



## RESEARCH ARTICLE

### Pathological and Molecular Studies on Antitumor effect of Curcumin and Curcumin Solid Lipid Nanoparticles

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#### ABSTRACT

Curcumin is widely known for its anticancer property but low aqueous solubility limits its use. In this study curcumin loaded solid lipid nanoparticles were used to overcome the defects of curcumin. The present study aimed at comparing the antitumor effect of nanocurcumin and curcumin. *In vitro* studies in DAL, A72 and HT29 cell lines confirmed the cytotoxic effect and *in vivo* studies were carried out in Dalton's Ascites Lymphoma in mice. Antitumor effect was assessed by various parameters like cell block technique, AO/PI staining, TUNEL assay, immunocytochemistry, immunofluorescence and Real time PCR. AO/PI staining and TUNEL assay revealed increased number of apoptotic cells in curcumin and nanocurcumin group. Immunofluorescence staining revealed the nuclear migration of Nf-kB in tumor control whereas it showed cytoplasmic expression in treatment group. Immunocytochemistry revealed increase in expression of Bax and Caspase 8 whereas Bcl2, Cyclin D1 and PCNA showed lesser expression in nanocurcumin as compared to curcumin group. The expression pattern of miR181a, pre-miR-182, miR155 and some of the potential targets in the apoptotic pathway were analyzed by real time PCR. The qPCR analysis revealed the down regulation of miR 181a, 155, pre-miR182, Nf-kB and Bcl2 as well as up regulation of p53, Caspase 8 and caspase 9 in treatment group in which nanocurcumin group showed better effect as compared to curcumin group. The curcumin solid lipid nanoparticles delivered curcumin to cancer cells effectively and increased the therapeutic effect by applying its functions through miRNAs, induction of apoptosis as well as inhibition of metastasis. Thereby it is useful in providing target specific therapy and reduces the side effects of common methods.

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#### INTRODUCTION

Though there is significant development in the field of clinical cancer therapy, cancer ranks the leading cause of death with 19.3 million new cases and 10.0 million cancer deaths worldwide in 2020 (Sung *et al.*, 2021). Development of cancer is associated with several genetic and epigenetic abnormalities. Understanding the target gene network and regulatory pathways might help in providing new therapeutic options in case of cancers. Recently, miRNAs are found to be one of the critical regulators in the process of carcinogenesis and tumor

progression. MicroRNAs are small noncoding regulatory RNAs that act as post transcriptional regulators. Binding of miRNA to target mRNA is critical in regulating the protein expression. The pattern of miRNA expression is extensively deregulated in cancer and affects genes involved in initiation, progression and metastasis. miRNA binding to mRNA can trigger either degradation of mRNA if there is full complementarity or lead to translational repression in case of partial complementarity (Xavier *et al.*, 2020). They can act either as tumor suppressors or as 'oncomiRs' by regulating the expression of oncogenes, tumor suppressor genes or

genes that are involved in cell proliferation and apoptosis (Baranwal *et al.*, 2010). Nuclear factor-kappa B belongs to *Rel* protein family and usually present as a heterodimer in the cytoplasm. Several reports suggest that activation of Nf-kB is associated with the overexpression of anti-apoptotic, premetastatic and proangiogenic genes (Yenmis *et al.*, 2021). Lymphoma, the most common hemopoietic neoplasm in humans (80%) and animals (83%) is commonly treated with multi drug chemotherapy protocols. miRNA alterations associated with clinical outcome were reported in many human and canine neoplasms even in canine lymphoma too (Craig *et al.*, 2019). Remission to chemotherapy is often achieved in case of lymphoma but prognosis is quite variable. Curcumin, a polyphenolic phytochemical obtained from *Curcuma longa* has been known as a beneficial compound in traditional medicine. Curcumin is well known for its anticarcinogenic action which is mediated through multiple signalling pathways associated with cell proliferation, angiogenesis, apoptosis, invasion and metastasis (Mohanty and Sahoo, 2010). Curcumin was found to regulate epigenetic alterations like modulation in the expression of oncogenic or tumor suppressor miRNA (Momtazi *et al.*, 2016). The pharmaceutical role of curcumin was found limited because of its low water solubility, rapid systemic elimination, inadequate tissue absorption, degradation at alkaline pH and reduced bioavailability (Mohanty and Sahoo, 2010). Many studies have confirmed that nanocarriers can enhance the bioavailability and pharmacokinetics of loaded drugs like curcumin (Gupta *et al.*, 2020). Among the various nanocarriers solid lipid nanoparticles consisting of a lipid core in which lipophilic compounds are entrapped are known for its high drug loading capacity, increased drug stability and controlled drug release with minimum side effects (Guorgui *et al.*, 2018). Herein we encapsulated curcumin in solid lipid nanocarriers. Present study is designed to study and compare the anticancer effect of curcumin and nanocurcumin and its underlying mechanism.

