Piperine attenuates the cancerous activity response in Neuro-2a cell line

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Abstract: Interactions of cancer cells with their microenvironment play a significant role in defining the severity of the disease. In search of novel compounds with anti-inflammatory and anticancerous capabilities, the effects of purified compound piperine were investigated in Neuro-2a cell line. The neuronal lineage of Neuro-2a cell line was confirmed by using antibody against β-III tubulin protein. The cells were treated with different concentrations of piperine (μM: 10, 50 and 100) for 48 hrs at 37ºC. A dose of 100 μM was selected that induces a 50% inhibition in the cell growth calculated by MTT and morphometery assays. The result shows that in the presence of piperine neurite outgrowth was decreased in a dose dependent manner. The gene expression of TN-C, TNfnD and TnfnC were significantly reduced whereas the expression intensities of TnfnA1, TnfnA2, CSPGs and Laminin were significantly elevated when compared to their respective untreated controls. Similarly proinflammatory marker COX-2 expression was significantly inhibited in the presence of piperine when compared to untreated controls. This is the first time we have illustrated that irrespective of increased expressions of CSPGs, a significant reduction in Tenascin-C and its TNfnD and TNfnC domains are necessary to inhibit the tumor progression. Taken together, the capabilities of piperine to induce an apoptosis by decreasing the neurite outgrowth, proliferation rate and expression of TN-C and COX-2 in Neuro-2a cell line confirmed for its anticancerous and anti-inflammatory potential.

Keywords: Neuro-2a cell line, TN-C, neurocan, brevican, COX-2, anti-tumor, anti-inflammatory, piperine, black pepper

INTRODUCTION

Neuroblastoma is the most common solid tumor in childhood (Tsutsumimoto *et al*., 2014; Brodeur, 2003; Maris & Matthay 1999;) and comprises of 7-10% of childhood cancer with an incidence of about 8 per million under the age of 15 (Heck *et al*., 2009; Mueller *et al*., 2012). However, in adults, there is an occasional risk of neuroblastoma development (Bayrak *et al*., 2012). Inflammatory responses are induced when developing neoplasm become chronic and associated with malignant cells, this interpreted into cancer related inflammation (Mantovani *et al*., 2008). Inflammation is involved in tumor progression as it initiates angiogenesis, provides neoplastic cells and disrupts immune system. Regardless of the present medical care, including surgical resection, adjuvant chemotherapy (Tanaka *et al*., 2012) and radiation, brain malignancies have poor clinical outcomes. Many studies have suggested the interactions between cancers and extra cellular matrix proteins/ growth factors (Swartz *et al*., 2012) play a vital role in the pathology of cancer metastasis (Dwyer *et al*., 2014). The tissue provide a rich environment to the cancer cells by releasing transforming growth factors β (TGFβ) (Hiraga *et al*., 2012), insulin-like growth factors (Yin *et al*., 1999), cytokines, receptors and extra cellular matrix proteins such as CSPGs, Neurocan (Zhendon *et al*., 2017), Brevican (Dwyer *et al*., 2014), TN-C (Yoshida *et al*., 2015) and laminin receptor (LRP/LR) leading to the remodeling of the extracellular environment of the tissue (Chetty *et al*., 2017). Cancer cells got activated and utilize the receptors, factors and proteins to produce even more quantities of hormones, growth factors and proteins which in turn favor their growth and create a cyclic connection between metastatic cancer cells and tissues (Yin *et al*., 1999). Uptill now there is no cure available for such types of cancer cells. Therefore in search of novel compounds those have abilities to combat inflammation and inhibit the proliferation of cancer cells purified compound piperine was used in the present study.

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MATERIALS AND METHODS

Cell line

Neuro-2a cell line was purchased from ATCC (American Type Cultured Collection) and was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Karachi, Pakistan) medium containing fetal bovine serum (10%, FBS, Sigma-Aldrich), Penicillin/Streptomycin (1%, Signa-Aldrich) and glutamax (0.5 mM, Sigma-Aldrich) in 5% CO_2 incubator at 37^{o}C.

Cell differentiation

To induce differentiation the Neuro-2a cells were seeded at concentrations of 2×10^3cells per well in 24 well plate

for 24 hrs in the growth medium. After 24hrs the growth media was replaced with the differential media containing fetal bovine serum (2%), penicillin/streptomycin (1%) and retinoic acid (3 µM) in DMEM. Differential media was refreshed after every 48 hrs. Post mitotic neurons are identified as cells containing neurites twice the diameter of their cell bodies**.**

Stock preparation of piperine

Piperine compound (98%) was bought from ACROS ORGANICS (CAS: 94-62-2). To prepare stock of piperine (20mM, F.W 285.34 g/mol) 5.7068 mg of piperine compound was dissolved in 1ml of methanol (100%).

NMR and mass spectroscopy

AVANCE AV-500 and 400 MHz instruments were used for recording of Proton NMR spectra. Recording of mass spectra the JEOL-MS instrument was used. The structure and mass of standard compound were characterized by NMR and MS techniques.

