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Influence of *Morinda citrifolia* on AKT induced regulation of Cox-2 in Experimental Glioma

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Foreword

The technical bulletin is the outcome from the concluded project, "Influence of *Morinda citrifolia* L. on AKT induced regulation of COX-2 in experimental glioma" funded by World Noni Research Foundation. I had the opportunity to watch closely the whole process of research work being carried out by well designed investigation by Dr. A. J. Vanisree, University of Madras, Maraimalai Campus, Guindy, Chennai.

The project is aimed to assess the expression of Akt , PTEN and COX-2 in glioma inflicted rat model when supplemented with Noni fruit juice thereby finding a way alleviate the effect of glioma.

I congratulate the author for her dedicated efforts to bring out these properties of Noni fruit and also for the preparation of WNRF Technical bulletin - 09.

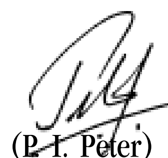
Chennai
September, 2012

(Kirti Singh)
Chairperson

About the Project

Noni is for wealth. Noni is for health. Noni is for well being. Empowering Noni Research is Empowering the world. WNRF encourages Noni Research workers by generous funding of the projects in different scientific disciplines. Clinical research funded by WNRF, enables the exploration of efficacy of Noni against various pathological conditions.

Several studies have highlighted Noni as a powerful healing agent of several complaints/diseases including cancer. It is said to affect the growth of cancer and precancer cells as reported by research personnel across the globe. The current WNRF funded project for the team based at Chennai, led by Dr. A.J. Vanisree, provides an additional proof for the property of Noni, which could act against the malignant features. The team attempted to explore the role of *Morinda citrifolia* (Noni) on the survival of glioma cells implanted in the rat brain and produced promising and strong data reflecting the potential of Noni which underwent three years of investigations. These data, I anticipate, could function as an effective platform that might be helpful for an effective translational research in future by the oncological research community.



Chennai
September, 2012

(P. I. Peter)
Chairman, Noni BioTech

Preface

Glioma, the tumor of glial cells, remains a challenge for oncologists and clinicians owing to its high invasiveness and also needless to say, its chemoresistant nature. Most of the current understanding of brain tumor pathology and its therapy has been gleaned through the animal models with a hope of predicting effective therapeutic response. The current study, funded by WNRF, Chennai, made an endeavour to provide an insight into the pathology of the animal model of glioma and also into the assuaging potential of *Morinda citrifolia* (Noni) on tumor burden in rats. Noni, the well known wellness drug possesses various therapeutic effects but the drug requires meticulous investigations for its property against cancers, especially in brain tumor, which demands special modes of therapy due to various reasons such as the existence of blood barin barrier *etc*. The study provides an additional documentation for Noni, by investigating its role against the survival of the glioma cells which are implanted in rat brain. It is the sincere hope of the author that the encouraging and promising observations made during the period of the investigation on experimentally induced glioma might be beneficial for future translational research-endeavours utilizing natural sources like Noni, by virtue of the property that negatively influences the survival of the cancer cells which would, obviously, solace the victims of brain tumor.

I take this opportunity to express my sincere gratitude to Dr. Kirti Singh, Chairperson and members of Research Advisory Board of World Noni Research Foundation for their critical suggestions and also for their effort to bring out an illustrated technical bulletin useful for Noni researchers especially in oncological field.

I also express my gratitude to Prof. P. I. Peter, the proponent of Noni, who encouraged and supported in all possible ways. The author sincerely expresses grateful appreciation for his dedication for wellness of the world.

I also express my genuine gratitude to Dr. K.V. Peter, Director and Dr. T. Marimuthu, Additional Director of World Noni Research Foundation, for continuous support and contribution. The valuable guidance and encouraging words are gratefully acknowledged.

Chennai
September, 2012 Dr. A.J.Vanisree

1. Introduction

Neoplasms of the neuroglial cells are called glial tumours/ glioma. Glial tumours are found predominantly in the brain arising from the astrocytes or oligodendroglial cells or ependymal cells of the brain and are divided into four stages by WHO classification scheme (Cavenee *et al.,* 1997). Gliomas are the most common primary intracranial tumours and strike earlier than other tumours (Shapiro *et al.,* 1995). They are challenging entity of tumours which represent more than 70% of all brain tumours (Ohgaki and Kleihues, 2005). They have morphology and gene expression characteristics similar to astrocytes, oligodendrocytes and / or a mixture of the two cell types (and their precursors). Many factors contribute to the aggressive nature of MGs. These include uncontrolled tumour proliferation, invasiveness into surrounding brain parenchyma, induction of tumoural angiogenesis, and inhibition of apoptotic pathways (Onda *et al.,* 1994).

About the model

The development of appropriate animal models of brain tumours is integral to the development of chemotherapy studies. The introduction of animal models in glioma research marks a major advance in the attempt to reach a better understanding of the biology of the tumour. The prevalent glioma model is still the injection model, where for reasons of convenience and reproducibility a fixed amount of cells of a known cell line, is injected into the animal brain using a micro syringe (Bjerkvig *et al.,* 1990). The rapidly proliferating rat C6 glioma cell line was originally induced in random-bred Wistar rats by exposure to N, N'-nitroso-methylurea (Benda *et al.,* 1999). Comparison of the C6-derived experimental models indicates that tumours grown from cells transplanted in Wistar rats have characteristics closer to human glioma than tumours grown in other rat strains (Chicoine and Silbergeld, 1995).

Intervention

Herbal and natural products of folk medicine have been used for centuries in every culture throughout the world. Scientists and medical professionals

have shown increased interest in this field as they recognized the true health benefits of these remedies. Contemplating the most prevailing drug resistant problems in the treatment of glioma and also the poor survival rate of the patients, it is conceived that it would be appropriate to gaze for supporting agents which might be complementing the therapy. There exists also an additional challenge for glioma treatment where the drug in question need to cross blood brain barrier. Hence, there is a need for new prototypes and new templates for use in the design of potential extracts. Among the medicinal plants discovered by the ancestors of Polynesians, *Morinda citrifolia* L (Noni) is one of the traditional folk medicinal plants which have been used for over 2000 years in Polynesia. It has been reported to have a broad range of health benefits for cancer, infection, arthritis, diabetes, asthma, hypertension, and pain (Zhang *et al.,* 1994; Wang *et al.,* 2002), of course, not against brain tumor. It has also been reported as non-toxic candidate and has ability to cross the blood brain barrier. Hence, the current investigation was aimed at elucidating the anti-glial tumor efficacy of methanolic extract of *M. citrifolia.*

2. State of knowledge

Cyclooxygenase (COX), a rate limiting enzyme of prostaglandins synthesis, is expressed in two isoforms, COX-1 (chromosome 9 (9q32-9q33.3)) and COX-2 (chromosome 1 (1q25.2-25.3)). COX-2 contains 10 exons and is approximately 8.3 kb with a 4.5 kb transcript. IL-1B is a major inducer of COX-2 up-regulation in the CNS (Kurzel *et al.,* 2002). COX-2 is undetectable in most normal tissues (Pamela, 2004). Normal astrocytes alone do not express COX-1, whereas COX-2 expression is minimal when compared with astrocytoma cell lines (Deininger and Schluesener, 1999). COX-1 and -2 expressions are present in all grades of astrocytoma, with COX-2 more often expressed than COX-1. It was found out that COX-2 levels serve as the most reliable indicator of aggressive gliomas (Inoue *et al.,* 1995). There is also compelling evidence that this enzyme may have a role in carcinogenesis in that COX-2-derived prostaglandins may modulate the production of angiogenic factors in colon cancer cells.