## MATERIALS AND METHODS

**Encapsulation of curcumin in solid lipid nanoparticles:** Curcumin solid lipid nanoparticles (CUR-SLN) (Curcumin- Sigma-Aldrich catalogue no: 1386) were prepared by Single emulsification solvent evaporation method (Pooja *et al.*, 2016). Physicochemical characterization confirmed that curcumin was encapsulated in Solid lipid nanoparticles (Gupta *et al.*, 2020; Kumari *et al.*, 2021).

**Invitro cytotoxicity studies:** Cytotoxicity was determined by MTT assay. DAL cell lines (Amala Cancer Research Centre, Kerala), A72 and HT29 cell lines (Department of Animal Biotechnology, Madras Veterinary College, Chennai) were procured. A72, DAL and HT29 cell lines were treated with different concentrations of curcumin and CUR-SLN (20,50,70 and 100µg/ml) for 24 hours and the absorbance was measured at 570nm.

**Induction of T cell Lymphoma:** Male BALB/c mice (20-25g) was procured from M/s Biogen Laboratory Animal Facility, Bangalore, and maintained under standard

laboratory condition. All the animal experiments in this study were carried out with the approval of Institutional Animal Ethics Committee, Madras Veterinary College (Approval number 04/SA/IAEC/2021). DAL cells were transplanted intraperitoneally into mice. Four groups, tumor control, methotrexate, curcumin and nanocurcumin each include 6 animals were used for assessing the treatment effect. Curcumin and nanocurcumin @100 and 50 mg/kg bodyweight were given orally from 24 hours of inoculation. Methotrexate, was administered @ 3.5mg/kg body weight. Development of tumor was assessed by increase in bodyweight, tumor volume and tumor weight. DAL transplanted mice were sacrificed on 17<sup>th</sup> day of inoculation. Ascitic fluid was collected for pathological and molecular studies.

**Cell block technique:** Cell blocks were prepared from the ascitic fluid (Shivakumaraswamy *et al.*, 2012). Sections of 4-5µm thickness were prepared and stained with H&E stain.

### Apoptosis evaluation

**Acridine orange/propidium iodide staining:** Ascitic fluid collected were centrifuged and washed with sterile PBS phosphate buffered saline. Cell suspension along with 1:1 ratio of acridine orange and propidium iodide solution was mixed well and kept in room temperature for 5 min. A small aliquot of this solution was placed on a slide and observed under blue filter in fluorescent microscope.

**TUNEL assay:** Cell blocks sections were prepared and staining was performed as per manufactures instructions in TUNEL staining kit.

**Immunofluorescence:** Cell block sections prepared using ascitic fluid was used for immunofluorescence staining. Slides were incubated with anti Nf-kB p65 polyclonal antibody conjugated with fluorescein isothiocyanate for 1 hour at room temperature. Cells were washed in PBS and observed under Nikon fluorescent microscope.

**Immunostaining:** Primary antibodies PCNA, Bcl2, Cyclin D1 (PathnSitu Biotechnologies Pvt Ltd, USA) Bax, Nf-kB and Caspase 8 (M/s Biogenex Pvt Ltd., USA) were procured. PolyExcel HRP/DAB detection system-one step-kit was used as universal secondary antibody. Cell block sections were deparaffinized and immunostaining with different antibodies were performed according to manufactures instructions and counterstained with Hematoxylin.

