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Mode of p38 and Nf-kβ Signalling Pathway on Induction of Apoptosis in Response to Flavonoids of Aloe Vera in Glioblastoma

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***Abstract*:** The phytochemical, Flavonoid has been shown apoptosis by numerous studies of cancer cell lines. Here, we have investigated the role of flavonoids of *Aloe vera* in regulation of apoptosis through p38 and Nf-kβ gene against human glioma-U87MG cells *in vitro*. The potential invasion of flavonoid on human glioma-U-87MG cells was examined by MTT assay and DNA fragmentation assay and expression of p38 and Nf-kβ gene was analysed by RT-PCR. Flavonoids significantly suppressed the apoptosis with IC_{50} 113.29±0.5µg/ml and confirmed by DNA fragmentation assay. The results suggest that flavonoids induce apoptosis with decreased expression of p38 and Nf-kβ gene and protein analysis with SDS-PAGE. Assessments of cell viability represent that U-87MG cells were very sensitive to flavonoids and indicating DNA and protein synthesis delayed. In conclusion, flavonoids of *Aloe* showed inhibitory effect on Glioblastoma a brain tumor cells by apoptosis through regulation of p38 and Nf-kβ pathway at transcription and post –transcription level.

***Keywords* -** Glioblastoma, *Aloe vera*, Flavonoids, p38, Nf-kβ, U-87MG cell lines, RT-PCR, SDS-PAGE.

I. INTRODUCTION

Brain tumor (GBM-Glioblastoma) is one of most serious malignant tumor of central nervous system **[1,2]** and characterised by their aggressiveness, angiogenesis, deregulation of apoptosis, proliferation and difficult to treat with conventional therapies such as surgery, radiotherapy and chemotherapy. Patients suffering with GBM have survival rate of one year **[3-7].** So, it's too difficult to treat GBM and therefore need to develop a novel therapy for prevent and treat Glioblastoma. Due to less issue, Plants and their extract's components could be alternative prevention for GBM. *Aloe* plant used as medicine in rural area and its flavonoids is used as drug for prevention of tumors because it can suppress brain tumor cells growth and progression and improved patient health from this lethal malignancy. Recently reported, that flavonoids isolated from *Aloe*, have antioxidant, anti-inflammatory and anticancer activity **[8-10]**. Flavonoids are polyphenolic natural compound introduced in a variety of fruits and vegetables. Aloe-emodin, aloesin, barbaloin, acemannan and anthreaquinone are major polyphenolic flavonoids, have been shown to inhibit the growth of brain, breast, ovary, lung and leukaemia cancer **[11-13]**.

It has been revealed that flavonoids possessed cell differentiation modulation and inhibited phosphorylation of MAPK and activation of expression of Nf-kβ **[10, 14, 16].** Apoptosis is a natural process that's controlled by genetically in normal cell development and regulated by expression of many genes included Mitogens and their receptor, RAS/MAPK and Nuclear factor kappa B cells (Nf-kB) [**17-18]**, which also involved in tumorigenesis and induced biological activity as metabolism, metastasis, apoptosis, and cell proliferation and differentiation. Phosphorylation of p38 expressed oxidative stress, inflammatory cytokines included tumor necrosis factor (TNF-α) and interleukin (IL-1β). The phosphorylated MAPKs activation occurred by functional mutation in RAS and hyper-activation of p38 pathway concerned neoplastic transformation. Since, flavonoids functions as p38 expression inhibitor [**19-21].** Afterward activation of p38 and Nf-kB, results in initiation of several gene including cyclooxygenase-2(COX-2), C-reactive protein (CRP), TNF-α and pro-inflammatory cytokines such as Interleukins (IL1, IL6) and employment of a major role in cancer **[22-23]**. The transcription factor Nf-kβ is become active on cytokine activation degradation of Ikβ whereas p38 MAPK activation diverse in upstream kinases as well as downstream substrates. Both p38 MAPK and Nf-kβ are

involved in regulation of transcriptional and post-transcriptional levels **[24]**.