Phosphatidylinositol 3-kinase (PI3K), a ubiquitous lipid kinase, is composed of a regulatory subunit (p85) and catalytic subunit (p110) (Jiang and Liu, 2008). PI3K catalyzes the phosphorylation of phosphoinositol-4,5 phosphate (PIP2) at the D3 position to form phosphatidylinositol-(3,4,5)-tri phosphate (PIP3) and activates various downstream elements including Akt/protein kinase B (PKB). PI3K regulates a number of important cellular processes such as cellular growth and transformation, membrane ruffling, actin rearrangement, vesicular trafficking and cell survival. Promotion of cell survival by the activation of PI3K/PKB occurs by the inhibition of proapoptotic signals and the induction of survival signals (Jones and Howell, 1997; Vanhaesebroeck *et al.,* 2001), which may contribute to malignant transformation. Inhibition of PI3K /PKB results in cell cycle arrest and differentiation in certain cell types, such as the human colon cancer cell lines HT29, Caco-2 (Wang *et al.,* 2001) and in glioma cells (Joy *et al.,* 2003). Protein kinase B (PKB/Akt), on the other hand, a 57-kDa protein-serine/threonine kinase serves a key role in mediating anti-apoptotic actions of growth factors on cell. Mammalian genomes contain three genes encoding Akts (termed Aktα/Akt1, Aktβ/Akt2 and Aktγ/Akt3). Akt3 is highly expressed in brain (Zinda *et al.,* 2001) and plays an important role in neuronal protection (Hui *et al.,* 2005). Stimulation of tyrosine kinase growth factor receptors activates PI3K, which leads to Akt activation. Akt activation is correlated with phosphorylation of Thr-308 at its catalytic domain and of Ser-473 at the C terminus. Over activation of Akt has been reported in a variety of cancers including glioma (Haas-Kogan *et al.,* 1998; Holland *et al.,* 2000). Activated Akt can phosphorylate a variety of substrates and thereby regulates important cellular processes, including cell-cycle progression, cell growth, cell survival, cell motility and adhesion, translation of mRNA into protein, glucose metabolism, and angiogenesis. (Knobbe *et al.,* 2002).

These two enzymes (COX-2 and PI3 K) and the related signaling components thus play a major role in aggressiveness of glioma and hence it is worth to analyze these targets during the drug trials. Further, an evidence suggests that, SC236, a COX-2 inhibitor is shown to induce apoptosis of gastric cell line through MAPK and Akt pathway (Fan *et al.,* 2004). These background knowledge functioned as a trigger for designing the objectives of the current study.

3. Objectives

Extract of *M. citrifolia*, selected for the study possesses several biological effects including anticancer property but whose molecular mechanism remains unclear. The present study was designed to investigate anti-glioma property of methanolic extract of *M. citrifolia* as a pioneering endeavour.

Specific objectives

- To assess the expression of Akt and PTEN in experimental glioma.
- To study the expression of COX-2 in glioma and to assess whether Noni has influence on the expression of Akt and COX- 2.
- To assess efficacy of Noni on the levels of phosphoinositides and prostaglandins in experimental glioma.

Work planned pertaining to the objectives

Role of methanolic extract of *M. citrifolia* against glioma model in the following aspects.

- Biochemical, histopathological and transmission electron microscopic analysis of brain tissues of *M. citrifolia* on *in vivo* glioma model.
- RT-PCR, immunoblotting and immunohistochemical analysis of proposed proteins in brain tissues of *M. citrifolia* on *in vivo* glioma model.
- HPLC analysis of PGs in brain tissues of *M. citrifolia* on *in vivo* glioma model.
- PI3K activity (measuring levels of Phosphoinositides)

4. Experimental Details

Materials and methods

Chemicals

Morinda citrifolia fruits were obtained from Noni Farm, Palliyagarm, Kancheepuram dt. NUT.MIX.F-12 (HAMs) medium (Invitrogen) was purchased from Bio Corporals, India. Fetal bovine serum (FBS), penicillin-G, streptomycin and trypsin were procured from Hi-Media laboratories, India. Xylazine was purchased from Indian immunologicals Ltd, India and ketamine was procured from Neon Laboratories, India. Primary anti- GFAP mouse monoclonal antibody was purchased from Santa Cruz, USA and anti-mouse FITC labeled secondary antibody was obtained from Genei, India. pAkt (Mouse monoclonal) and PI3K (Rabbit polyclonal) were purchased from Calbiochem, USA. COX-2 (mouse polyclonal) and ?-actin (mouse monoclonal) and TRI reagent from M/s Sigma chemical company, USA. All other chemicals were purchased from M/s SRL chemicals, Mumbai, India.

C6 Glioma cell lines

Rat C6 glioma cell line was obtained from the NCCS, Pune, India and maintained in monolayers in 100-mm dishes at 37 ºC under humidified 5 % CO2 - 95 % air. The cells were cultured in NUT.MIX.F-12 (HAMs) medium supplemented with fetal bovine serum (FBS 10 % final concentration), penicillin-G (50 unit/ml) and streptomycin (50 µg/ml). Cells were harvested during the log phase with a solution of 0.05% trypsin and 0.02% EDTA, and resuspended in HAM's F-12 medium supplemented with FBS to a final concentration of 105 cells per 10 µl (microlitre) for the implantation.

Chemicals

Animals

Male Wistar rats, weighing between 250 - 300 g, were purchased from Kings Institute, Guindy, Chennai, India and maintained under controlled environmental condition. The animals were provided with pellet food (Gold

Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai and water *ad libitum.* This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (IAEC No. 01/012/08).

Protocol

Animals were divided into five groups with six animals in each group.

Group I (Control): Animals were injected with 10 µ1 of MEM supplemented with 10 % FBS.

Group II: Animals were injected with cell suspension of C6 glioma cells (10 µ1 of MEM supplemented with 10 % FBS containing 105 cells) under a controlled pressure.

Group III: Animals served as drug control (500 mg of Noni fruit extract administrated orally / kg of bw for 30 days).

Group IV: Rats were induced glioma as mentioned in group II and treated with Noni fruit extract as in group III (from the second day of tumor implantation).

Group V: Rats were induced glioma as mentioned in group II and treated with celecoxib (COX-2 reference drug) 20 mg administered orally / kg of BW for 30 days (for the comparison of COX-2 expression). Rats were induced glioma as mentioned in group II and treated with 5 µl wortmannin (PI3K reference drug) 20 µmol/L administered intracranially at single dose (for the comparisons of PI3K/Akt expression).

Tumor Implantation

Tumor implantation was carried out by the method of Nobuhisa *et al.* (1993). Rats were anesthetized by injection of xylazine (10 mg/kg, IM) and ketamine (100 mg/kg, IP). The animals were placed in a stereotactic surgical frame (Instrument & Chemicals Pvt Ltd, Ambala city, India). A small burrhole was drilled into the right side at a location defined by the following stereotactic

coordinates: - 0.8 mm to the bregma; 4 mm medial to lateral; 5mm dorso ventral from the skull surface for the injection of C6 glioma cell suspension with a Hamilton microsyringe. The craniotomy was sealed with bone wax and overlying skin incision was closed.

Biochemical analyses

At the end of the experimental period, the animals were subjected to cervical decapitation, 100 mg of the whole brain tissue was weighed, uniformly homogenized with 1.0 ml of 0.5 M phosphate buffer, pH 6.9 and the homogenate was used for the following biochemical assays. Protein content was estimated by the method of Lowry *et al.* (1951)

Assays of marker enzymes

The marker enzyme analyses were done from the brain tissues of glioma induced, drug treated and control rats. The activity of LDH in brain tissue was estimated using the method of King *et al.* (1965) CK by the method of Okinaka *et al.* (1961) 5' nucleotidase by Fini *et al.* (1992) G6PD by the method of Baquer and Mc Lean (1972) and HK was assayed by the method of Brandstrup *et al.* (1957).