Immunostaining results were evaluated semi quantitatively by H-score method. Immunopositive cells were counted in five scattered fields and the staining intensity (0,1+,2+, 3+) for each cell was determined in a fixed field and final score was assigned using the formula given by Yenmis *et al.* (2021).

$$H \text{ score} = [1 \times (1+\% \text{ of cells}) + 2 \times (2+\% \text{ cells}) + 3 \times (3+\% \text{ of cells})]$$

**Quantitative real time PCR analysis:** The expression levels of miR 181a, pre-miR 182 and miR 155 and target genes like Nf-kB, p53, Caspase 8 and 9 and Bcl2 were assessed using qPCR. Total RNA was isolated from ascitic

fluid cell suspension by Trizol method and cDNA was synthesized. All the reactions were performed in triplicate and the fold change was measured using  $2^{-\Delta\Delta ct}$  equation.

**Statistical analysis:** To test the statistical significance One-way ANOVA was applied.

## RESULTS

**Antiproliferative effect:** The cytotoxic activity of free curcumin and curcumin solid lipid nanoparticles on different cancer cell lines like A72, DAL and HT29 were evaluated by MTT assay. Curcumin and curcumin nanoparticles inhibited the cell proliferation in a concentration dependent manner. The  $IC_{50}$  value was used to evaluate the cytotoxic effect of CUR-SLN. The  $IC_{50}$  value for curcumin and nanocurcumin in A72 cell line was 54.46 and 52.91  $\mu\text{g/mL}$ , in DAL cell line was 29.89 and 26.01  $\mu\text{g/mL}$  and in HT29 cell line was 63.147 and 58.73  $\mu\text{g/mL}$ , respectively (Fig. 1). CUR-SLN has low  $IC_{50}$  value as compared to curcumin indicating better cytotoxic potential.

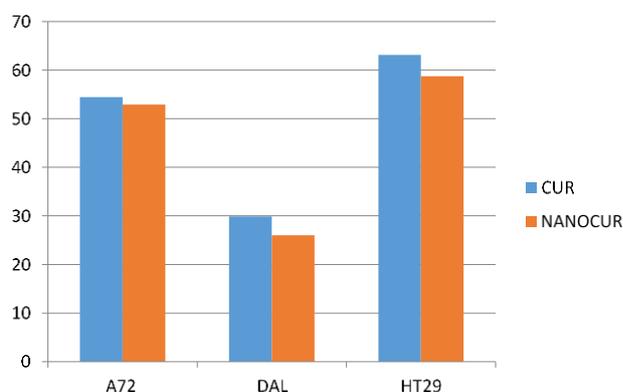
**Induction of apoptosis:** In treatment groups, cells showed typical features of apoptosis like cell shrinkage, membrane blebbing, cytoplasmic vacuolation, chromatin condensation and nuclear fragmentation. Percentage of apoptotic cells were more in nanocurcumin group (Fig. 2A, B, C and D).

In Acridine orange/ Propidium iodide double staining assay viable cells with intact membrane appear green, early apoptotic cells appear green with membrane blebbing, late apoptotic/necrotic cells give red fluorescence. In tumor control, the percentage of live cells with green fluorescence were more. However, in treatment group increased percentage of apoptotic and necrotic cells have been observed (Fig. 2E, F, G, H and 3).

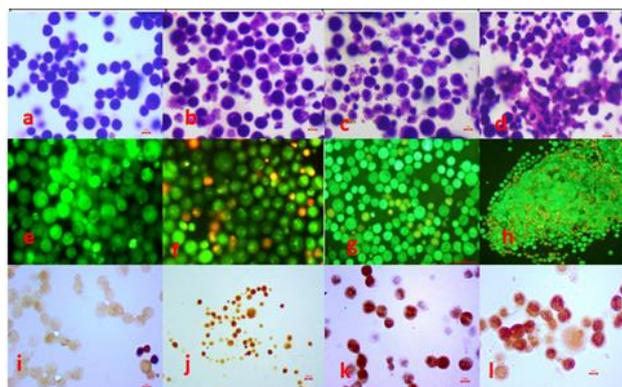
In this study, only few TUNEL positive cells were observed in tumor control group. In the treatment group 60 to 70 % of the cells showed TUNEL positive staining (Fig. 2I, J, K and L).