The major option for inhibit cancer morbidity and mortality is chemotherapy. Aggressive nature of GBM is due to high proliferation rate and resistance to chemotherapeutics drugs like temozolomide, cannabidiol, doxorubicin and carmustine etc. So, it is necessary to find new alternative treatment to amendment therapeutic effect against GBM with low side effect. Still, the growth inhibitory activity of flavonoids is to be studied on GBM and report described anti-neoplastic nature of flavonoids against U87MG cell line but had negligible effect over normal cells **[10, 16]**. Anticancer activity of Aloe's flavonoids was correlated with decreased level of p38 and Nf-kβ expression in U87MG cells. Therefore, we suggest that flavonoids might be used in therapy of brain tumor. We observed that flavonoids decrease the viability of GBM cell. The low concentration 50µM of Aloe flavonoids expressed growth inhibition against U87MG cells. There are dozens of local plants and their constituents (also called secondary metabolites) as alkaloids, terpenoids, flavonoids, steroids, Saponins, polysaccharides; Glycosides, xanthone etc. may correlate with someone cellular signalling pathways. Flavonoids are specially isolated from leaves **[16]** and have been observed to increase apoptosis and inhibit invasion and metastasis by suppressing of p38 MAPK and Nf-kβ pathway with flavonoids. It does also have been found for association between above signalling pathway and flavonoids of *Aloe*. However, the present studies addressing the effect of flavonoids of *Aloe* on expression of p38 and Nf-kβ activation in GBM before and after treatment.

II. MATERIAL AND METHOD

Drug Preparation

Aloe vera was collected from Chandigarh region and all the chemicals used were analytical grade. Plant's leaves were cut in small pieces and dried under absence of light. After dried and grinding, 100gm of sample was placed in Soxhlet with solvent system (Methanol: Water) in 60/40 ratio at 60^{o}C for 24 hr and crude extract obtained. The flavonoids are quantified with Aluminium chloride (spectrophotometric). In brief, 1ml of extract was added in methanolic aluminium chloride (2%) and after 15min, determined the absorbance at 430nm. The crude extract was dried and make a solution with ethanol and poured into a column (200 x 5.0mm) packed with silica and again washed with 70% ethanol. The eluted flavonoids was collected and concentrated at 40^{o}C. High performance liquid chromatography (reversed phase, C18, 250mm x 416mm, Shimadzu) was used to identify the separated flavonoids according to their retention time (RT). The mobile phase consisted Toluene: Ethyl acetate: formic acid (7:5:1), injection volume was 20µl, flow rate was 1.0ml/minutes and wavelength was 350nm. Then purified flavonoids were kept at -20^{o}C for further study **[25-26].**

Cell Line and Drug Treatment for Cell Cytotoxicity

The human Glioblastoma (U87 MG – *JOB CODE - NCCS2007*) cell lines were provided from NCCS (National centre for cell science), Pune, India and grown in Dulbecco's modified Eagle's medium (DMEM) with content supplement of 10 % FBS (Fetal bovine serum) and 1% penicillin-streptomycin antibiotics in tissue culture flasks containing 5% CO_2 and 95% humidity at 37^{o}C. The cells were sub-cultured at 3-5 days interval for achieve cell count till 2 x 10^4. Then saline fraction of flavonoids was prepared in increasing concentration from 25, 50, 100, 200 and 400µg/ml. The drugs were treated in 96 well plate containing monolayer of U87MG cell lines. Negative control was U87MG cell line and Positive control was treated with Cisplatin (20mM). After incubation at 37^{o}C for 24hrs, remove spent media and after washing with Phosphate buffer (PBS), add MTT reagent (0.5mg/ml) and again incubate for 3hrs and after solubilisation with saline, read the absorbance on ELISA reader at 570nm with reference wavelength 630nm in triplicate. The IC_{50} value calculated by linear regression equation $Y = MX \pm C$. where, Y = 50, M and C values were obtained from the viability graph.

Apoptosis Analysis by Cell Viability

With final IC_{50} concentration treated cells, cell growth and viability were measured by cells count using a haemocytometer. In brief, 0.5 ml of the trypan blue solution (0.4%) was transferred to a test tube, 0.3 ml of PBS and 0.2 ml of the trypsinized cell suspension were added. The final solution was mixed and allowed to stand for 5 min. Then load cell suspension onto both chambers of the haemocytometer. Cells were examined and counted in triplicates under light microscope at 40X10. Percentage cell viability was calculated by the formula: Cell viability = No. of viable cells (unstained cells) × l00 / Total no. of cells (stained and unstained).

