruments limited, UK), 1ml of quercetin nanosuspension 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.[10]

Scanning electron microscope(SEM) analysis of QNS

The surface morphology of the prepared QNSwas determined for by using scanning electron microscopy (JEOL model JSM-6390LV). A drop of QNS 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 grid 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 study of QNS

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 nanosuspension. FTIR analysis of pure quercetin and quercetin Nanosuspension 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 QNS

Saturation solubility is defined as the maximum quantity of a compound (solute) that can be dissolved in a certain quantity of a specific solvent at a specified temperature .However it is well known that the saturation solubility also depends upon the modification of polymorphic compounds and the particle size if the particles is less than 1µm are present. Solubility studies were performed using a shaker (orbital shaker incubator, GeNei, Bangalore). Excess of pure quercetin was added in 20 ml DI water and stored at 37 ± 0.5 ^{0}C. After 24 hours of shaking, suspensionswere filtered and analyzed using UV spectrophotometer at 370 nm (UV- 1700

Pharmaspec, Shimadzu). Experiments were carried out in triplicate, and solubility data wereaveraged.[11]

Stability studies of QNS

Stability is an important factor in case of nanosuspension. In case of suspensions the possibility of sedimentation and cake formations are more. The particles will settle down and it would not redisperse uniformly after shaking. So the dose will be changed, bioavailability will be less which leads to the decreased pharmacological output. When we are formulating the drug in to nanometer range the particle size will be less than 1µm, so it would not sediment rapidly it will suspend in the solvent more time and redispersion will be fast. The stability was measured at different temperature and find out at which temperature it would be more stable. The prepared formulation was keptat room temperature and 4°C for six months at specific time period (0,1,2,4,6 months). A portion of the sample were taken and subjected to particle size, zeta potential and polydispersity index analysis for the determination of stability.[12]

***In vitro* dissolution studies of QNS**

The *in vitro*dissolution rate of the QNS as well as the purequercetin 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 purequercetin and Quercetin Nanosuspension 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. [13]

% of drug release = [Concentration x DF x 900/strength of formulation] x 100

Construction of Calibration curve for pure quercetin

Pure quercetin stock solution was prepared by dissolving 1g of pure quercetin in 100ml of methanol. 1 ml of stock solution was taken and made up the volume to 10ml with methanol. From solution, required concentration of 2,4,6,8 and 10mg/ml were prepared using methanol.

***In vitro*Antioxidant Activity of QNS**

Antioxidant activity can be evaluated by scavenging of the stable DPPH radical. The model is extensively used as it is less time consuming than the other methods. DPPH can accept an electron and hydrogen radical and can be converted in to a stable diamagnetic molecule. DPPH contain an odd electron, and so it has a strong absorption at 517nm.when this electron becomes paired off, the absorption decreases with respect to the electro take up. Such change in the absorbance produced

in this reaction has been widely applied to assess the capacity of numerous molecules to act as free radical scavengers. Free radical scavenging activity of all the prepared quercetin nanosuspension formulation was determined by DPPH assay method.

Antioxidant activity of QNS: 2.5ml of 0.3mM DPPH solution was added to 0.5ml of different concentrations of the QNS. Another series of solution were prepared by dissolving 2ml of DPPH with prepared concentrations of standard ascorbic acid. The above solution was allowed to react at room temperature for 30 min. The absorbance values are measured at 517nm. The absorbance of DPPH solution also taken as control. Ascorbic acid is used as reference standard.[14]

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 QNS against Dalton Lymphoma Cell lineby MTT assay**

The 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) dye reduction assay was conducted to diagnose the cytotoxic activity of Quercetin and formulated Quercetin nanosuspension at different concentrations. 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) andincubated in a carbon dioxide incubator with 0.1% DMSO(vehicle). Cells in the exponential phase was harvested and counted using a haemocytometerwith the aid of trypan blue solution. The cell suspensions were dispensed in triplicate into 96-well culture plates at optimized concentrations. After 48 h incubation of Dalton's lymphoma (DL) cells in various concentrations(2.5mg/ml, 5mg/ml and 10mg/ml) of QNS, cells were incubated with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide) reagent for 48 h in the incubator. Dimethylsulfoxide (DMSO) was added to dissolve the precipitate formed inside the living cells. The colouredformazanproduct was assayed spectrophotometrically at 570 nm using ELISA plate reader which corresponds to the cell viability.[15]

Statistical analysis

Student t-tests wereperformed for comparing the statistical difference among *In vitro* methods.