**Inhibition of Nf-kB translocation:** Immunofluorescence indicated that curcumin and nanocurcumin effectively inhibited the translocation of Nf-kB p65 from cytoplasm to the nucleus whereas in untreated proliferating cell it was located in the nucleus (Fig. 3A and B).

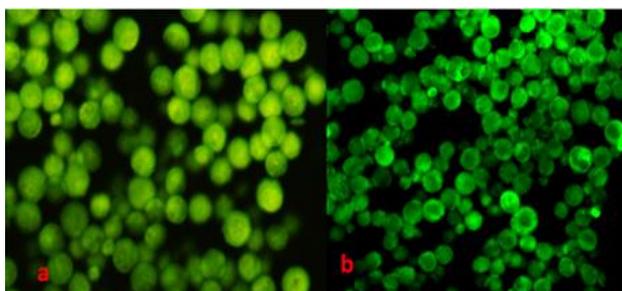
**Immunocytochemistry:** The immunoreactivities of Bax, Bcl2, Caspase 8, Cyclin D and PCNA were compared between tumor control methotrexate, curcumin and nanocurcumin group. The analysis revealed that Bax and Caspase 8 positivity was significantly increased in treatment groups in which nanocurcumin group showed increased expression as compared to curcumin group. The antiapoptotic marker Bcl2 expression found decreased in treatment groups. Our results also indicated that the expression of Cyclin D and PCNA was also decreasing in treatment groups as compared to tumor control and nanocurcumin group showed significant decrease in expression of Cyclin D and PCNA as compared to curcumin group (Fig. 4).



**Fig. 1:**  $IC_{50}$  value of curcumin and nanocurcumin A72, DAL and HT29 cell line.



**Fig. 2:** A Cellblock-Tumorcontrolgroup-LymphoblastcellsH&E5 $\mu\text{m}$  b. Cellblock-Methotrexategroup-ApoptoticcellsH&E5 $\mu\text{m}$  c. Cellblock-Curcumin group-Nuclearcondensationandmembraneblebbing inapoptoticcellsH&E5 $\mu\text{m}$  d. Cell block-Nanocurcumin group-nuclear condensation e. AO/PI staining-Tumor control group-viable cells f. AO/PI staining-methotrexate group-late apoptotic/necrotic cells g. AO/PI staining-Curcumin group-earlyapoptoticcellswithmembranedisruptions h. AO/PI staining-Nanocurcumin group-Earlyapoptoticcellswithmembranedisruptions i. TUNEL assay-Tumorcontrolgroup-fewpositivecells5 $\mu\text{m}$  j. TUNEL assay-methotrexate group More number of positive cells 10 $\mu\text{m}$  k. TUNEL assay-curcumin group-More number of positive cells 5 $\mu\text{m}$  l. TUNEL assay-Nanocurcumin group-more number of positivecells5 $\mu\text{m}$ .



**Fig. 3:** A Immunofluorescence-NuclearlocalizationofNf-kBintumour controlgroupb. Cytoplasmic localization in treatment group.

**Real time PCR:** To further analyze the anticancer effects, we evaluated the expression of miR 181a, pre-miR-182 and miR 155. Our analysis indicated downregulation of miR 181a, pre-miR 182 and miR 155 in treatment groups as compared to the tumor control group. The miR181a was markedly lower in all the three groups and degree of decrease is higher in DAL+ nanocurcumin compare to control group. The expression of miR 155 was significantly

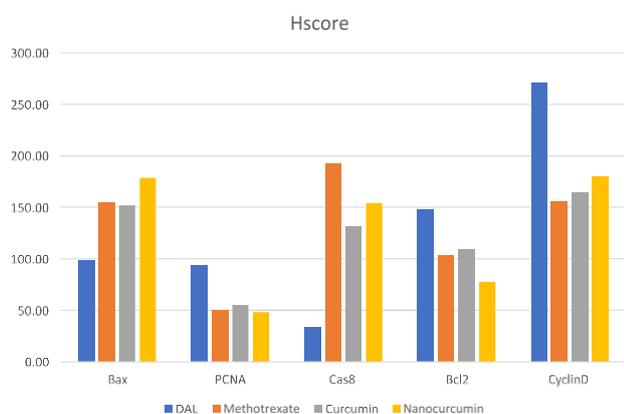


Fig. 4: H-score–Expression of apoptotic and proliferative markers.