Neurite outgrowth assay

The effects of piperine on neurite outgrowth were evaluated by Neurite outgrowth assay. Neuro-2a cells were cultured in 24 well plate at a concentration of 2 $x10^3$ cells per well. Differentiated cultures were treated with different concentrations of piperine (µM: 10, 50 and 100) on DIV-7. Neurite lengths were measured as neurites, which is 1.5 folds longer than the cell body by using optical vision pro software under a Nikon microscope (Nikon TE2000-U, Tokyo, Japan). A mean average length of 10 longest neurites and sum of all neurite lengths was calculated.

MTT assay

MTT (3-(4,5-dimethylthaizol-2-yl) -2,5-diphenyltetrazolium bromide) assay was performed to investigate the effects of piperine on the growth of Neuro-2a cells. The cells were seeded at a concentration of 9 x 10^3 cells per well in 100µl of culture media in 96 well plates. After 24 hrs the media was replaced with differentiated media containing different concentrations of piperine (µM: 300, 200, 150, 100, 75 and 50). Methanol (1%) was used as vehicle/control. After 48 hrs media was removed carefully and 10 µl MTT (5 mg/ml) was added to each well and incubated for 4 hrs at 37°C in 5% CO_2. MTT dye was removed and 100 µl DMSO was added in each well in order to dissolve formazan crystals. The absorbance was measured in the microplate reader at 590 nm and a graph between % growth inhibition and concentration was plotted. Percent inhibition was calculated from (% inhibition=100-[(treated-negative control/positive control-negative control)*100]. IC50 value was identified by EZ fit software.

Immunocytochemical studies

Cells were plated at a concentration of 1x 10^3 cells per well on glass coverslip in 4 well plate and differentiated for 7 days. The differentiated cells on DIV-7 were treated with 100 µM of piperine. After 48 hours of incubation cells were immunostained by using antibodies against β-III, COX-2, CSPGs, LN and TN-C proteins and pro-inflammatory marker COX-2. In case of ECM proteins (CSPGs, LN, TN-C) cultures were first incubated with primary antibodies for 1 hrs and then fixed with 4% paraformaldehyde (PFA) for 10 to 15 mins. Whereas in case of β-III tubulin protein and COX-2 cells were fixed with PFA (4%) and then it was incubated with the primary antibodies for 1 hr. Cultures were then stained with secondary antibodies (568 and 488) for 45 mins. followed by nuclei staining with DAPI for 15 mins. All the dilutions and washings were done by using Krebs Ringer's buffer pH 7.3 containing bovine serum albumin (KRH/0.01% BSA). Coverslips were mounted using immunomount for overnight in 4 °C in dark.

2.8 RT-PCR

Total RNA from Neuro-2a cells with or without piperine treatment were isolated by using EZ-10 DNAaway RNAMiniprep Kit (Cat# BS88133). Followed by the synthesis of cDNA by using TOPscript™ cDNA synthesis kit (Cat # EZ005S) was used for reverse transcribing total RNA to cDNA. RT-PCR Master Mix (2X) (Cat # K0171) was used for RT-PCR reaction by using primer sequences as described previously (Siddiqui *et al*., 2009). RT-PCR was carried out on MJ mini thermocycler (BIO RAD). Cycling conditions were as follows: 5 min denaturation step at 94°C, followed by 34 cycles of 30 s at 94°C, 30 s at given annealing temperatures (table 1), 1 min at 72°C# an extension step and 72°C for 5 min a final extension step. The bands were visualized and resolved in 1.5% agarose gel (Biotechnology grade I, BIO WORLD) prepared in Tris acetate buffer (TAE) pH 8.3. The gel was run at 80 V for 30 mins.

STATISTICAL ANALYSIS

IBM SPSS statistics 19.0 software was used for the statistical analysis. Data were analyzed by Mann Whitney U-test and paired sample two-tailed test. Experiments were performed in triplicates. A P value of **p <0.05 and ***p<0.001 were considered as significant.

RESULTS

Effects of piperine on neurite outgrowth

The effects of piperine were investigated on the inhibition of Neuro-2a cell outgrowth. Therefore differentiated Neuro-2a cells were cultured in the presence of different concentrations of piperine (µM: 10, 50 and 100) for 48 hrs and were stained with β III-tubulin antibody (fig. 1A). The data show that the higher concentration (100 µM) of piperine significantly ($p<0.05$) inhibited the effects on the average length of the 10 longest neurites whereas no significant differences were observed at lower

Table 1: Dose dependent inhibition on the proliferation rate induced by piperine

S. No.	Piperine Concentrations (µM)	Mean	% Inhibition
1	50	0.022008	20
2	75	0.018892	33.59
3	100	0.070715	52.1275
4	150	0.043615	88.53
5	200	0.003858	91.36
6	300	0.015351	97.53