DNA Fragmentation

Before and after treatment, U87MG cells were centrifuged and washed with PBS and re-suspend the obtained pallets in DNA lysis buffer (200mM Tris, 100mM EDTA, 1% SDS, pH 8.00) and 10µl (20mg/ml) proteinase K was added and incubate sample at 37^{o}C for two hr and follow up extraction with phenol/chloroform and precipitate DNA sample in ethanol. After washing with 70% ethanol, DNA sample were re-suspended in TE buffer. Then Electrophoresis applied with 1% agarose gel with running buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, 0.5µg/ml Ethidium bromide, pH 8.00) for 2hr at 10 V/cm **[27-28]**.

RNA Preparation and RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to confirmed the over expression of p38 and Nf-kβ in U87MG cell line. Total RNA was isolated from before and after treatment, U87MG cells with Trizol reagent

(INVITROGEN) in accordance to standardized protocol as such manufacturer suggested with small modification. Reverse transcription was performed on 5µl of total RNA using RT-PCR kit (Thermo-fisher) containing syber green in 25µl of reaction volume. This was carried out in triplicate and analysed using selected program Rotor-Gene Q Series Software version 2.3.1 (Build 49) QIAGEN. The primers were as follows-

p38
F-5'-TTGACTCAGATGCCGAAGATGAAC-3'
B-5'-TAGGCAAAGTAGGCATGTGCAAG-3'

Nf-kβ
F-5'-ACCTGAGTCTTCTGGACCGCTG-3'
B-5'-CCAGCCTTCTCCCAAGAGTCGT-3'
At the end of reaction, the melting curve analysed for specificity of p38 and Nf-kβ expression.

Protein isolation with cell lysis & SDS-PAGE
Before and after drug treatment, cells were lysed to isolate protein for SDS-PAGE performed. We used a modified method (). In brief, cells were harvested and lysed with lysis buffer (50mM Tris, 150mM NaCl, 20mM EDTA, 0.5% triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerol-phosphate and pH - 7.50) and lysed cells were centrifuged at 15000 rpm for 30 min at 4^{o}C and collect the supernatants as protein and determined the concentration by Lowry method for performed SDS-PAGE. Total proteins (20µg/ml) were separated with 10% SDS-PAGE electrophoresis.

Statistical Analysis
The parameters in this study were subjected to statistical analysis using Microsoft excel spread sheet. The results are expressed as mean ± error of mean using Microsoft excel. For statistical assessment of the data, origin8 software was used. P-value of data was signified with <0.05.

III. RESULT

Drugs
The extraction of flavonoids observed in *Aloe vera* by extraction method and confirmed with high performance liquid chromatography. The level of total Phenolic content and flavonoids were 32.43 and 62.59 respectively.

Table 1 – Total phenolic content and Flavonoids of *Aloe vera*

Plant's Name	Plant's part	Quantity	Extract	Total Phenolic Content	Flavonoids
		gm	Gm	µg of TAE/serving	µg of CE /serving
Aloe vera	leaves	100	5.44	16.97	21.84

Effect of flavonoids on GBM cell viability
The effect of flavonoids was assessed by MTT assay on U87MG cell line, incubated for 24hr with different doses of flavonoids (25, 50, 100, 200, 400µg/ml) in compare to 20mM Cisplatin. It's performed to study mitochondrial/ non-mitochondrial dehydrogenase activity for flavonoids as cell cytotoxicity. The result revealed that IC_{50} value for flavonoids of *Aloe* was 113.29±0.5µg/ml against U87MG cell lines and was toxic in nature for GBM. At initial concentration of flavonoids of 25µg/ml, U87MG cell viability decreased consistently from 70.49 to 19.95% at concentration of 400µg/ml (P<0.05).