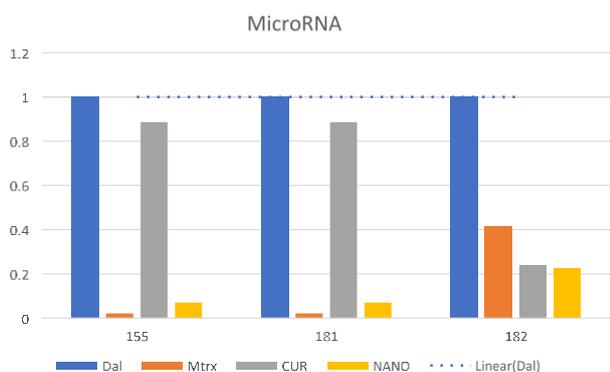


Fig. 5: Realtime PCR: Effect on expression of miRNA.

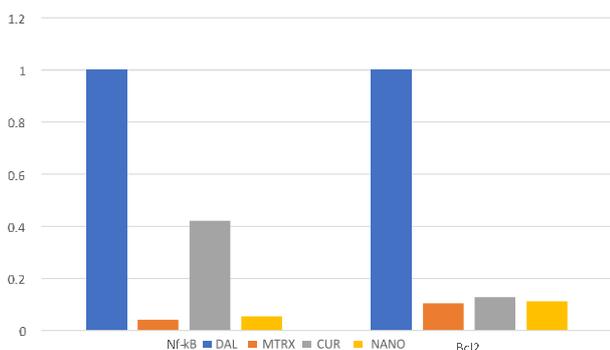


Fig. 6: Realtime PCR: Effect on expression of Nf-kB and Bcl2.

lower in all three groups compared with control group. The expression of pre-miR182 was significantly lower in all the treatment groups and degree of decrease more in nanocurcumin group (Fig. 5). There was significant upregulation of Caspase 8, Caspase 9 and P53 in treatment groups whereas Nf-kB and Bcl2 were significantly down regulated. (Fig. 6 and 7).

## DISCUSSION

Natural extracts like curcumin, major component of turmeric root was suggested to have chemopreventive effect against cancer. The anticancer properties of curcumin were reported in various malignancies like lung, breast, ovary, colorectal, prostate and liver which could be attributed due to disruption in various cellular signalling pathways like Wnt/ $\beta$  catenin, P13k/Akt, JAK/STAT, MAPK, P53 and Nf-kB signalling pathways involved in

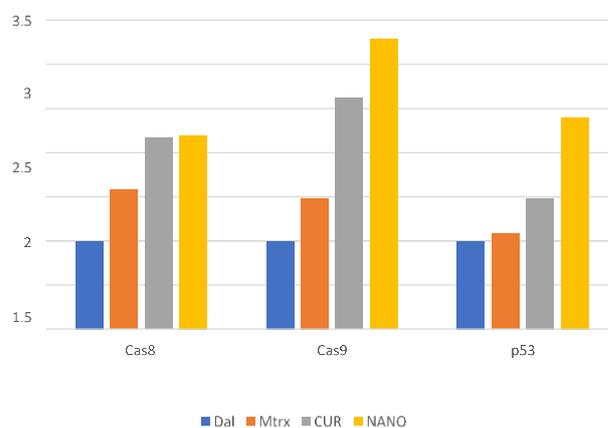


Fig. 7: Real time PCR: Effect on expression of Cas 8, Cas 9 and p53.

proliferation, angiogenesis and apoptosis (Zhou *et al.*, 2021). However, clinical application of curcumin is limited due to less bioavailability and rapid elimination. As compared to the conventional drug carriers nanoparticles have various advantages such as excellent reliability, bioactivity, improved penetration and retention impact and precise targeting properties. Even though it has a lot of advantages, there are some disadvantages like inefficient sophisticated equipment for the synthesis, difficulty in assessing the safety and restrictions to some compound (Rahman *et al.*, 2022). Solid lipid nanoparticles are widely used nanocarriers for drug delivery. Its suitability for large scale production and sterilization makes it more useful.