Estimation of lipid profile

Total lipid was extracted from the brain sample according to the method of Folch *et al.* (1957). Tissue cholesterol content was estimated by the method of Parekh and Jung *et al.* (1970). Free cholesterol was estimated by the method of Leffler and Mc Dougald *et al.* (1963). Phospholipids were estimated by the method of Rouser *et al.* (1970) after digesting the lipid extract with perchloric acid. Triglycerides were estimated by the method of Rice *et al.* (1970). Free fatty acid content was estimated by the method of Horn and Menahan *et al.* (1981).

Immunofluorescence study for GFAP

Immunofluorescence was performed on the portion of brain specimen fixed in 10% buffered formalin and embedded in paraffin wax. Sections were cut at 5 μm in thickness. The tissue sections were deparraffinized in two changes

of xylene at 60ºC for 20 min each and hydrated through a graded series of alcohol, the slides were incubated in a citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven for antigen retrieval. The sections were then allowed to cool to room temperature and then rinsed with TBS and treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. Non-specific binding was blocked with 3% BSA at room temperature for 1 h. The sections were then incubated with diluted primary antibodies GFAP (1:100) at 4ºC overnight. The slides were washed with TBS and then incubated with anti-mouse FITC-labeled secondary antibody at a dilution of 1:500 for 30 min in room temperature. Then they were washed thrice with TBS and mounted in glycerol: PBS mix (1:9). Then slides were viewed under fluorescence microscope using wavelength of 488 nm.

Immunohistochemistry of COX-2

Immunohistochemistry was performed on the portion of brain specimen fixed in 10% buffered formalin and embedded in paraffin wax. Sections were cut at 5 µm in thickness and carried out following the method of Zhang *et al.* (1994)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The mRNA expression of COX-2, PTEN, Akt and PI3K were analysed using RT -PCR. Total RNA extraction from brain tissue was performed using TRIZOL reagent which is based on the acidic phenol-chloroform method and about 5µg of total RNA was taken for the synthesis of cDNA. The reaction mixture for the synthesis of cDNA contains 5 µg of total RNA, 1 µl of random hexamer (0.2 mg/µl) and 12.5 µl of nuclease free water. Then the contents were mixed for 3-5 seconds and spun at 70ºC for one minute and snap frozen on ice. To the mixture, 4.0 µl 5X reaction buffer-Reverse transcriptase, 0.5 µl Ribonuclease inhibitor (20units/µl), 6.0 µl DEPC treated water and 4.0 µl $MgCl_2$ (25mM) were added. Then the reaction mixture was kept at 25ºC for 5 min. To the reaction mixture, 5 µl of Reverse transcriptase (40 units/ µl) was added and maintained at 25 ºC for 10 min and suddenly transferred to 42 ºC for 60 min and the reaction was stopped by heating the mixture to 70ºC for 30 min and snap frozen. The resulting mixture contains

the cDNA product which was used for PCR analysis. The reaction mixture consist of Millipore water - 4.95 µl, 10X PCR -1.0 µl 10mM, dNTPs -0.2 µl, Primer forward-1.0 µl (5 pmoles/ µl), Primer Reverse-1.0 µl (5 pmoles/ µl), Taq polymerase-0.05 µl, Template-1.0 µl. PCR was performed using the following profile: Initial danaturation temperature - 95°C -5 min, Cyclic denaturation temperature - 95°C -30 sec, Annealing temperature - varies for different primers- 45 sec, Extension temperature -72°C -30 sec, Stage 2 was repeated for 29 cycles and Final extension temperature -72°C-3min. PCR product analyses were done using the image quant densitometer (Bio RAD Co).

Table 1. Nucleotide sequences of sense and antisense primers designed for RT-PCR

Primer	Sequence	Size (bp)	Source	Tm (°C)
COX -2	5?-TGGTGCCGGGTCTGATGATG-3' 5?-GCAATGCGGTTCTGATACTG-3'	253	Gustafson-Svärd et al., 1996	59
PI3K	5?- CACCTGGACTTGTGGAACCT -3' 5?- GAATCAGAATCCTCCGGACA-3'	233	Primer-3 software	60
Akt	5?-TGGTTCGAGAGAAGGCAAGT-3' 5?-AAAAACAGCTCTCCCCCATT-3'	213	Primer-3 software	59
PTEN	5?-ACACCGCCAAATTTAACTGC-3' 5?-TACACCAGTCCGTCCTTTCC-3'	169	Primer-3 software	57
?-Actin	5?-ACCACAGCTGAGAGGGAAATCG-3' 5?-AGAGGTCTTTACGGATGTCAACG-3'	276	Gong et al., 2002	60

Western blot analysis of pAkt, PI3K, PTEN and COX-2

COX-2, PTEN, pAkt and PI3K protein expressions were analyzed using a protocol, adapted from Fiddo *et al.* (1995). 50 g of total protein containing tissue homogenate was mixed with 2x sample buffer and boiled for 5 min. The sample mixture was run on 12% SDS-PAGE gels in 1x running gel buffer at 100 V for 2.5 h and electro transferred to a PVDF membrane at 30 V for 1 h 30 min. The membrane was blocked in blocking buffer containing 5% BSA for overnight. After overnight, the blocked membranes were incubated with primary antibodies diluted in TBS-T: pAkt (1:1000), PI3K (1:1000),

COX-2 (1:2000), PTEN (1: 1000) and β-actin (1:5000) overnight at 4ºC, with gentle shaking. The membranes were washed with TBS-T gently and subsequently incubated with appropriate secondary antibodies (anti-rabbit or anti-goat IgG) linked to HRP at a 1:4000 dilution for 1 h. β-actin served as internal control to check for equal loading of protein. The bands were visualized using luminol reagent and intensity of each band was determined using an image analyzer (quantity none software from Bio Rad).

Estimation of PGE2

The tissue PGE2 was extracted by the method of Bligh and Dyer, (1959) with the final proportions of 0.13 g of tissue, 1.6 ml of H2O, 2 ml of methanol, and 2 ml of CHCl3. After removal of the first CHCL3 extract, the H2O. methanol phase and solid interface were acidified with HCl (final concentration 0.01 M) and re-extracted with 2 ml of CHCl3. An additional CHCl3 extraction of the acidified H2O.methanol phase was performed to maximize the recovery of PGs. The combined CHCl3 extracts which contained butylated hydroxytoluene were back-extracted with H2O (1 volume of H2O/3 volumes of CHCl3) until the pH of the H2O was neutral. The CHCl3 extract was concentrated under a stream of N2 and aliquots were taken for HPLC. The recovery of standard PGE2 was 85%. The HPLC system was normal phase using a 25 cm x 4.6 mm zorbax C18 column with 5-µm particles. The solvent system was 0.1% TFA in water and acetonitrile (v/v/v) at a flow rate of 0.7 ml/min.

Measurement of PIP3 (PI3K activity assay)

Brain tissues were homogenized in the presence of protease inhibitors. Protein concentrations were determined. The amount of PIP3 produced was quantified by PIP3 competition enzyme immunoassays according to the manufacturer's protocol (Ecelon, UT). The enzyme activity was expressed as amounts of PIP3 (picomoles per milliliter).

Statistical analysis

All the grouped data were significantly evaluated with SPSS/15 software. Hypothesis testing methods included one-way analysis of variance (ANOVA)

followed by least significant difference (LSD) test. P values of < 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± SD for six animals in each group.

5. Experimental Findings

Magnetic resonance imaging

All animals which were injected with C6 cells were investigated on MRI after 30 days of injections. There were visible tumors in the animals scanned on MRI. The rim of the tumors could be seen images with contrast; the tumors were easily visualized as high signal intensity areas at the site of injection (Fig. 1). Necrosis was not observed, and no cystic parts could be detected within the tumors.