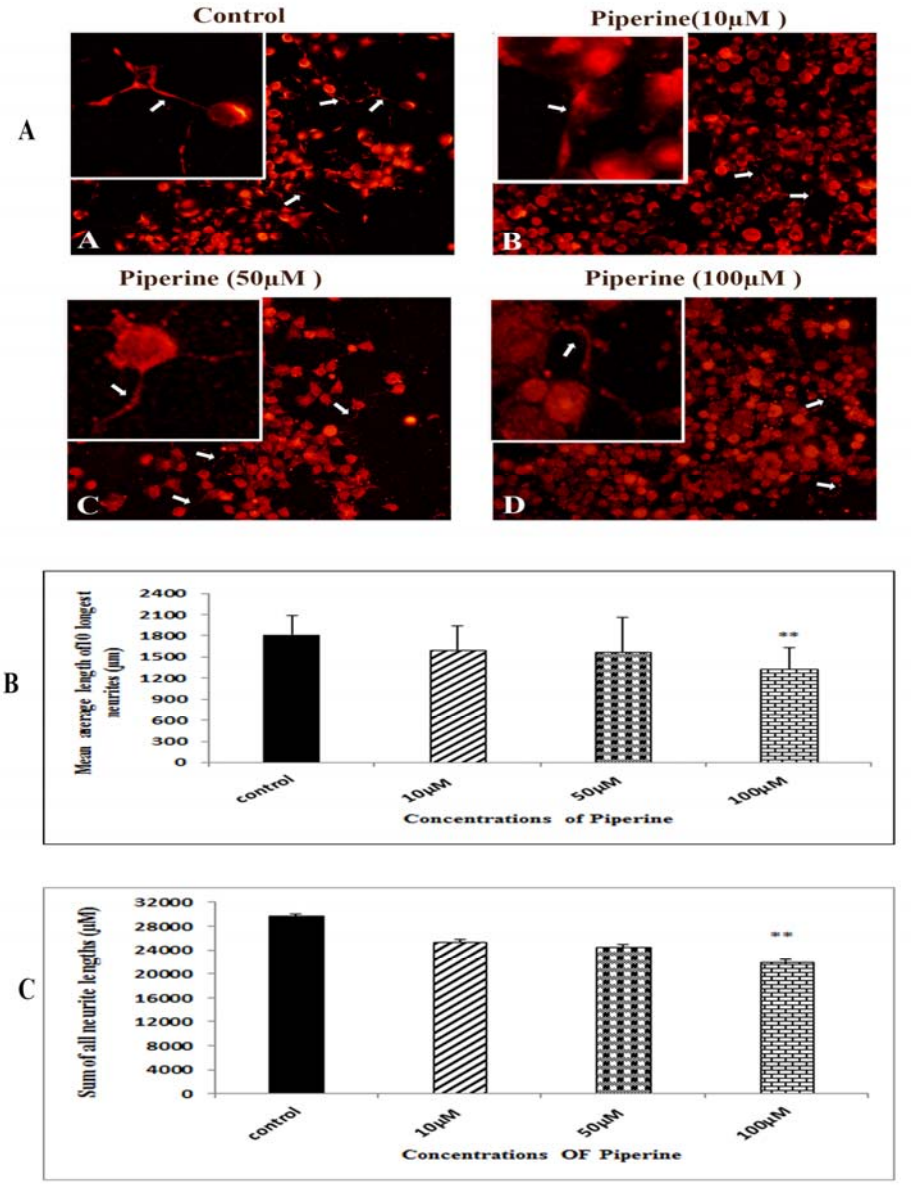

Fig. 1: Piperine reduces neurite outgrowth in a dose dependent manner. A) Neuro-2a cells were cultured in the presence of piperine at various concentrations (10 µM, 50 µM and 100 µM) for 48 hrs and visualized by using β-tubulin-III antibody. B) Effects of piperine on mean average length of neurites after 48 hrs. Statistical analysis revealed by using Mann Whitney U- Test. Data was represented as Mean ± S.D. **p <0.05 were considered as significant from the controls. C) Effects of piperine on the sum of all neurite length after 48 hrs. Statistical analysis revealed by using Mann Whitney U- Test. Data was represented as Mean ± S.D. **p <0.05 were considered as significant from the controls. Arrows represent neurites. Scales = 25 µm, 40 µm. Number of experiments= 3.