The p-values that were 0.05 or less, the difference wasconsidered significant.

RESULT AND DISCUSSION

Formulation of Quercetin Nanosuspension (QNS)

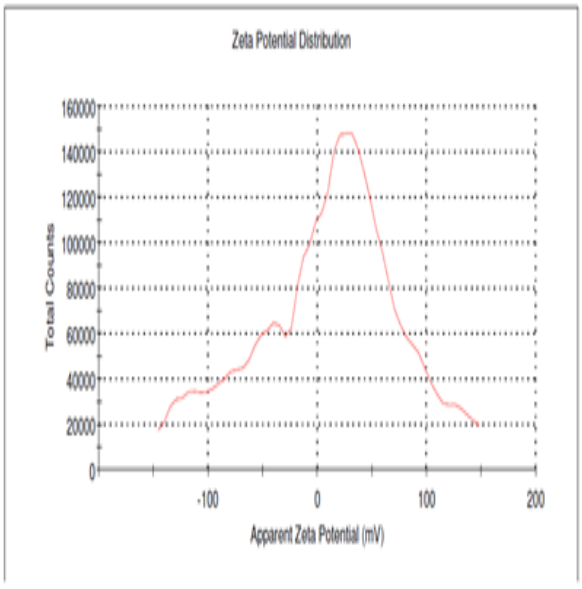
Quercetin Nanosuspension was prepared by homogenization method. In this method, Homogenization involves the forcing of the suspension underpressure through a valve having a narrow aperture.The homogenizer instrument was operatedat pressures of 1500 bars to produce the QNS.Therefore, this study indicated that homogenization method is cost effective and could be suitable for large scale production.

Characterization of QNS

The particle size distribution of formulated QNS showed range from ~ 200 to 300 (d.nm)and PdI was found to be 0.070. The zeta potential values of formulated Quercetin nanosuspension were obtained as 3.69mV (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 nanosuspension is essential as it gives an idea about the physical stability of the nanosuspension. The zeta potential of a nanosuspension is governed by both the stabilizer and the drug itself. In order to obtain a nanosuspension exhibiting good stability, for a nanosuspension a minimum zeta potential of ±30mV is required.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -3.69	Peak 1:	34.2	68.8	36.5
Zeta Deviation (mV): 132	Peak 2:	-63.2	20.3	23.4
Conductivity (mS/cm): 0.487	Peak 3:	-124	6.6	12.0

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 219.9	Peak 1:	229.0	100.0	38.93
PdI: 0.070	Peak 2:	0.000	0.0	0.000
Intercept: 0.675	Peak 3:	0.000	0.0	0.000



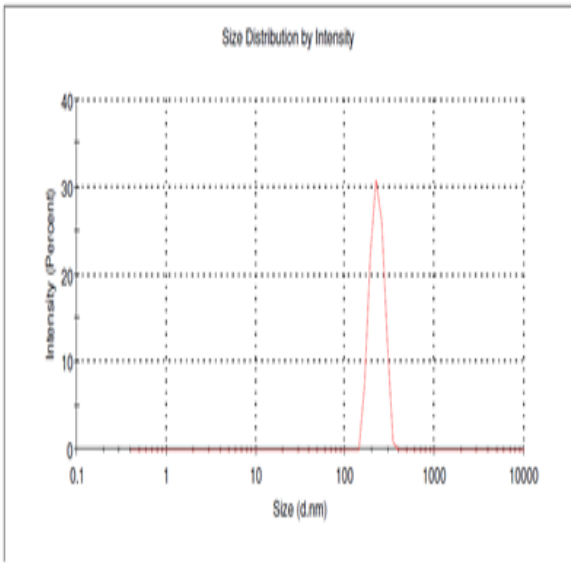


Figure 1: Particle size distribution &zeta potential analysis of QNS

SEM Analysis of QNS

Particle size of the QNS formulation was found to be within the range of ~160-200nm (Figure 2). Therefore the formulation is in nanoscale.The particle size of the formulated QNS was in nanometer range.Nanogrinding is a critical process which used to obtained appropriate particle size reduction and stability of nanosupension. The mean particle size and width of the particle size distribution are important characterization parameters as they govern the saturation solubility, dissolution velocity and Physical stability.