Histopathology

Histopathological studies showed promising outcome as observed in the panel of figures below. Fig. 2a Brain histology showing normal architecture of control rats (10X). Fig. 2b and c (10X) Histology of brain from group II. The tumor has uniform, dense cellularity and is well vascularized with fibrillary background. The tumor-brain boundary is distinct, with local infiltration of tumor cells. Fig. 2e represents histology of brain of group III rat. The architecture that resembles normal one with occasional tumor infiltration, extensive necrosis and cellular regions was encountered, while Fig. 2d represents normal histology (10X) of brain from the drug control group.

Transmission Electron Microscopy

To identify more clearly the types of cells undergoing apoptosis and to better visualize vascular changes during tumor formation and treatment, ultrastructural analysis was performed by TEM. Figure 3a (7,000 X) represents the electron microscopic appearance of normal rat brain. Fig. 3b (15,000 X) represents the electron microscopic pattern of brain implanted with tumor. Neoplastic cells have multiform nuclei, well developed golgi apparatus, enlarged mitochondrias and number of polysomes.

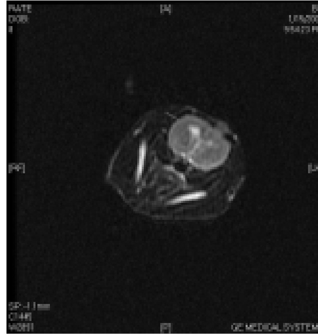


Fig. 1 MR imaging of glioma induced rat brain (30th DPI)

Arrow indicate the run of the tumor, the tumor was easily visualized as high signal intensity area at the side of injection

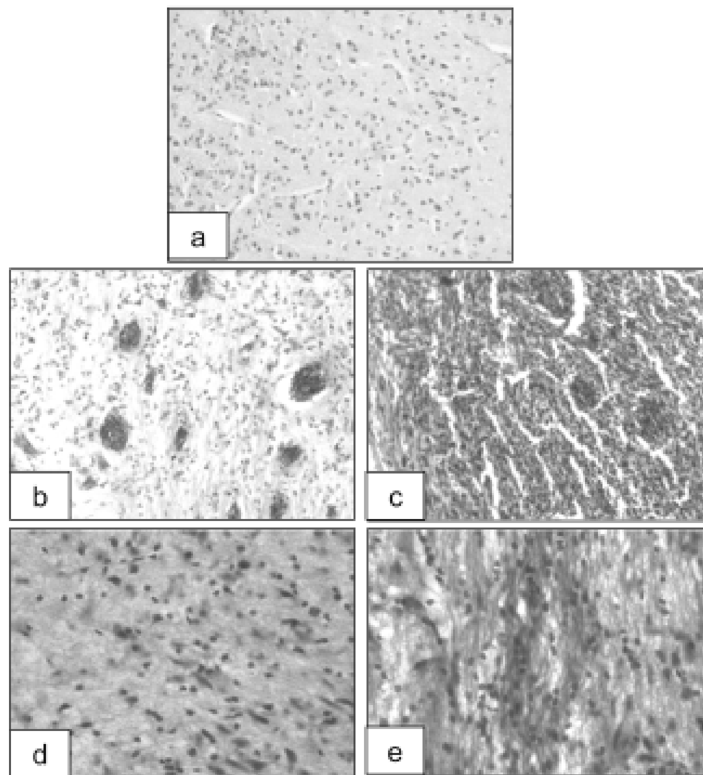


Fig. 2 Histological pattern of brain tissue sections of control and experimental groups of rats.

a) Brain from control animals showing normal architecture (10 x) . b) C6 glioma cells implanted brain showing hyperchromatictumor area and tumor infiltration (10 x). c) Tumor area of brain from the glial tumor bearing animals with fibrillary background, characteristic of fibrillaryastrocytoma (40 x). d) Histological image of brain from drug control animals showing normal architecture as that of control animals (10 x). e) Brain histology of Noni treated, tumor bearing animals depicting an architecture with lesser intensityof tumor histology (10 x) with acellularregion.

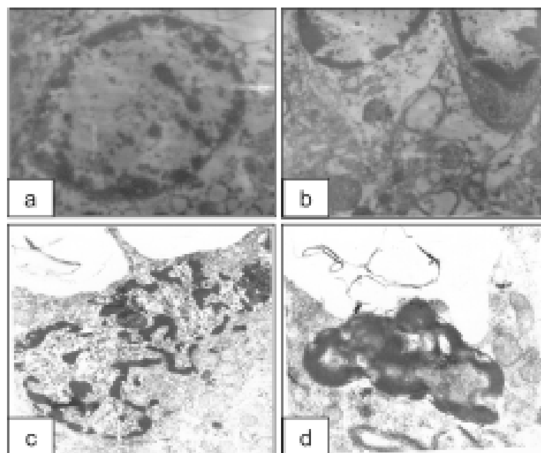


Fig. 3. Transmission electron microscopic appearance of the brain tissues of control and experimental groups of rats

Figure a (7000 X), represents the electron microscopic pattern of normal brain. Figure b (15,000 X) represents the electron microscopic pattern of brain implanted with tumor. Neoplasticcells have multiform nuclei, well developed golgiapparatus, enlarged mitochondria and number of polysomes Figure c and d (10,000 X) show the transmission electron microscopic pattern of rat brain that underwent drug treatment after tumor induction. The figure represents an occasional pericytewith features of apoptosis. These cells demonstrated shrinkage, increased cytosolicdensities, disintegration of nuclear membrane and condensed nuclear chromatin.

Figure 3C (10,000 X) and 3d (30,000 X) show the transmission electron microscopic pattern of rat brain that underwent drug treatment after tumor induction. The figures represent occasional pericytes with features of apoptosis. These cells demonstrated shrinkage, increased cytosolic densities, vacuolization, and condensed nuclear chromatin. The cytoplasm of apoptotic cells also contained small ultra condensed mitochondria and small vesicles.

Biochemical assessments

The results (Table 1) showed the effect of drug on the levels of total protein in the brain samples of control and experimental animals. There was a significant ($p<0.05$) increase in the level of the protein in the brain tissue of the glioma induced Group II animals when compared with control and treated animals. On administration of drug, there was significant ($p<0.05$) decrease in the level of total protein in the drug treated group (Group III) when compared with brain tissues of the glioma induced group II animals. There was no significant difference between control and drug control groups.

Table 2 showed the effect of drugs on the activities of GST, 5'ND and CK in the brain samples of control and experimental animals. There was significant ($p<0.05$) increase in the activities of these enzymes in the brain tissue of

the glioma induced Group II animals when compared with control and treated animals. On administration of drug, a significant ($p<0.05$) decrease in the activities of these enzymes in the drug treated group (Group III) when compared to brain tissue of the glioma induced group II animals was encountered. There was no significant difference between control and drug control groups.

The expression of GST isoenzymes is variable in various pathological conditions, including neoplasia. The GST- pi expression was reported to be increased in glioma; change in GST isoenzymes expression can play an important role in the susceptibility of CNS to carcinogenesis (Usarek *et al.,* 2005); malignant glioma such as astrocytoma and glioblastoma has strong expression of the same. Hara *et al.* (1981) showed the existence of strong expression of GST in both astrocytoma and GBM. Similarly, CK activity was also seen in all the tissues but mostly in the brain with increased activity in many cancer conditions (Julie and Abbal, 1998); it has an active role in the ATP regeneration in brain.

The 5' ND is an endonuclease which is expressed on normal and neoplastic glial plasma membranes (Ludwig *et al.,* 1999). The inhibition of 5' ND might result in a decrease in extracellular adenosine production with a consequent reduction in tumour progression (Wink *et al.,* 2003). In the present study, the activities of these enzymes which are related to inactivation of drug and chemo resistance were found to be comparatively higher in group II. The reverse picture was observed in drug treated groups. The observed reduction in the activities of these enzymes in the Noni treated groups might be interpreted as the influence of noni on intensity of tumor burden. This effect may also help in suppression (partially) of chemo resistance, the common problem in the treatment of glioma.