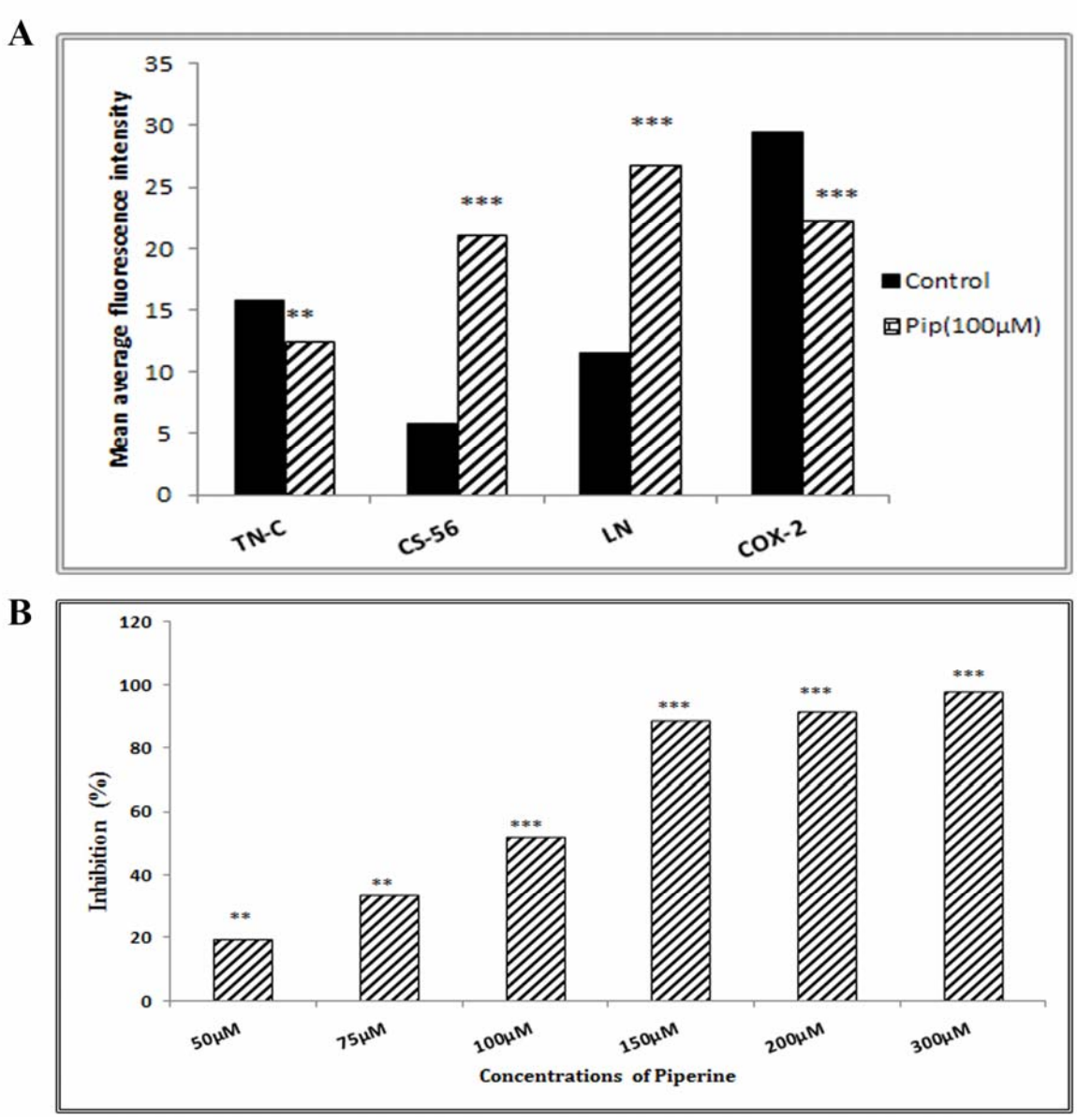

Fig. 2: A) Expression intensities of ECM proteins and COX-2 at DIV-9. Means are ± S.D. Statistical difference was analyzed by paired sample t-test. **p<0.001 was considered as significant. B) Effects of piperine on growth inhibition of Neuro-2a cell. Cells were treated with various concentrations of piperine (µM: 50, 75, 100, 150, 200 and 300) for 48 hrs. Data represented as Means ± S.D. Statistical differences were revealed by student's T-test. **p<0.05 and ***p<0.001 were considered as significant when compared to controls. Number of experiments= 3.

concentrations (10 and 50 µM). Furthermore piperine also decreased the sum of the total neurite lengths in a dose dependent manner (p<0.05). However, only higher dose of piperine (100 µM) showed a significant reduction in the sum of all the neurite lengths (fig. 1B,C).

MTT assay

Cells in the presence of different concentrations of piperine (µM: 50, 75, 100, 150, 200 and 300) were tested to select the cytotoxicity dose at which growth and proliferation rate was inhibited by 50% (fig 2B). The data show that as concentration increases the proliferation and viability of the cells decreases significantly (p<0.05). The highest level of reduction (97.53 %) was (p<0.001) found at 300 µM whereas 50 % inhibition was established at 100µM (table 1). Hence IC50 value of piperine compound was calculated and selected as 98.68µM (100µM) after 48hrs.

Effects of piperine on the expression of ECM proteins

To study the effects of piperine on the expression of different ECM proteins in Neuro-2a cells, the cells were incubated with piperine (100 µM) for 48 hrs. The expression levels of ECM proteins were identified by