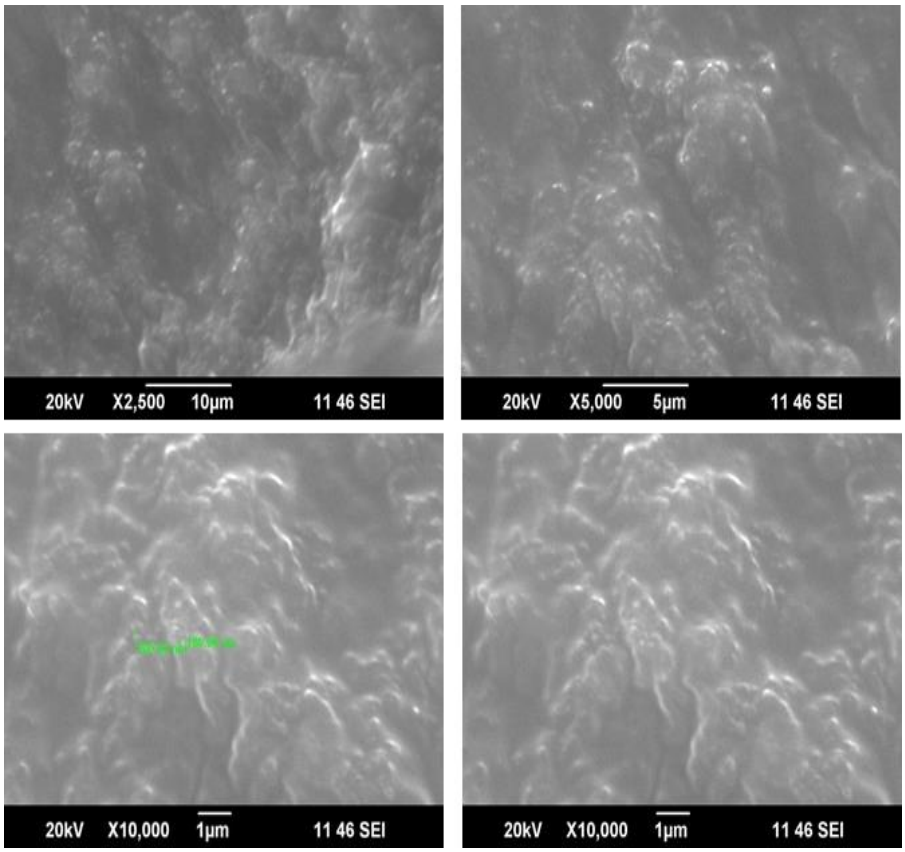


Figure 2: SEM Analysis of QNS

FTIR study of QNS

The FTIR of pure Quercetin and the prepared QNS were analyzedfor the comparison.FTIR peak between 3416-3290 showed presence of OH stretching. The FT-IR shows one of the unique characteristic peaks of pure Quercetin was observed at 3283.94 (OH-streching vibration).In formulated QNS indicated that stretching vibration peak at 3456, showed presence of OH-group.FTIR of pure quercetin a peak at 1661.95 and formulated QNS peak at 1643.43 showed presence of C-O-stretching. (1663-1609). FTIR of pure quercetin a peak at 1461.14 and formulated QNS peak at 1465.96 showed presence of C=C stretching vibration (1517-1312). FTIR study indicated that no interaction of materials used during the preparation of QNS (Figure 3).