The results presented in Table 3 highlight the effect of drug on the activities of enzymatic antioxidants SOD and catalase in the brain of control and experimental groups. There was significant ($p<0.05$) increase in the activities of these enzymes in the brain tissue of the glioma induced Group II animals when compared with those of control and treated animals. On administration of drug, a significant ($p<0.05$) decrease in the

Table 1 : The level of protein in the brain of control and experimental groups

Particulars	Group I	Group II	Group III	Group IV
Protein	22.32±1.46	67.53±12.12*	35.54±1.15*	23.93±1.78NS

The values are expressed as mean ± SD (n=6). Protein mg/g of tissue,Statistical significance represented as *p<0.05. Group II (glioma bearing rats) vs Group I.Group III Vs Group II .NS - Not Significant. Group IV vs Group I.

Table 2 : The activities of markers like GST, 5' ND, CK in the brain of control and experimental groups

Particulars	Group I	Group II	Group III	Group IV
GST	17.44±1.53	35.59±1.43*	25.51±1.52*	17.04±1.73NS
5' ND	4.63±0.84	15.85±1.05*	9.66±0.75*	4.73 ±0.72NS
CK	152.21±12.40	213.54±31.25*	175.36±11.42*	156.67±18.25NS

The values are expressed as mean ± SD(n=6).GST- units/mg protein, 5'ND AND CK- nmoles of pi liberated / min/mg protein.Statistical significance represented as *p<0.05.Group II (glioma bearing rats) vs Group I,Group III vs Group II NS - Not Significant. Group IV vs Group I.

Table 3 : The activities of enzymatic antioxidants like SOD and Catalase in the brain of control and experimental groups

Particulars	Group I	Group II	Group III	Group IV
SOD	11.78±0.85	20.45±1.20*	16.73±0.88*	11.99±1.20NS
Catalase	5.44±0.74	14.42±1.04*	10.23±1.43*	5.54±1.08NS

The values are expressed as mean ± SD(n=6).SOD - units/min/mg protein, Catalase-µmoles of H2O2 formed/min/mg protein.Statistical significance represented as *p<0.05. Group II (glioma bearing rats) vs Group I, Group III vs Group II, NS - Not Significant. Group IV vs Group I.

activities of these enzymes in the drug treated group (Group III) when compared to brain tissue of the glioma induced Group II animals was observed suggesting the action of components of Noni on tumor cell redox status. There was no significant difference between control and drug control groups.

The levels of antioxidants were reported to be increased in glioma (Pameeka *et al.,* 2007). The antioxidant enzymes are also known to be factors involved in radio resistance and chemo resistance (Grant and Ironside, 1995). Increased SOD activity in the meningioma and glial tumors has been reported (Tuna *et al.,* 2002). High expression of Cu / Zn SOD caused the suppression of both types of fas induced cell death (Jayanthi *et al.,* 1999). The activity of catalase was also found to be increased in the glioma (Popov *et al.,* 2003). In this study, the activity of these enzymes was found to be decreased in drug treated group which might be due to the action of the active components of Noni.

The results presented in Table 4 showed the effect of Noni on the levels of cholesterol, free cholesterol, ester cholesterol, triglycerides, free fatty acid and phospholipids in the brain tissue of control and experimental groups. The levels of these components increased significantly ($p<0.05$) in the brain tissue of the glioma induced Group II animals when compared to control and noni treated animal.

The increased level of total cholesterol in the induced group showed that the process of carcinogenesis is associated with alteration in the lipid metabolism affecting cellular function and growth. Altered lipid levels between primary tumors and metastasis were reported to be varying significantly (Chuanting Li, 2007). Increase in cholesterol was reported in different grades of glioma correlates well with histological vascular proliferation (Tosi *et al.,* 2003). Changes in lipid composition may play a role in structural and functional membrane alteration in neoplastic cells (Ledwozyw and Lunicki, 1992). The data obtained in the current study suggested that the modifications in the levels of lipid components which are said to be gradually, accompanied by a progressive increase in the malignancy of the tumor (Tosi *et al.,* 2003) are also responsible for functional variation connected with neoplastic growth.

The lipid components were maintained to the levels very much comparable to that of normal on administration with Noni in group III rats. This restoration of lipid levels could be due to strong hypolipidemic activity of the Noni. This suggests that noni might exert effect on neoplastic induced perturbation of cell lipids.

The results presented in Table 5 showed the effect of drug on the levels of hexose, hexosamine, sialic acid and fucose in the brain of control and experimental groups. There was a significant ($p<0.05$) increase in the levels of these PBC in the brain tissue of the glioma induced Group II animals when compared with that of control and treated animals. On administration of drug, a significant decrease ($p<0.05$) in the levels of these PBC in the drug treated group (Group III) was observed when compared to that of glioma induced Group II animals. There was no significant difference between control and drug control groups.

The levels of PBC were altered in tumor condition. The abnormal level of these PBC is the major indicator of pathogenesis. The increased levels are associated with advanced stages of cancer (Baxi, 1991). In this study, the elevated levels of PBC were found to be significantly decreased in noni treated groups, suggesting the intervening role of noni on this oncological feature.

Table 4 : The levels of cholesterol, free cholesterol, ester cholesterol, triglycerides, free fatty acid and phospholipids in the brain of control and experimental groups

Particulars	Group I	Group II	Group III	Group IV
Cholesterol	25.31±1.89	32.27±1.40*	22.04±1.25*	25.48±1.96NS
Free cholesterol,	20.73±1.27	16.43±1.06*	18.35±1.03*	20.94±1.83NS
Ester cholesterol	4.58±0.62	15.84±0.34*	3.69±0.22*	5.21±0.21NS
Triglycerides	84.28±0.87	122.45±1.60*	92.70±1.54*	79.84±5.13NS
Free fatty acid	33.73±1.17	62.82±1.81*	42.83±1.17*	34.52±1.31NS
Phospholipids	77.66±1.46	98.03±1.54*	85.71±2.30*	74.61±1.97NS

The values are expressed as mean ± SD(n=6).Lipid profile mg/g of tissue. Statistical significance represented as *p<0.05.Group II (glioma bearing

rats) vs Group I, Group III vs Group II, NS - Not Significant. Group IV vs Group I.

Table 5 : The levels of Hexose, Hexosamine, Sialic acid and Fucose in the brain of control and experimental groups

Particulars	Group I	Group II	Group III	Group IV
Hexose	153.60±1.80	214.32±1.16*	174.45±1.86*	154.82±1.85NS
Hexosamine	143.93±2.08	196.68±3.29*	161.22±12.8*	145.97±1.52NS
Sialic acid	214.54±1.32	253.67±2.13*	242.00±1.20*	213.24±2,41NS
Fucose	97.47±1.67	155.65±1.30*	132.86±2.17*	114.02±1.43NS

The values are expressed as mean ± SD(n=6).PBC mg/g of tissue. Statistical significance represented as *p<0.05.Group II (glioma bearing rats) vs Group I,Group III vs Group II. NS - Not Significant. Group IV vs Group I.

Immunofluorescence of GFAP

Glial fibrillary acidic protein (GFAP) is a member of the class III intermediate filament protein family. It is heavily and specifically expressed in astrocytes and certain other astroglia in the central nervous system, in satellite cells in peripheral ganglia and in non myelinating Schwann cells in peripheral nerves. Antibodies to GFAP are therefore very useful as markers of astrocytic cells. In addition, many types of brain tumor, presumably, derived from astrocytic cells, heavily express GFAP.