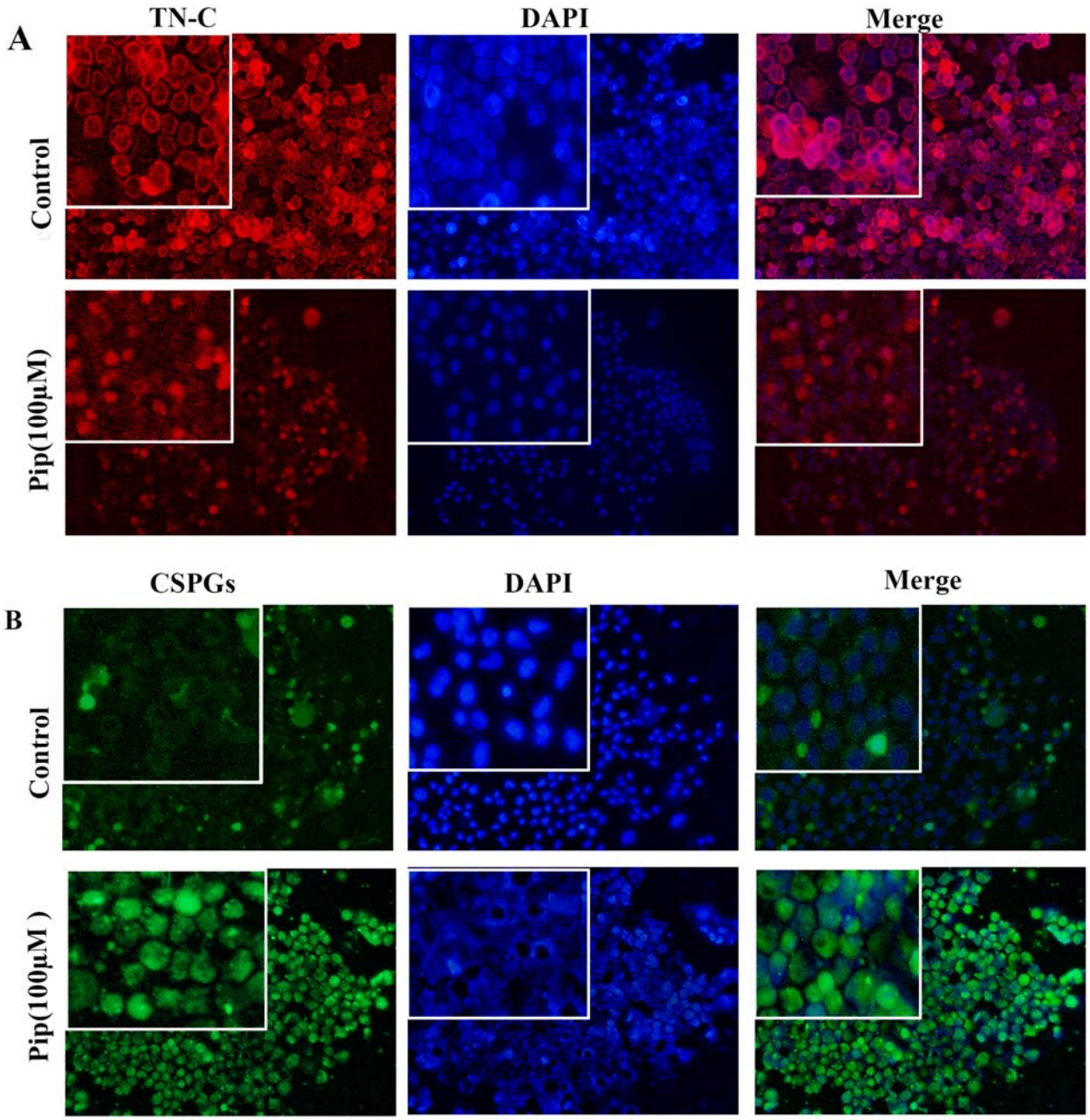

Fig. 3: Expression of TNC and CSPGs in Neuro-2a cells in piperine at DIV-9. A) TNC (red). B) CSPGs (green). Nuclei were stained with DAPI (Blue). Scales = 25 µm, 40 µm. Number of experiments= 3.

using antibodies against TN-C (fig. 3A), LN (fig 4A) and CSPGs proteins at DIV-9 (fig. 4B). The data show that TN-C protein expression was found on the periphery of the cells and devoid from the center and mark a sharp and clear boundary around Neuro-2a cells. The result demonstrates a significant ($p<0.001$) decrease (63.65%) in the levels of TN-C (fig. 2A) after piperine treatment at DIV-9 (fig. 3A) when compared to untreated controls. On the other hand the surface expression of CSPG significantly ($p<0.001$) increased in the presence of piperine. The percent increase was calculated 353.26% when compared to untreated controls (fig. 2A). Similarly the expression of LN (fig. 2A) was also found to be significantly ($p<0.001$) high when compared to untreated controls. The morphology of the cells changes from round to irregular in the presence of piperine when compared to untreated controls (fig. 2A).

Effect of piperine on proinflammatory marker

Cox-2 is a proinflammatory marker. It is highly expressed in various cancers and inflammatory conditions. The present data shows that in the presence of piperine there is a significant decrease ($p<0.001$) in the intensity of COX-2 expression (fig. 4B). There was 46% reduction in intensity of COX-2 when compared to the untreated controls (fig. 2A). As COX-2 expression increases in