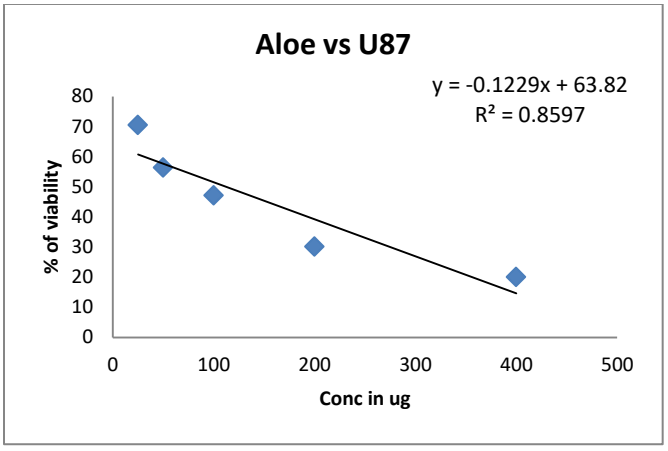

Figure 1- Flavonoids decrease the viability of Glioblastoma (U87MG) cell lines

Effect of flavonoids on GBM cell proliferation and apoptosis
Morphology of cell lines were examined in microscope and observed that cells are shrinkage, with increasing of concentration of flavonoids, percentage of dead cell increased and detects high percentage of apoptosis. We ensure that growth inhibitory effect was due to cellular apoptosis. MTT assay revealed that apoptosis in Glioblastoma is a concentration dependent. In untreated cells, apoptosis was negligible but when it was treated with 25 µg/ml flavonoids then, it's also observed that IC_{50} of flavonoids were highly significant. There was also morphogical changes observed that associated with apoptosis like membrane blobbing, nuclear condensation, DNA fragmentation in treated cells. Aloe's flavonoids represented dose dependent effect. So, our data suggest that flavonoids should be selective towards brain tumor than normal.