GFAP were present in hypertrophic reactive astrocytes, which were numerous in early neoplastic proliferations, in microtumors of the white matter, and in those collected at the periphery of large tumors (Mauro *et al.*, 1983). In another investigation of GFAP in brain tumours, varying amounts of fibrillary acidic protein were seen in every glioma. In ependymal and oligodendroglial tumours, a high number of positive neoplastic elements were reported (Gullotta *et al.*, 1985), GFAP positive were also the peri-vascular cells of a so-called astroblastoma. In pilocytic astrocytomas, Rosenthal fibers were in part GFAP positive and in part negative. Intraleptomeningeal growing tumour cells presented usually a very strong positivity. In recurring

oligodendrogliomas, the number of GFAP positive tumour cells was the same in the primary tumour and in its recurrence (Gullotta *et al*., 1985).

Immunofluorescent pattern of GFAP of the experimental groups was presented below. Fig 4a and c represent that of the control and drug control (*Morinda citrifolia* alone) groups showing very low level of expression as evident from the less intensity fluorescence in rat brain cells.

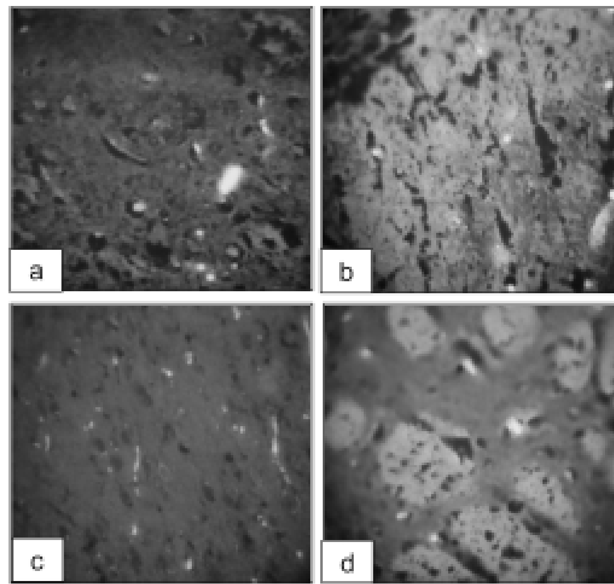


Fig. 4. Immuno fluorescence of GFAP in brain tissues of control and experimental group of rats

a: Control (Group 1); b: Glioma induced (Group 2); c: Drug control (Group 3);
d: Tumor induced + Noni treated (Group 4). a and c present similar fluorescent pattern.
Tumor induced intense fluorescence pattern appearing in b was found to be reduced in d.

GFAP positive was detected in tumor bearing rats with high intensity of expression. (Fig.4b). In contrast, group IV showed GFAP positive with low intensity of expression as represented by a comparative lesser intensity of fluorescence (Fig 4d). ($p < 0.05$)

Figure 5 and Figure 6 represent mRNA and protein expression pattern of the proposed enzymes with the internal controls.

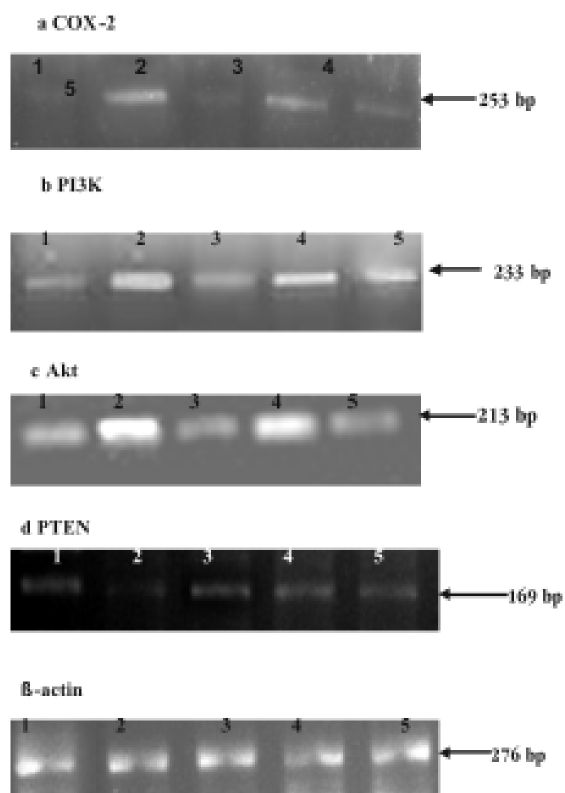


Fig. 5. Representing mRNA Expression of cox-2, P13K, Akland PTEN in brain tissues from teh control and experimental rats.

Beta actinserved as internal control. Lane 1: Marker Lane 2: Control (Group 1); Lane 3: Glioma induced (Group 2); Lane 4: Drug control (Group 3); Lane 5: Tumor induced + Nonitreated (Group 4). Lane 6: Tumor induced +reference drug (Group 5). Values are expressed as mean ±S.D. ($n = 6$). '*'symbol represents statistical significance at $p < 0.05$. Comparisons are made as (a) Group 1 *vs.*Group 2, (b) Group 2 *vs.*Group 4, (c) Group 1 *vs.* Group 4, (d) Group 4 *vs.* Group 5 and †-non significant Group 1 *vs.* Group 3.

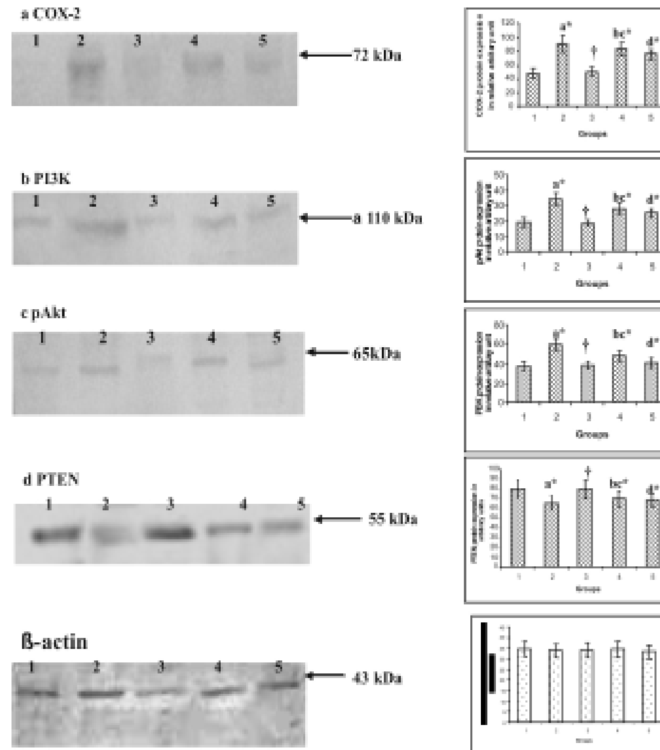


Fig. 6 : Representing protein expression of COX - 2, P13K, pAKland PTEN respectively, in brain tissues from the control and experimental rats.

Beta actinserved as internal control. Lane 1: control (Group 1); Lane 2: glioma induced (Group 2); Lane 3: drug control (Group 3); Lane 4: Tumor induced + Nonitreated (Group 4), Lane 6: Tumor induced +reference drug (Group 5).Values are expressed as mean ±S.D. (n = 6). '*'symbol represents statistical significance at $p < 0.05$. Comparisons are made as (a) Group 1 *vs.*Group 2, (b) Group 2 *vs.*Group 4, (c) Group 1 *vs.* Group 4, (d) Group 4 *vs.*Group 5 and †-non significant Group 1 *vs.* Group3.