Previous report suggests that curcumin loaded solid lipid nanoparticles has enhanced antitumor activity in breast cancer (Wang *et al.*, 2018). Here, we studied the effect of curcumin and CUR-SLN on cell proliferation in DAL, A72 and HT 29 cell lines by MTT assay. The results indicate that both curcumin and CUR-SLN inhibited cell proliferation and viability in a concentration dependent manner. The  $IC_{50}$  value of nanocurcumin was found lower than curcumin after 24 hours. The result suggested that CUR-SLN had higher cytotoxic effects than curcumin which might be due to improve cellular uptake of CUR-SLN (Mohanty and Sahoo, 2010). It seems that formation of curcumin nanoparticles increased the effect on cells and proved its anti-proliferative effect.

Induction of apoptosis is an important marker of cytotoxic antitumor agents. The inhibitory effect of curcumin and nanocurcumin was studied on DAL bearing mice. DAL cells showed cell shrinkage, membrane blebbing, cytoplasmic vacuolation, chromatin condensation and nuclear fragmentation, typical characteristics of the apoptotic cells as described by Shtilbans *et al.* (2010). These apoptotic morphological changes membrane blebbing and cell shrinkage were also observed in AO/PI double staining (Aziz *et al.*, 2018). TUNEL method is used to determine the apoptotic cells demonstrate the DNA fragmentation a typical characteristic of apoptosis (Zhivotosky and Orrenius, 2001). TUNEL assay is a widely used method for detection of apoptotic cells label 180-200bp multiple DNA terminals resulting from DNA fragmentation. Number of apoptotic cells was highest in nanocurcumin group. These results confirmed that curcumin and nanocurcumin induce cell death via apoptosis.

Nf-kB exists in the form of a heterodimer consisting of p50 and p65 subunit sequestered in the cytoplasm in an inactive form which on activation gets translocate to nucleus. Compounds inhibiting translocation of Nf-kB could be extremely useful in treatment of cancer. The results of Nf-kB immunostaining in the present study indicate that treatment of curcumin and nanocurcumin inhibited the translocation of Nf-kB p65 in DAL cells. Consistent with our findings Belakavadi and Salimath (2005) observed inhibition of nuclear translocation in EAT cells on treatment with curcumin. This indicates that Nf-kB is one of the potential targets of curcumin and nanocurcumin to elicit growth suppressive activity in Dalton's Ascites Lymphoma.

Cell proliferation is one of the important criteria for indicating cancer prognosis. The evaluation of cell proliferation can be partially done by counting mitotic figures. PCNA is another parameter used for assessing proliferation. Expression of cyclin D1 and PCNA are reported to be related (Caputi *et al.*, 1999). Higher expression of cyclin D1 was found to be correlated with tumor progression (Diehl, 2002). In this study nanocurcumin reduced both PCNA and cyclin D1 expression better than curcumin which could be due its effect on Nf-kB activation. Consistent with our findings, decreased cyclin D1 expression with curcumin nano disk was reported previously (Singh *et al.*, 2011).

There are two types of apoptotic pathways in mammalian cells extrinsic and intrinsic pathway in which caspase 8 and caspase 9 act as the mediators, respectively. Activation of these caspases initiates the activation of caspase 3 (Aziz *et al.*, 2018). The present study revealed significant downregulation of Bcl2 protein and upregulation of pro apoptotic protein Bax. Similar results were reported previously in Dalton's Ascites Lymphoma model (Debnath *et al.*, 2018). Cytoplasmic expression of Caspase 8 was also increased in nanocurcumin and curcumin treated group as compared to DAL control group. Consistent with our findings, previous reports also suggest that curcumin regulates the expression of caspase 8 in lymphoma cells (Wu *et al.*, 2005). P53 is a transcription factor that regulates cell cycle that is when there is damage to the DNA p53 stops cell cycle at G1 and G2 check points allowing DNA repairing proteins to activate. If it fails, Bax gene is activated and apoptosis is triggered. Both Bcl2 and Bax are the transcriptional targets for p53. It is important to assess the expression of p53 along with Bcl2 and Bax to determine the pathway of apoptosis. Previous reports suggested that inhibiting Nf-kB activation can regulate the expression of various other genes which are involved in cell proliferation, apoptosis and metastasis (Aggarwal *et al.*, 2006). Gene expression studies revealed significant down regulation of Bcl2, p53 and Nf-kB genes and up regulation of Caspase 8 and 9 indicate that nanocurcumin induced apoptosis of DAL cells by activating both extrinsic and intrinsic pathway. Based on the observations it was confirmed that nanocurcumin induced apoptosis by acting through Nf-kB and p53 signaling mechanisms.