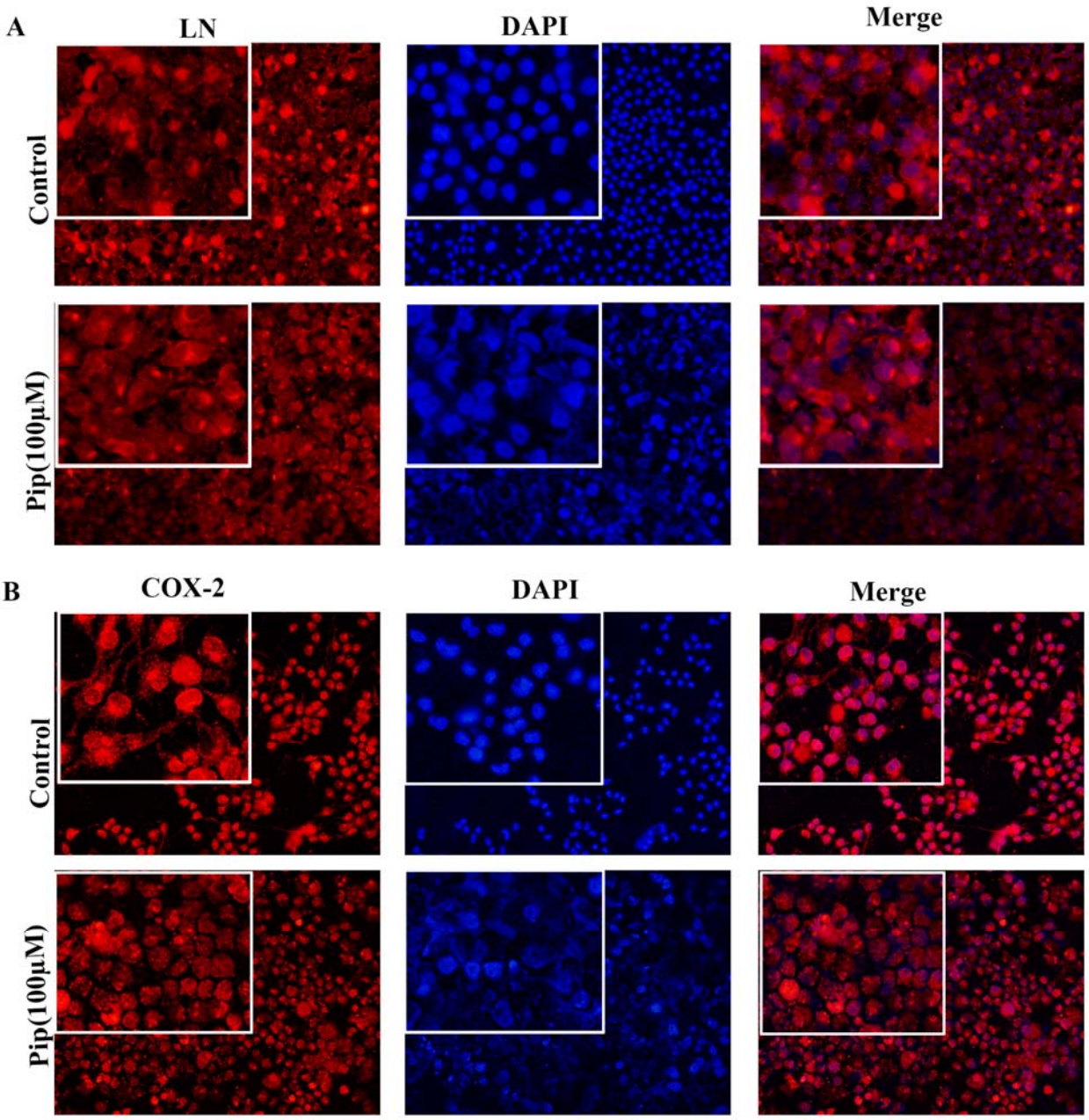

Fig. 4: Expression of laminin and COX-2 in Neuro-2a cells in piperine at DIV-9. A) Laminin. B) COX-2. Nuclei were stained with DAPI (Blue). Scales = 25 µm, 40 µm. Number of experiments= 3.

inflammatory and cancerous states decreased in expression means there is a decrease in inflammatory process and increase in apoptosis of cell.

Effects of piperine on mRNA expression of ECM proteins

The mRNA expression of ECM proteins after the treatment with piperine was analyzed by RT-PCR by using primers against the conserved region of Tenascin-C such as TNfn05-06, TNfnA1, TNfnA2, TNfnB, TNfnC, TNfnD and laminin beta 1 subunit (Lnβ1), and CSPGs such as Neurocan (Ncan) and Brevican (Bcan). β-actin was used as a positive control showing constant expression in both treated and untreated controls (fig. 5A). The data show that piperine significantly decreased ($p<0.05$) the expression of TNC-05-06 (0.4 folds) along with the expression of domain TNfn-C (0.5 folds), and TNfn-D (0.3 folds). Whereas there is a significant increase ($p<0.05$) in the expression of TNC-B (0.49 folds), TNC-A2 (0.2 folds) and TNC-A1 (0.16 folds) when compared to their respective untreated controls (fig 5B). In contrast to this the levels of CSPGs, neurocan and brevican was found to be significantly elevated by both Neurocan (0.35 folds) and Brevican (0.32 folds) when compared to their respective untreated controls. Expression of Laminin (Lamb1) (0.5 folds) also