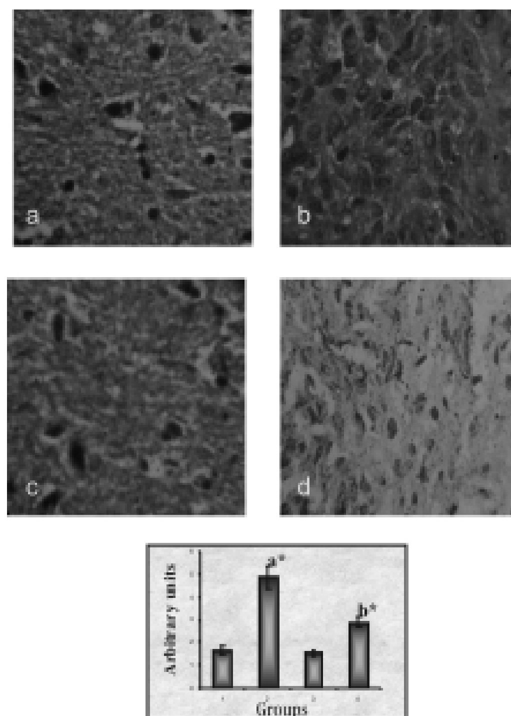


Fig. 7 : Immuno histostaining pattern of COX-2 in the brain tissues of control and experimental animals.

a: Control (Group 1); b: Glioma induced (Group 2); c: Drug control (Group 3); d: Tumor induced + NONI extract treated (Group 4) e: Densitometric pattern of the expression of Cox-2. Values are expressed as mean ±S.D. ($n = 6$). '*' symbol represents statistical significance at $p < 0.05$. Comparisons are made as (a) Group 1 (control) *vs.*Group 2 (gliomainduced) and (b) Group 2 (gliomainduced) *vs.*Group 4 (Noniextract treated) (100 X).

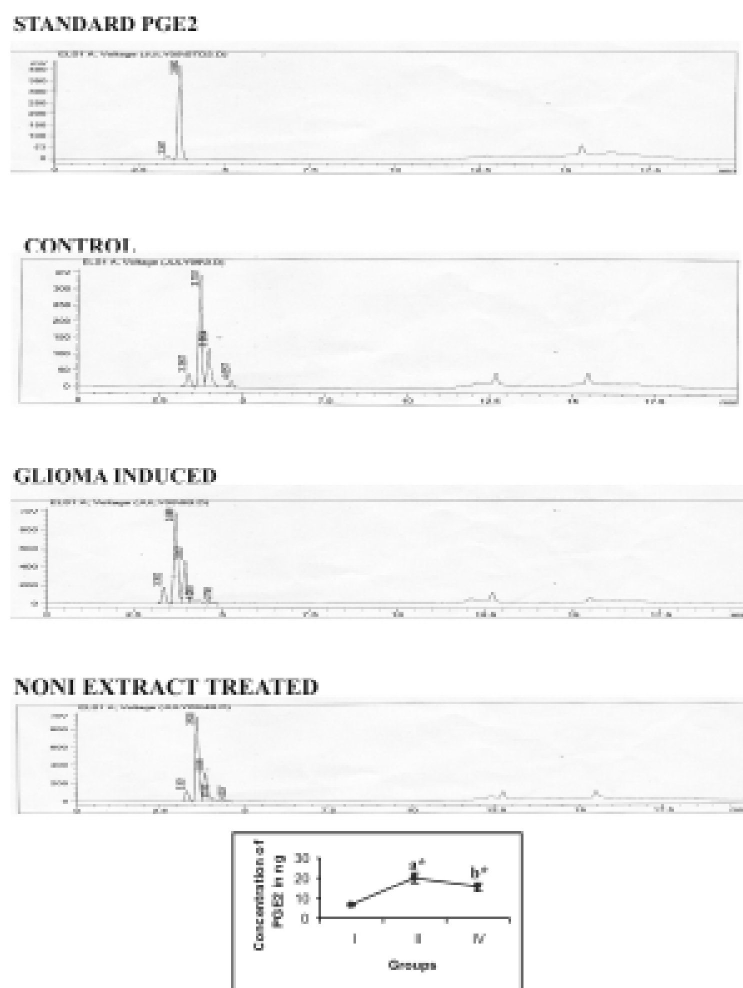


Fig. 8 : Effect of Nonion PGE2 in brain tissues of the control and experimental groups

a: Standard PGE_2. b: Control (Group 1), c: Glioma induced (Group 2); d: Tumor induced + Noni treated (Group 4). Area under the peaks (PGE_2) was determined to represents the concentration of PGE_2.
* symbol represents statistical significance at $p < 0.05$. Comparisons are made as (a) Group 1 *vs.* Group 2 and (b) Group 2 *vs.* Group 4.

Expression of COX-2

RT- PCR analysis and Western blotting were performed to determine the expression of COX-2 at the level of mRNA and at the level of protein in control and experimental groups. COX-2 mRNA and protein exhibited low level expression in Noni fruit extract treated group when compared to that of glioma induced group and was comparable to and close to the expression in celecoxib (COX-2 inhibitor, reference drug) treated groups (Figure 5a and 6a).

Immunohistostaining pattern of COX-2 in the brains of control and experimental animals is presented in Fig.7. Glioma induced group II showed significantly higher ($p < 0.05$) intensity of immunostaining in the extended vascular network when compared with brain of group I control animals. Noni treatment significantly decreased ($p < 0.05$) the COX-2 positive cells when compared with that of C6 cells implanted rats (group II).

COX-2 Activity (PGE2 levels)

The HPLC analysis of PGE2 reveals that the production was increased in glioma induced group when compared to that of control rats ($p < 0.05$). The administration of Noni fruit extract to glioma induced group markedly reduced ($p<0.05$) the levels of PGE2 (Figure 8).

Expressions of PI3K and Akt

RT- PCR and Western blotting analyses were performed to determine the expression of PI3K and Akt at the level of mRNA (Fig 5) and at the level of protein (Fig 6) in control and experimental groups. Both of PI3K and Akt mRNAs and proteins showed significantly high level of expression in glioma induced rats (group II) when compared to those of control rats. In Noni fruit extract treated group, there was a significantly ($p < 0.05$) low level of expression PI3K (Fig. 5b and 6b) and Akt (Fig. 5c and 6c) both at the level of mRNA and protein when compared to that of glioma induced group and was comparable to and close to that of wortmanin (a PI3K inhibitor, reference drug) treated groups.

Figure 9 showed the expression of PTEN (mRNA and protein) in brain tissues of control and experimental groups. The intensity of expression of PTEN mRNA and protein was comparatively low in C6 glioma cells implanted rat brain when compared to that of control (group I).

PI3K activity

Figure 10 depicts the effect of Noni on the activity of PI3Ks as a measure of PIP3 in control and experimental rats. The increased activity of PI3Ks as