Dysfunction of miRNA is one of the important problem in cancer development. Micro RNA profiling studies reported aberrant expression of miR181, miR182 and miR155 in various malignancies even in hemopoietic

tumors in humans and animals (Craig *et al.*, 2019). MicroRNA can function either as oncogenic or tumor suppressors. Recent studies reported that miR 181 can function as a tumor suppressor gene or oncogene depending on the target cell (Weng *et al.*, 2015). The overexpression of miR 181a was found associated with chemoresistance in T cell lymphoma (Yan *et al.*, 2015). In canine lymphoma it was found upregulated in T cell lymphoma and used for predicting the immunophenotype (Craig *et al.*, 2019). In the present study, down regulation of miR181a was observed in methotrexate (0.041-fold), curcumin (0.085-fold) and nanocurcumin (0.0316-fold) as compared to the tumor control group. It was found consistent with the previous report supporting its oncogenic function in lymphoma. Lin *et al.* (2018) suggests that miR 181 acts as a tumor suppressor and mediate Nf-kB pathway and induced apoptosis in ovarian cancers. In contrast to this miR 181 acts as an oncogenic in DAL and can be correlated with the expression of Nf-kB. Nanocurcumin effectively downregulated the expression of oncogenic miRNA 181a and regulated the apoptosis through Nf-kB pathway suggesting that microRNA 181a can act as a suitable therapeutic target for curcumin in the treatment of Lymphoma. Increasing evidences suggest that miR182 is oncogenic in nature in various malignancies. It was found associated with glucocorticoid resistance in lymphoblastic malignancies which is considered as a poor marker of prognosis (Yang *et al.* 2012). Pakizehkar *et al.* (2020) predicted some apoptotic potential target genes (Caspase 8, Caspase 9, TP53 and Bax) for miR 182 by *in silico* studies. They suggested that miR182 is a potential target for curcumin loaded polymerosome nanoparticles in breast cancer. They found that p53, Caspase 9 and Bax upregulated and with downregulation of miR182 suggesting that they act as potential targets of miR182. Caspase 8 didn't show any change in their study. Consistent to their findings in our study also pre-miR-182 was downregulating and p53 and Caspase 9 was upregulating. P53 can induce apoptosis by transcriptional activation of Bax and downregulating Bcl2 which supports our result. In contrast to their observation Caspase 8 found upregulated in our study which was consistent with their *in-silico* study results suggesting that Caspase 8 can be a target for miR182. Babar *et al.* (2012) observed the overexpression of miR155 in lymphoid tissues resulted in disseminated lymphoma and nanoparticle-based therapy targeting miR155 inhibited tumor growth in mouse model. In agreement with the previous reports in our study also it acts as an oncogenic and down regulated on treatment indicating that miR155 also can act as a potential therapeutic target for curcumin.

**Conclusions:** In summary, curcumin encapsulated in solid lipid nanoparticle administered at 50mg/kg potentiates its anti-tumor effect by inducing apoptosis and inhibiting cell proliferation by targeting miRNA expression and regulating Nf-kB and p53 signaling pathways.

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**Authors contribution:** P. Krishnaveni: Carried out the experiment, Preparation of manuscript M. Thangapandiyan: supervised the work, Correction of manuscript. P. Raja: Analysis of molecular work. G.V.S Rao: Correction of the manuscript.

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