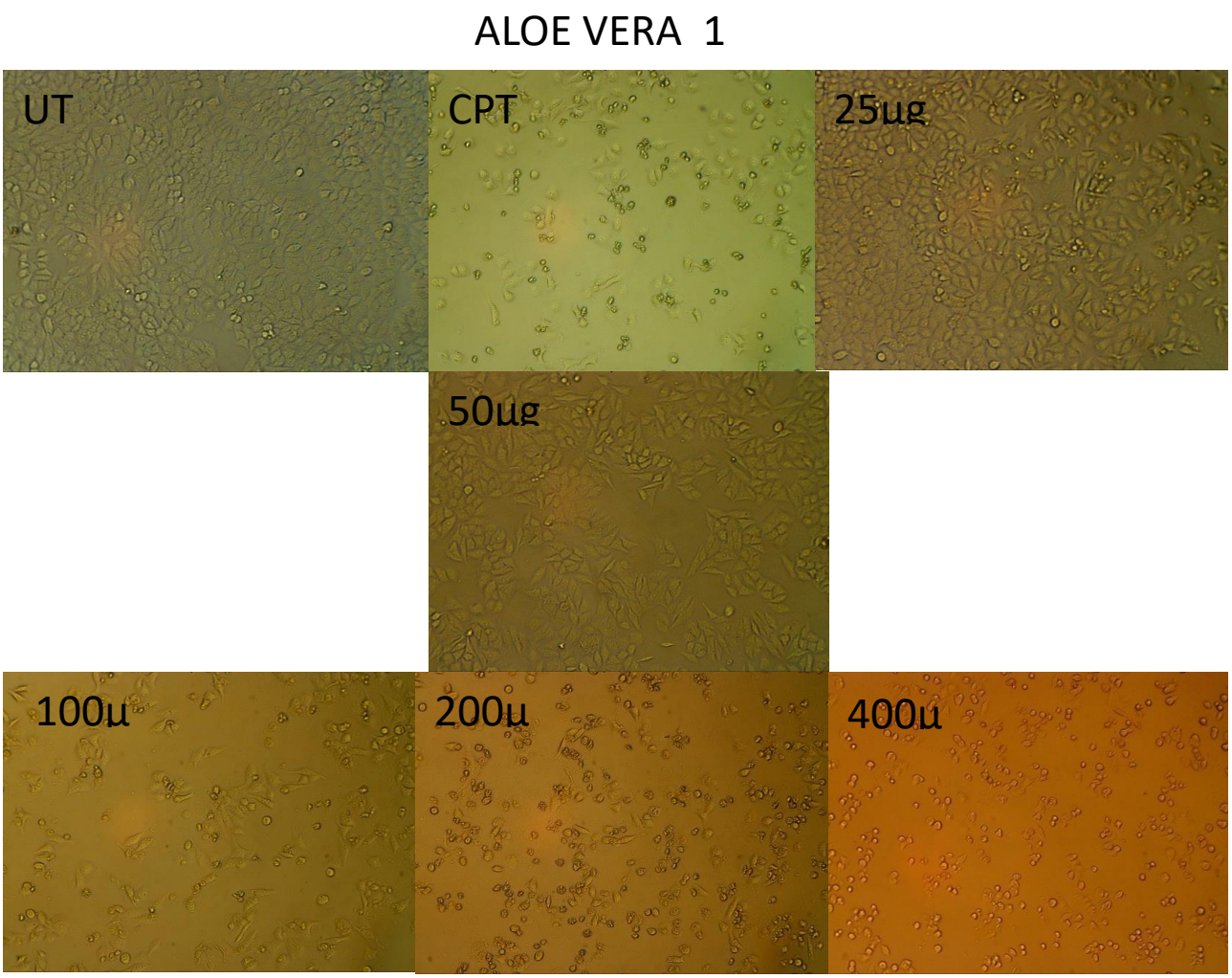

Figure – 2 - Tumor inhibition with flavonoids (UT- Untreated, CPT- Cisplatin & increasing concentration of flavonoids 25-400µg) against U87MG cell lines.

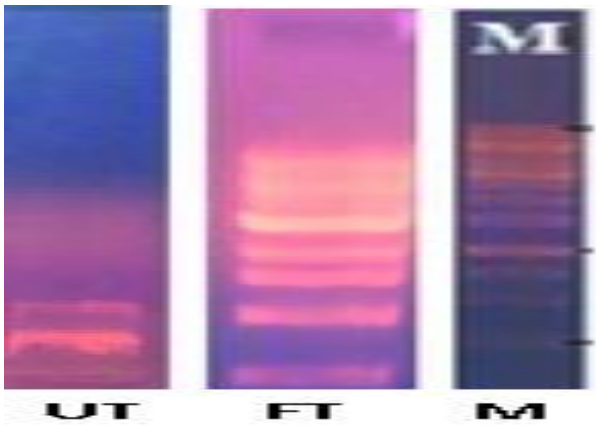

Figure – 3-DNA Fragmentation of Untreated and flavonoids treated against DNA marker

Signalling Pathways involved the growth inhibition activity of flavonoids

p38 and Nf-kβ genes are main factor for regulation of apoptosis and expressed against housekeeping β-Actin gene and normalized in normal cells. As Real Time PCR evaluation of flavonoids (139.02±0.5µg/ml) treated U87MG cell lines showed a significant dose and time dependent decreased expression of p38 and Nf-kB. As evident from result, now clear that p38 play a key role in modulation of apoptosis. The activation of MAPK and Nf-kβ decreased expression from normal expression with significant dose of flavonoids in GBM. These results suggest that flavonoids induce apoptosis with activation of caspase -3 through increased ratio of Bax/Bcl-xL by decreased expression of p38.

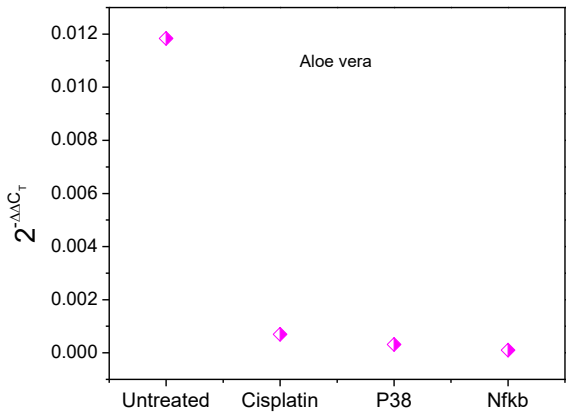

Figure 4 – Real time PCR analysis of p38 and Nf-kB gene in U87MG cell lines show the suppression of gene expression in treated cells with flavonoids and Cisplatin compared to Untreated.

