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Original Article

Elucidation of the Anti-inflammatory, Anti-proliferative and Epithelial Mesenchymal Transition Inhibiting Potentials of *Cichorium intybus* Extract on Human Cancer Cell Line(s)

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ABSTRACT

Background: *Cichorium intybus* L. is cultivated in various temperate regions of the world for use of its different parts (mostly roots/seeds) as medicines or food. However, the anti-inflammatory, anti-proliferative and Epithelial Mesenchymal Transition (EMT)-inhibiting potential of *C. intybus* extracts have not been extensively studied at molecular level using suitable human cancer cell-line models. **Objectives:** Elucidation of the anti-inflammatory, anti-proliferative and EMT inhibiting potential of *C. intybus* extract using human cancer cell-line models. **Materials and Methods:** We prepared Methanolic Extract (MKE) of *C. intybus* leaves and studied its anti-inflammatory, anti-proliferative, and anti-EMT activities in THP-1 and U87MG cell lines through real time expression analysis and various functional assays including cell viability, cytotoxicity, colony forming and wound healing assays. **Results:** MKE significantly reduced the LPS-induced transcription of proinflammatory cytokines; TNF-α, and IL-6 from macrophage turned THP-1 cells. Also, expression of TGF-β gene was significantly downregulated by MKE in both THP-1 macrophages and U87MG cells. In U87MG glioma cells, MKE significantly downregulated the expressions of proliferation (Ki67, and PCNA), and EMT markers (LIX1, VIM, ZEB1, SNAIL, and SLUG), which are associated with aggressiveness, histological grade and poor prognosis in glioma. **Conclusion:** It should be emphasized that this is the first study done on examining the potential of *C. intybus* anti-inflammatory, anti-proliferative, and anti-EMT activities against the THP-1 macrophages and U87MG glioblastoma cells. Our findings strongly suggest *C. intybus* leaves as an efficient source of pharmaceutical formulations that can contribute to the treatment of inflammatory diseases and cancer and could add in improving human health significantly.

Keywords: *Cichorium intybus*, Anti-EMT, Anti-inflammatory, Anti-proliferative, Anti-neoplastic.

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INTRODUCTION

Cellular plasticity is defined as the ability of cells to change their phenotype in a reversible manner. An example of cellular plasticity is illustrated in early metazoan embryo formation, where the epithelial cells acquire mesenchymal properties, in a process known as epithelial-to-mesenchymal transition. The EMT suggests that an epithelial cell transforms into a mesenchymal cell with the capacity to migrate and invade surrounding tissues.[1] EMT in carcinogenesis, in contrast to other EMT types, is aggressive and uncontrolled rather than mild and controlled.[2] For instance, EMT may be responsible for the inflammatory responses that arise during the development of cancer and is, in turn, regulated by the inflammatory microenvironment. For example, the close relationship between inflammation and tumour development is demonstrated by ulcerative colitis, hepatitis, chronic gastritis and pancreatitis, with their respective connections with colon, liver, gastric, and pancreatic carcinomas.[3] TGF-β, acts as a master molecule in the induction of EMT program.[4] In case of chronic inflammatory conditions, TGF-β1 triggers the reactivation of EMT developmental processes, disturbing the epithelial homeostasis, destabilizing the extracellular matrix, and promoting the progression of cancer towards metastatic state.[5] Owing to the association between inflammation and EMT, it would be interesting to consider particular anti-EMT medications to treat chronic inflammatory conditions in addition to anti-inflammatory therapy and vice-versa. There are mounting evidences supporting the fact that phytoextracts or their isolated bioactive compounds have the ability to control EMT by acting

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Table 1: List of real time primers used in the present study.