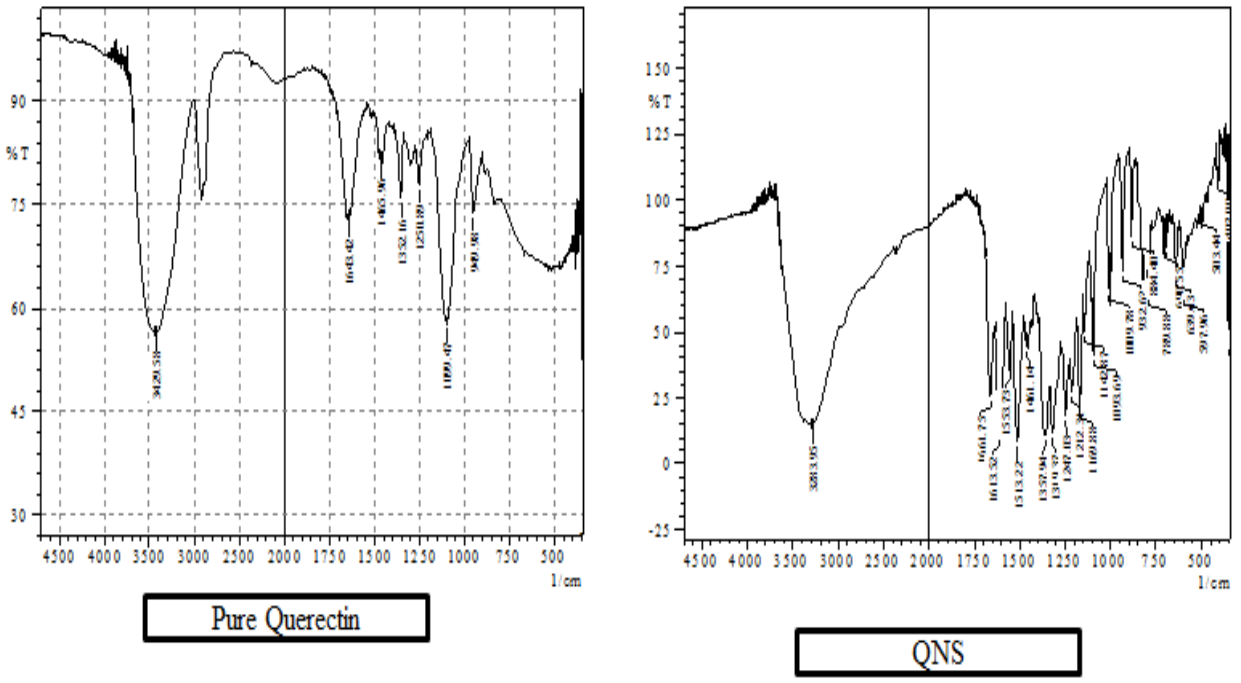


Figure 3: FTIR spectrum of QNS

Solubility studies of QNS

Solubility of pure quercetin after 24 hr shaking was found to be 2.5±0.2081µg/ml. Solubility of quercetinnanosuspension after 24 hr shaking was found to be 15.41±0.6683µg/ml (Figure 4). The study indicated that nanosuspension increase the saturationsolubility as well as dissolution velocity.From the solubility of original pure quercetin was extremely low being only 2.5µg/ml. There was the significant enhancement in the solubility of the nanosuspension produced by homogenization method. This is because the saturation solubility increases withdecreasing particle size below 1000 nm.