Analysis of Expression of p38 and Nf-kβ proteins

As shown in figures SDS-PAGE, protein expression of p38 and Nf-kβ in untreated and treated U87MG cell lines. When compared the result with control then confirmed the results from RT-PCR. Flavonoids treated cells death was investigated by monitoring of apoptotic proteins of U87MG cell lines with decreased expression of proteins at SDS PAGE. As shown in fig, exposure of flavonoids decreased concentration of protein after 24hr treatment. Further results revealed that flavonoids induced Caspase 7 and promote morphological changes of apoptotic cells. In apoptotic cells, figure showed that flavonoids induced cell's proteins appear as degraded. **[29-30].** The above results indicate that plant pigments induced cell deaths in U87MG by apoptosis with modulation of signalling pathway.

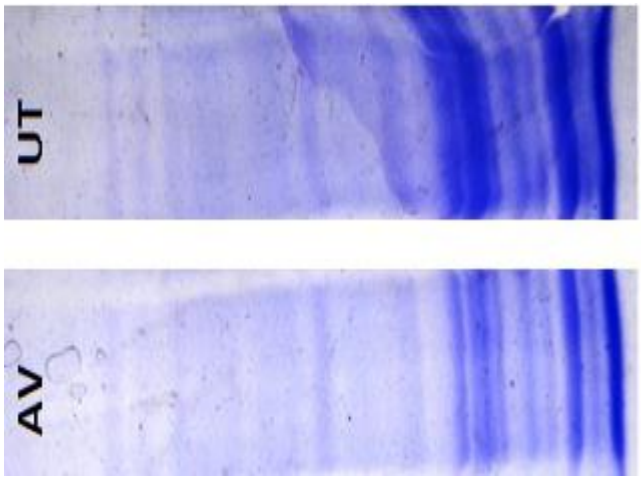

Figure – 5- Protein analysis expressed the decreased the protein level in flavonoids treated U87mg cell lines.

IV. DISCUSSION

The Phenolic compounds are most ubiquitous plants metabolites. Flavonoids are hydroxylated known phenolic substances synthesized by plant. *Aloe* could be regarded as medicine due to flavonoids content in them. Flavonoids play an important and varied role as secondary metabolites. There are of particular importance in the human diet as there is evidence that they act as free radical scavengers, antioxidants, and diuretic, antiviral, antibacterial, antimicrobial, anti-inflammatory, anti-tumor, anti-platelets agents **[8**, **31]**. Here, many chemotherapeutics drugs were cytotoxic against normal cells. Therefore, it's considerable for cure cancer and should be valuable alternative source **[32-33]**. Number of in vitro studies represents the treatment and prevention of brain tumor (Glioblastoma) carried by flavonoids and affects the initiation, promotion, metastasis and proliferation of GBM **[34]**.

In present study, we have analysed the effect of flavonoids at U87MG cell lines. These antioxidant flavonoids assessed in-vitro growth and anti-proliferation of U87MG cells using cytotoxicity assay. It's expressed the reduction of cell viability as others have reported **[31, 35-37]**. Our results indicate, DNA fragmentation increased with apoptosis increased as compared to control **[38]**. To define the role of flavonoids in brain tumor invasion and metastasis, we used U87MG cell line because this is highly invasive and metastatic in nature. U87 cells are responsive to flavonoids for suppressive effect of growth and increased apoptosis. As shown in figure, expression of p38 and Nf-kβ exhibit less in treated as compared to untreated. Although there is not yet precise mechanism for understood regulate the GBM invasion and metastasis. It has been suggested that many signalling pathway including p38 and Nf-kβ to promote the invasion of brain tumors. The most important constituents of *Aloe vera* are aloin, barbaloin, anthranol, cinnamic acid, aloetic acid, emodin, chrysophanic acid, resistanol, and enzymes. Some of them are flavonoids, can affect the initiation, promotion as well as progress stage of brain tumors [**34, 38]**.