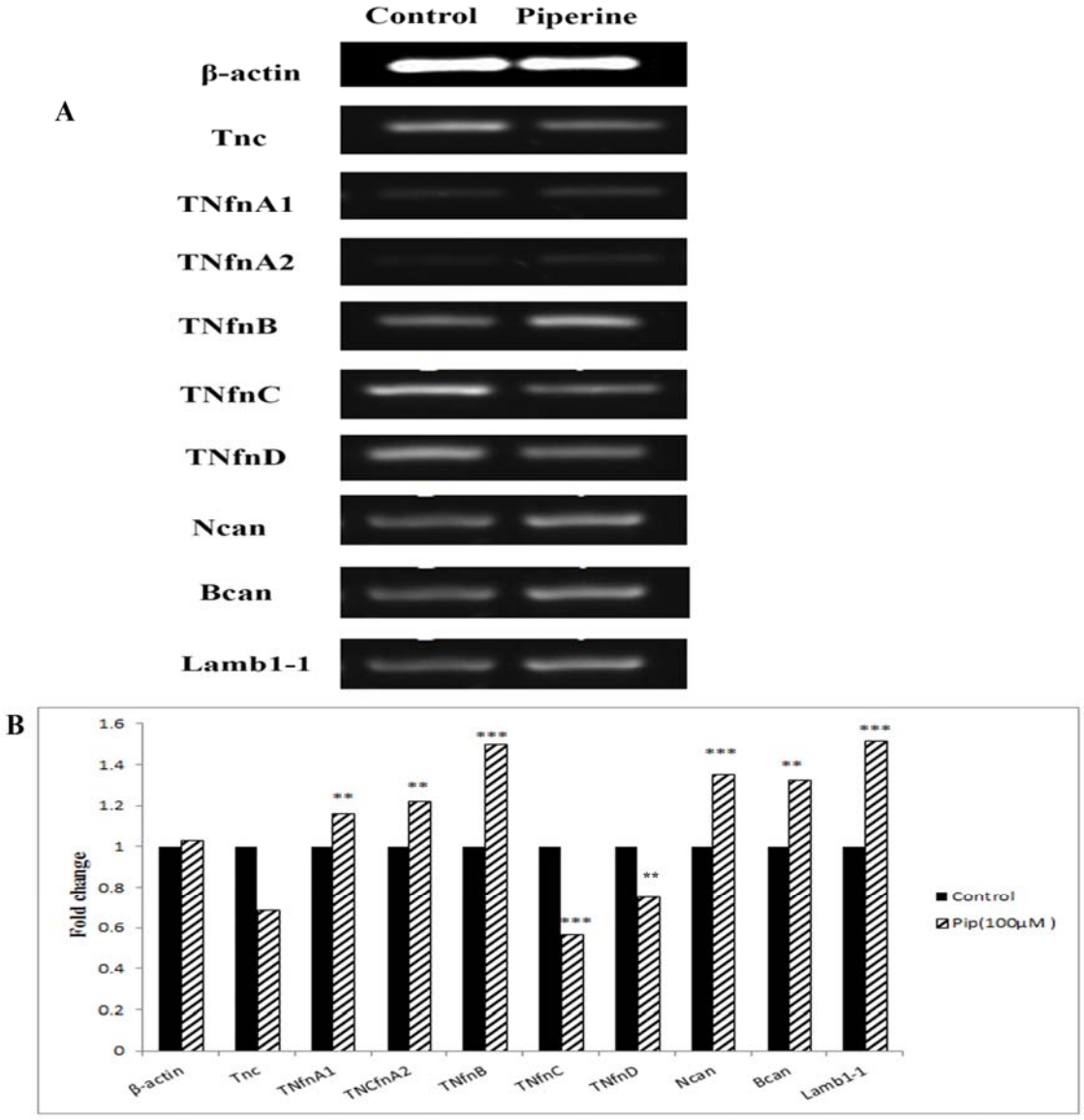

Fig. 5: A) RT-PCR analysis on ECM gene expression after 48 h treatment of Neuro-2a cell lines with piperine compound. β-actin was used as a positive control. B) Gene expression intensities of ECM and COX-2 proteins. *** $p < 0.001$ and **$p<0.05$ were considered as significant. Band size= 500 bp. Number of experiments= 3.

significantly increased ($p<0.05$) as compared to their respective untreated control. Nevertheless the mRNA expression of TN-C and its TN-fnIII-domains were found to be significantly elevated along with the expressions of Laminin β 1 subunit, Neurocan and Brevican when compared to the their respective untreated controls (figs. 5A, B).

DISCUSSION

Chronic diseases such as cancer, herbal medicines are widely used as a supplementary therapy (Olaku and White, 2011). In Asian countries King of spices, black pepper is largely used as a medicinal herb (Mehmood and Gilani, 2010). Natural purified compound piperine from black pepper found to displays nephroprotective effects in rat model of lead acetate induced nephrotoxicity (Sudjarwo *et al*; 2017). However its effects on the neuroblastoma cell neurites and proliferation rate have not been identified.

In the current study we have incorporated neuroblastoma cell line, Neuro-2a. This cell line is derived from neuroectoderm and has a neuronal fate. According to Tremblay and Co-worker (2010) this cell line has been identified to express Nurr-related factor 1 (Nurr 1) with less expression levels of tyrosine hydroxylase (TH) and dopamine upon differentiation in differentiated medium containing DMEM and 0.5% fetal bovine serum. The cell

line also exhibited significantly high levels of TH and number of dopaminergic neurons in the presence of dibutyryl cyclic adenosine monophosphate (dbcAMP). However no further increase in the TH and number of dopaminergic neurons was found upon differentiation induced by retinoic acid (Tremblay *et al*., 2010).