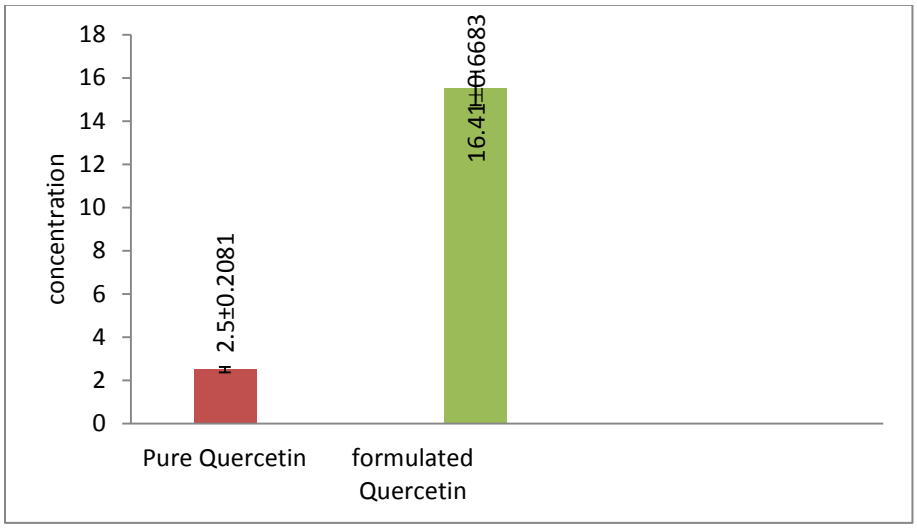


Figure 4:Solubility study of QNS

Stability studies of QNS

Nanosuspensions were stored at 4°C and at room temperature respectively. Particle size, polydispersity index (PdI) and zeta potential were monitored over six months (Table 1). Stored at the lower temperature, the particle size showedno 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 an increased PdI can be attributed to the widening of particle size distribution. The particle size distribution indicated uniformity initially but due to the storage the particles aggregation might have occurred and so particle size was increased and uniformity was reduced. The stability would be only obtained when it stored at refrigerated temperature.

Table 1: Stability study of QNS

Duration (month)	Particle size		PdI		Zetapotential	
	4^0 c	RT	4^0c	RT	4^oc	RT
0	229	230	0.070	0.088	-3.69	-3.69
1	260	290	0.168	0.176	-2.77	-3.53
2	427	520	0.172	1	-1.56	-3.65
4	563	680	0.188	1	-3.19	-2.47
6	856.7	954	1	1	-1.44	-2.22

***In vitro* dissolution study of QNS**

The percentage drug release for nanosuspension is 36% at 15 min then gradually increases and reached 85.5% at 180 min. In case of normal pure quercetin it is 4.95% at 15 min and 16.2% at 180 min(Figure 5).

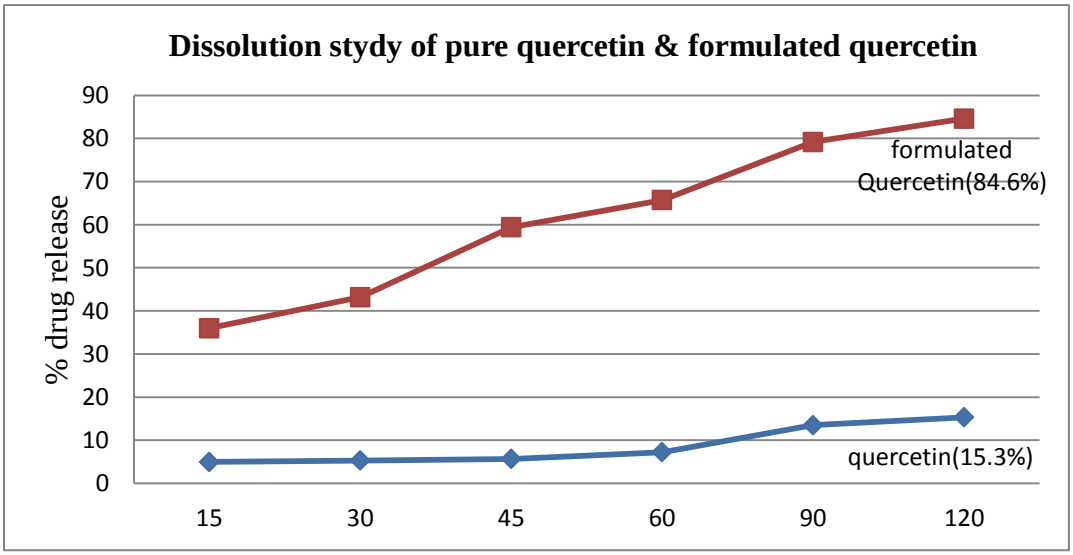


Figure 5: *In vitro* Dissolution Study of QNS

Dissolution study showed that release of drug will be more in quercetin nanosupension compare to that of normal pure quercetin. This results suggested that dissolution rate of QNS has been

increased. This study indicated that nanosuspension has an important advantage over other techniques, that it can increase the dissolution velocity as well as the saturation solubility.

***In vitro* Antioxidant Study of QNS**

The free radical–scavenging properties of the original pure quercetin and pure quercetin nanosuspension are shown in table as well as graph. The observed scavenging effect of pure quercetin on the DPPH radicals decreased in the following order: lyophilized nanosuspension > original pure quercetin. From the graph (Figure 6) it is clear that QNS showed significant antioxidant activity compared with that of standard ascorbic acid.