Some studies revealed that flavonoids of *Aloe* have mutagenic and genotoxic effects and that were confirmed with DNA fragmentation assay and RT-PCR. *Aloe*-emodin, one of flavonoids has anticancer and cytotoxic activity against brain tumor, lung cancer and liver cancer. In our study, we have found that cytotoxicity of *Aloe's* flavonoids could be through modulation of apoptosis against brain tumors (U87MG) cells. Both gene and protein expression for p38 and Nf-kβ were significantly different in without and with treatment. In our studies, we found that flavonoids induced p38 and Nf-kβ gene expression significantly reduced and these observations showed that p38 and Nf-kβ gene expression suppressed with flavonoids and regulate

transcription gene. p38 and Nf-kβ are major pathway groups for regulation of cell death programming included morphological changes, DNA damage, and gene and protein expressions through induction of apoptotic pathway.

Previous studies revealed to lead to reduce the cell proliferation with cell cycle arrest at many stages. Cell cycle is an important regulatory phenomenon to regulate cell growth and differentiation with cascade's enzyme and activation-inactivation of cyclin and cyclin-dependent kinases (CDKs). Cell cycle arrest and apoptosis can be initiated by flavonoids of *Aloe*. It does also enhance the expression of p21 and p27 to block cell cycle at G1 phase and led to apoptosis in cancer. It's also found that flavonoids were effective in prevention of neuronal apoptosis via phosphorylation of p38 signalling pathway and Nf-kβ regulation [14,23].

p38 signalling pathway is a regulators to lead oncogenes expression. There is evidenced that p38 participate in cox-2 gene expression by hypertonic solution and lipo-polysaccharides and p38's inhibitor stopped cox-2 mRNA. Nf-kβ activation can be regulated by multiple mechanisms and cross references represents that Nf-kβ activation is regulated by p38 **[39].** It's also reported that Nf-kβ and its associated cytokines initiate inflammation and cancer causing factors and is activated by degradation of Ikβ to lead physiological response. Nf-kβ is a transcription factor, responsible for gene involved in growth, cell survival, inflammation and differentiation. Flavonoids reduced p38 and Nf-kβ expression that modulated by p38 MAPKs. With inhibition of p38 pathway significantly reduced the expression of Nf-kβ due to low binding of TBP to TATA box [**40-41]**.

Previous studies **[42-43]** expressed that flavonoids compound inhibited angiogenesis in cancer. VEGF is key regulator in tumor development, maintenance and promotes angiogenesis by stimulating tumor growth and metastasis **[44]**. Recent studies showed that flavonoids inhibited angiogenesis, indicated the inhibition potential in tumor growth **[35]**. Some researcher showed that expression, activation and translocation of Nf-kβ is regulated by Akt pathway and suppression of Nf-kβ expression with flavonoids in U87MG cells, indicated the role of Nf-kβ and Akt pathway in cell proliferation and apoptosis **[36]**. Our conducted work agree with in vitro studies, that flavonoids reduced number of cells with increasing concentration, growth rate, reduced viability of cells **[37]**. On sum-up, previous studies and our results represent the potential of flavonoids to find and development of new drugs. The challenge of chemotherapy in tumor is that chemotherapeutic drugs are toxic for normal cells. So, flavonoids will be alternative in Glioblastoma treatment.

V. CONCLUSION

In present investigation, we observed the role of flavonoids in regulation of brain tumor invasion. Flavonoids of *Aloe* could have cytotoxic and genotoxic activity against GBM. *Aloe* constituents showed morphological changes, DNA damage, gene and protein associated apoptosis of GBM. Based on above works, we concluded that p38 and Nf-kβ activation are significantly inhibited by 139µg/ml flavonoids and this dose is achievable. Our results demonstrated that flavonoids play an inhibitory role in brain tumor invasion by decrease the expression of p38 and Nf-kβ signalling pathway through induction of apoptosis. So, flavonoids regulate the growth of tumor, angiogenesis, apoptosis and modulation of cell regulatory pathway as p38 and Nf-kβ signalling pathway. Therefore, it should be considered a potential anticancer agent and continue more research both in-vitro and in-vivo.

Conflict of Interest

None

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