The present study shows that piperine significantly reduces neurite outgrowth from the differentiated Neuro-2a cells (fig. 3.1). Previously we have showed the stimulatory effects of Vitex negundo and Calotropis procera extracts on hippocampal neurite outgrowth (Siddiqui *et al*., 2018; Kamal *et al*., 2018). Neurite inducing capabilities of a compound makes it a very strong neurotrophic molecule and have beneficial effects on the neurite regeneration, plasticity (Min *et al*., 2006) and differentiation. However in the present study we observed a dose dependent decrease in the neuroblastoma neurite outgrowth, proliferation and apoptotic rate as we increased the concentration of the purified compound piperine from 50 to 300 µM. This shows that it induces an antiproliferative, antimetastasis and anticancerous effects in the Neuro-2a cell line.

Person and colleagues (2017) showed that an increased expression of β-III tubulin inhibit the activity of microtubule-targeting anticancerous drug taxanes. Therapies that reduce the expression of β-III tubulin from various brain tumors or pancreatic adenocarcinoma could be helpful in treating the patients with cancers. The present study shows that piperine at 100 µM reduces the expression of β-III tubulin from the Neuro-2a cells suggesting its anticancerous potential.

Besides β-III tubulin levels the expression of different ECM proteins are also shown to be involved in tumor formation and growth. Targeting ECM in cancerous tissue may also affect ECM components in healthy tissue (Cathcart *et al*; 2014). In the current study we have analyzed the expression of Tenascin-C, Laminin, CS-56 stub, Neurocan and Brevican in differentiated Neuro-2a cells. The result show that TN-C in the presence of piperine was significantly reduced when compared to the untreated controls. Tenascin-C is a hexabranchion dual natured molecule that exerts neurite outgrowth and neurite guidance in different types of cells to which they interact. These effects depend upon the age of the cells and the type of the cells. As TN-C has many functional domains it can exert neurite outgrowth, neurite guidance, cell motility, cell growth, angiogenesis, and microtubule remodeling (siddiqui *et al*., 2008; 2009). Earlier studies have shown its involvement in the formation of many brain tumors esp. of fibrotic stroma and astrocytoma (Imre *et al*., 2012).

In intracerebral metastases of non-small cell lung cancer cells high levels of Tenascin-C act as a significant prognostic marker of decreased overall survival in both univariate and multivariate analysis (Onion *et al*., 2018). Significant decrease in TNfnD, TN-05-06 and TNfnC were observed in the current study. It has been shown previously that TnFnD is involved in neurite outgrowth (Siddiqui *et al*., 2009; 2008) while TnfnC is involved in the guidance and TN-05-06 is designed to identify the conserved region of TN-C protein. A decreased expression can be correlated with the treatment of cancer. TNfnA1, TnfnA2 and TnfnB domains of TN-C increases in the presence of piperine as A1 and A2 domain of TNC inhibits retinal neurite growth (Siddiqui *et al*; 2009) we suggest that piperine inhibits TNfnD and increases TNfnA1 and TNfnA2 and exerts its antitumor activity in Neuro-2a cells.

Proteoglycans during tumor formation participates by changing the activity of effective molecules such as growth factors and cytokines. Neurocan functions in axon guidance and neurite outgrowth as it decreases neural adhesion and outgrowth in vitro and expression pattern in vivo (XIAO *et al*., 2001) and is involved in malignant phenotypes (Zhendong *et al*., 2017). Brevican regulate inhibitory function of CSPGs by causing maturation of mossy fibers in cerebellum and cell surface binding (Yamada *et al*. 1997). In present study expression of CSPGs (Neurocan and brevican) was significantly increased in Neuro-2a after the treatment with piperine. Numerous studies have shown that an increased expression of CSPGs in response to insult is a protective mechanism that limits the area of damage and its spread. Similarly elevated levels of Neurocan are found after injury, its function is to inhibit neurite regeneration (Klump *et al*., 2016). However the data clearly shows the loss of the expression of CSPGs from the periphery in to the center of the cells. Could be due to the translocation of the receptors from the periphery in to the center or due to cell membrane rupture leading cell apoptosis. Nuclei staining also show cell apoptosis and fragmentation.

A proinflammatory marker COX-2 expression is found in non-epithelial, inflammatory and stromal (Agrawal *et al*., 2018) cells. Recently (Niranjan *et al*., 2018) it has been shown to be involved in the development of Parkinson's disease (PD). It functions in migration and growth of neuroblastoma cells in vitro (Varga *et al*., 2014). An increased expression of COX-2 is associated with the tumor progression and recurrence (Agrawal *et al*., 2018). Therefore in present study the expression of COX-2 significantly decreased after piperine treatment proving that it has anti-inflammatory and antitumor potentials.

CONCLUSION

Natural purified compound piperine from black pepper found to inhibit neuroblastoma cell line Neuro-2a proliferation, neurite outgrowth and induces apoptosis by

reducing the expression of β-III tubulin, TN-C, TNfnD and pro-inflammatory marker COX-2 while it increases the expression of TNfnA1 and TNfnA2, Neurocan and Brevican. By modulating microtubules, TN-C protein and pro-inflammatory COX-2 expression levels, piperine has proved to have antitumor and anti-inflammatory potentials on Neuro-2a cell line.

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