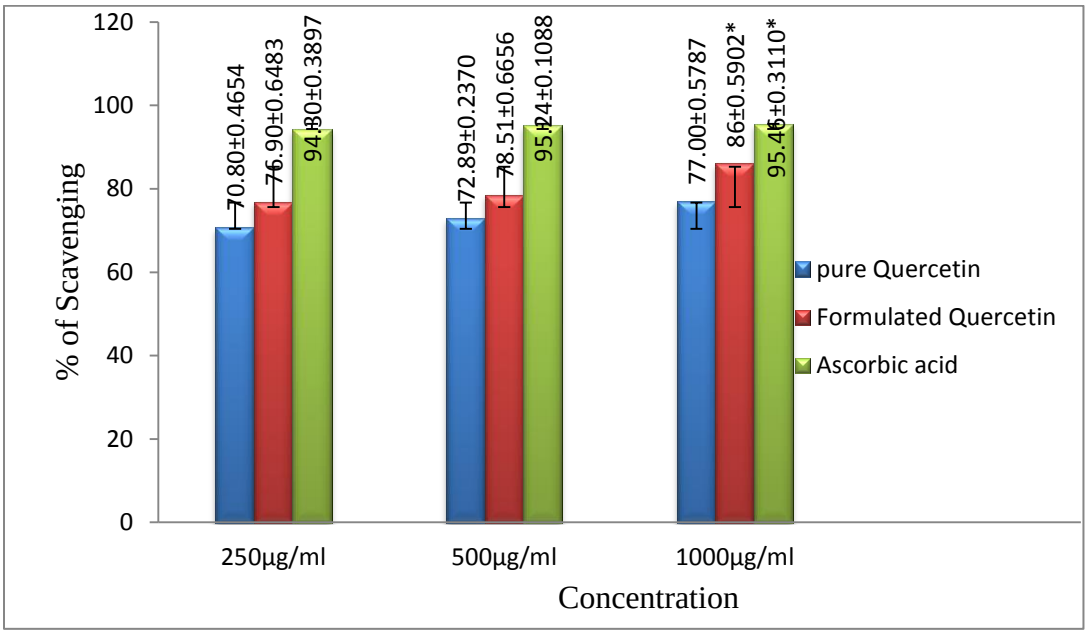


Figure 6: *In vitro* antioxidant activity of QNS by DPPH method

***In vitro* cytotoxicity study of QNS against Dalton Lymphoma Cell line by MTT Assay**

The maximal %of inhibition after 48 h exposure was obtained with 10 mg/mlof formulated QNS with a percentage of inhibition of nearly (96.90%). Using the DLAcells 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 QNS exhibited dose-dependent anti-tumor activity with DLA cells. The percentage of inhibition will be more in 10 mg/ml (10X) dilution for all sample (K1,K2, K3).when we are analyzing the report it is clear that percentage of inhibition was more for QNS than pure quercetin (Table 2).

Table 2: Percentage inhibition of QNS against DLA cells

K_1	K_2	K_3	Pure quercetin
85.67±0.12 %*	88.59±0.48 %*	96.90±0.56 %*	38.64±0.41 %

K_1 - 2.5mg/ml; K_2 - 5mg/ml; K_3 -10mg/ml. * - < 0.05 (p-value)

CONCLUSION

Nanosuspensions are mainly seen asvehicles for administering poorly water soluble drugs. Nanosuspension formulation have been largely solved the solubility as well as dissolution problems toimprove drug absorption. It has therapeutic advantages, suchas simple method of preparation, less requirement ofexcipients, increased saturation solubility and dissolution velocity of drug.Quercetin Nanosuspension (QNS) was prepared by using homogenization method.The particle size distribution of formulated QNS showed range from ~ 200 to 300 (d.nm)and PdI was found to be 0.070. The zeta potential values of formulated Quercetin nanosuspension were obtained as (3.69mV) using homogenization method. FTIR study showed that there is no interaction materials used in the preparation of QNS.Solubility study indicated that QNS enhanced the solubility of the quercetin produced by the homogenization method. Stability study showed that at low temperature the stability of nanosuspension can be maintained for six months. *In vitro* dissolution study exhibited that significantly increase the dissolution rate by QNS.In addition, QNS exhibited significant antioxidant activity using DPPH method and anti-tumor activity against Dalton Lymphoma cells at dose dependent manner. This study concluded that QNS exhibited potent antioxidant activity as well as anti-tumor activity.

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