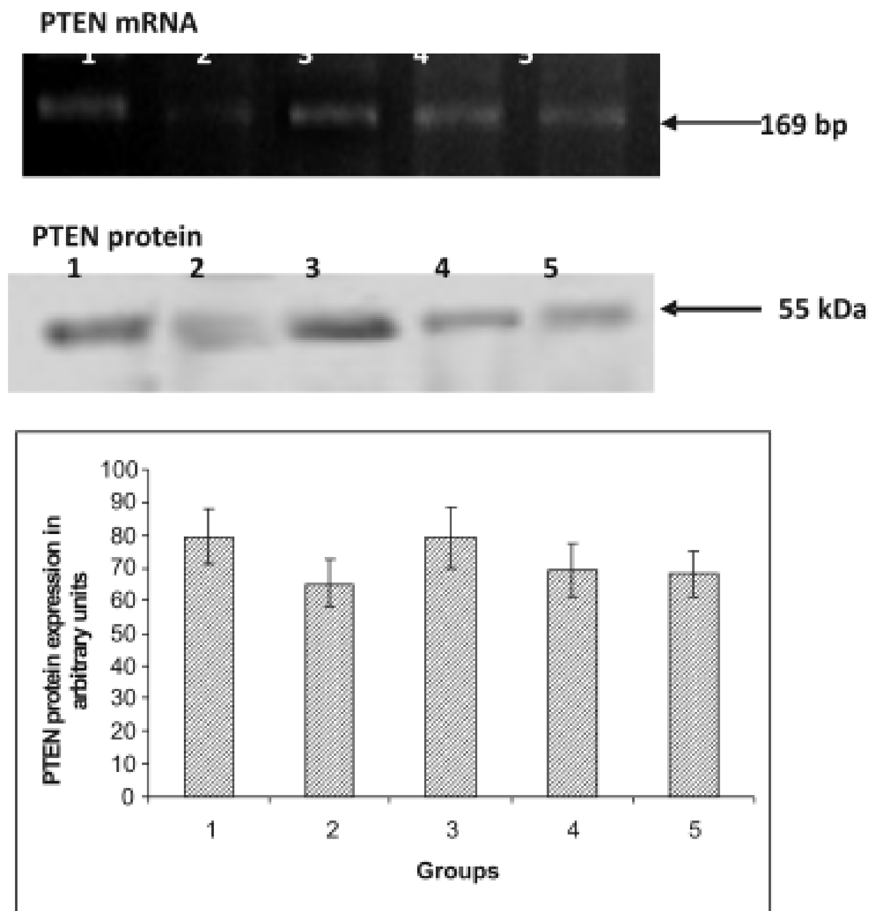


Fig. 9 : Expression of PTEN. Lane 1= Control, Lane 2= Induced, Lane 3= Noni extract control, Lane 4= Noni extract treated, Lane 5= Ref drug treated; Values are expressed as mean±S.D. ($n = 6$). '*' symbol represents statistical significance at $p < 0.05$. Comparisons are made as (a) group I (control) vs group II (glioma induced) and (b) group II (glioma induced) vs group IV (C6 implanted + Noni treated).

a measure of PIP3 in glioma induced rat (Group II) was significantly decreased upon Noni treatment (Group IV). The reduced activity of PI3K, thus prompting to interpret the results as negative influence of noni on this protoncogene and hence on the related malignant features of the cell which is well supported by histological and TEM results in the current study.

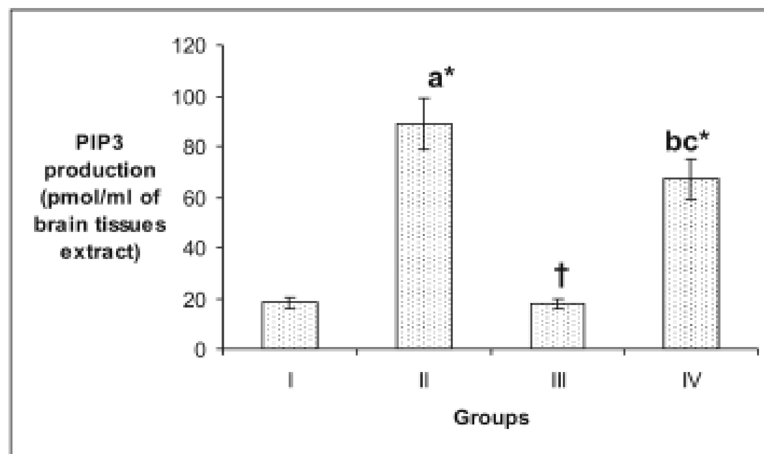


Fig. 10 : Effect of Noni on the activity of P13K as a measure of PIP3 in control and experimental rats.
Results are expressed as mean ± standard deviation ($n = 6$). * Represents statistical significance of the data at $p < 0.05$; Comparisons are made as (a) Group I *vs.* Group II, (b) Group II *vs.* Group IV, (c) Group I *vs.* Group IV and †- non significant Group 1 *vs.* Group 3.

Studies by Badie *et al.* (2003) suggest that intracranial tumours express more COX-2 than models of subcutaneous C6 glioma which is consistant with current study showing an over expression of COX-2 (mRNA and enzyme) as well as high level of PGE2 production in glioma induced group. This elevation can influence the mechanisms involved in carcinogenesis, like angiogenesis, inhibition of apoptosis, stimulation of cell growth as well as the invasiveness of C6 glioma cells (Lupulescu, 1996).The elevated cox2 activity and PGE2 levels (in group II) were found challenged by Noni treatment in the study. This effect of Noni was comparable and close to that of celecoxib induced COX-2 inhibition. Thus, COX-2, an important molecular target for anticancer therapies (Pamela, 2004) and an enzyme which is involved in mitogenesis, cellular adhesion, and invasion by its metabolite (Kurzel *et al.,* 2002; Hwang *et al*., 2004) such as PGE2 in human cancer cells, was targeted by Noni in

the current study. Further, the observed marked reduction in the production of PGE2 which also plays a major role in the cytoprotection (Smith, 1989), suggests a challenge by noni against the invasion of C6 glioma cells in rat brain.

The activation of PI3K/Akt pathway which has a key role in regulating cell cycle processes (Thaler *et al.,* 2009; Nogueira *et al.,* 2009), angiogenesis (Dimmeler and Zeiher, 2000), anti-apoptotic functions (Nicholson and Anderson, 2002) has been reported. It has also been reported that increased PI3K activity due to gene amplification and mutation of catalytic and regulatory subunits of PI3K is common in high grade glioma, (Mizoguchi *et al.,* 2004) and that inhibition of PI3K is cytotoxic in several human glioma cell lines *in vitro* (Guillard *et al.,* 2009). In this study, glioma implanted rat brain registered an increased expression of PI3K/Akt in group II rats leading to a positive influence on the cell survival mechanism. On the observation of the PI3K/Akt expression in group IV rats, it is prompting to interpret the results as a potential of noni to act against the survival of tumor cells.

Earlier in a study, Noni extract was reported as dual inhibitors of both COX-2 and 5-LOX enzymes which are potential candidates for neuroprotection, anticancer and anti-inflammation. (Su *et al.* 2001). pAkt is said to increase the expression of COX-2 (St-Germain *et al.*, 2004). As Akt has been reported to the influence COX-2 expression, the observed reduced expression of pAkt in group IV (noni treated rats) is speculated to be involved in COX-2 regulation in glioma.

6. Summary and Conclusion

The prevailing shortcomings of conventional therapies against glioma, an aggressive brain tumour prompted the present investigation to screen *M. citrifolia*, for its anti-glial tumour activity anticipating its constructive role in intervention along with the existing therapy. The investigations carried out exhibited the following observations :

- The MRI and histopathological experiments confirm the implantation of c6 cell in the rat and thus the development of glioma model.

- Glioma induced rats showed significant variations in the activities of pathophysiological enzymes and antioxidant enzymes, whereas treatment with Noni extract restored their activities almost close towards that of normal animals.

- Histopathological features of glial tumour rat brain were relatively attenuated in drug treated rats reflecting the potential of methanolic extract of *M. citrifolia* against glioma induced tissue variations.

- Transmission electron microscopic study presents potential of methanolic extract of *M. citrifolia* as depicted by the signs of apoptosis in otherwise programmed cell death escaping tumor cells.

- Results reveal the modulating effect of methanolic extract of *M. citrifolia* on the activity of PI3K (proto-oncogene).

- Results reflected the potential of methanolic extract of *M. citrifolia* against glioma with respect to the expression of COX-2, PI3K and Akt (both at the level of mRNA and protein) as well as the product of COX-2, namely PGE2, a major prostaglandin essential for progression of tumor.

Conclusion

The block of PI3K/Akt as observed from the relatively reduced expressions of PI3K and Akt on *Morinda citrifolia* (Noni) administered glioma model and perhaps the Akt regulated expression of COX-2, which also registered a decreased expression in the study were putatively seem to be involved in mediating the tumor attenuating effect of Noni, as supported by biochemical, microscopical observations, on C6 glioma cells induced brain changes in rats.

The current investigations, for the first time, reveal the potential of *M. citrifolia* (Noni) in minimizing the glial tumor related changes in rat brain, perhaps by its influence on the survival of cells of aggressive glioma.

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