GAPDH forward	5'- CTCCTCTGACTTCAACAGCG-3'	[12]
GAPDH Reverse	5'-GCCAAATTCGTTGTCATACCAG-3'	
Ki-67 forward	5'-GCTGGCTCCTGTTCACGTA-3'	
Ki-67 Reverse	5'-CCTGTACGGCTAAAACATGGA-3'	
PCNA forward	5'-GGCGTGAACCTCACCAGTATG-3	
PCNA reverse	5'-TTCTCCTGGTTTGGTGCTTCA-3'	
TNF-α forward	5'-CCCAGGCAGTCAGATCATCTTC-3'	[3]
TNF-α reverse	5'-AGCTGCCCCTCAGCTTGA-3'	
Il6 forward	5'-GAACTCCTTAAAGCTGCGCAGA-3'	
Il6 reverse	5'-ATC CAGTTCCTGCAGAAAAAGGC-3'	
Il-1β forward	5'-CCACTCTACAGCTGGAGAGTG-3'	
Il-1β reverse	5'-CCAGGAAGAAGACGGGCATGTT-3'	
TGF-β forward	5'-GAC ACGCAGTACAGCAAGGTCCT-3'	[12]
TGF-β Reverse	5'-TTGCAGGAGCGCACGATCATGT-3'	
Snail forward	5'- TAGCGAGTGGTTCTTCTGCG-3'	
Snail reverse	5'- CTGCTGGAAGGTAAACTCTGGA-3'	
Slug forward	5'-ATTAGAACTCACACGGGGGAG-3'	
Slug reverse	5'-GCCAGATTCCTCATGTTTGTGC-3'	
VIM forward	5'-TGC CCTTAAAGGAACCAATGAGTC-3'	
VIM reverse	5'-ATTCACGAAGGTGACGAGCCAT-3'	
ZEB forward	5'-CAG ATTCCACACTCATGAGGTCTT-3'	
ZEB reverse	5'-TCC AGCCAAATGGAAATCAGGATG-3'	
LIX1L forward	5'-GCT TTGGGAGTTTCCAGTTTTGCC-3'	[12]
LIX1L reverse	5'-CCC TGTATTTGGGTTGTCAGCTTC-3'	

as antioxidants, anti-fibrotic, anti-inflammatory and anti-cancer agents.

Cichorium intybus L. (chicory or kasni) is one such plant that is well-known for its hepatoprotective, anti-inflammatory and anti-cancer activities in different parts of the world. In traditional and Unani medicine, kasni has been used for the treatment of diarrhoea, fever, spleen and liver enlargement, jaundice, rheumatism, and gout.[6] Due to the presence of numerous medicinally significant chemicals such as alkaloids, saponins, sesquiterpene lactones, inulin, coumarins, flavonoids, unsaturated sterols, all parts of this plant have tremendous pharmacological relevance.[7] *C. intybus* has been shown to have anticancer potential against a number of cancer cell lines, including amelanoic melanoma C32, renal adenocarcinoma and prostate and breast cancers. However, majority of them have just been focussing on the anti-proliferative potential of *C. intybus* extract(s) on these cells. Furthermore, to the best of our knowledge, there has not been any study done till date to comprehensively study the anti-inflammatory, anti-proliferative, and EMT-inhibiting properties of *C. intybus* using human leukemia monocytic and human glioblastoma cell lines. Therefore, this study aims to find a correlation between the anti-inflammatory, anti-proliferative, and EMT-inhibiting potential of *C. intybus*. Studying EMT-inhibiting potential is crucial since it has been directly linked to the development of drug resistance and metastasis in a variety of tumour types.[8,9] To study these phenomena and effect of *C. intybus* extract, we used THP-1 human leukemia monocytic cells and U87MG human glioblastoma cells, as *in vitro* cancer models.

MATERIALS AND METHODS

Materials

Extract preparation

C. intybus leaves were collected from Haldwani, Uttarakhand, India. The extract was prepared according to the method described previously.[10] The extract yielded 1.3 g residue which was dissolved in 2 mL DMSO (25%) and stored at 4°C until further use.

Cell culture

THP-1 human leukemia monocytic cells and U87MG human glioblastoma cells were purchased from National Centre for Cell Science, India and cultured in RPMI-1640 and minimum essential cell culture medium supplied with 15% FBS. Both the cell lines were maintained at 37°C with 5% CO_2 in CO_2 incubator.

In vitro anti-inflammatory activity of phytoextract

THP1 cells (1 X 10^5/well) were stimulated with Phorbol 12-myristate 13-acetate (PMA:20 ng/mL) for differentiation into macrophages for 48 hr.[11] The THP1 macrophages were induced with 100 µg/mL Lipopolysaccharide (LPS) to stimulate

inflammatory responses and treated with different concentrations of MKE (20 and 200 µg/mL) and equivalent DMSO-dilution (solvent control) for 24 hr. After the treatment, the cells were collected in trizol for RNA isolation. After cDNA synthesis from RNA (as described below), the expression modulation of TNF-α, IL-6, IL-1β and TGF-β gene were determined using real-time PCR.

RNA isolation and real time PCR

RNA isolation was carried out using Direct-zol RNA miniprep kit, as per the manufacturer's instructions. cDNA was prepared using Thermo Scientific Maxima H Minus First Strand cDNA synthesis kit using random hexamers. Real time PCR was performed using SYBR green PCR master mix (Thermo Fisher Scientific) using specific primers. The relative gene expression was analyzed using $2^{-\Delta\Delta CT}$ method.[12] The expression levels of various genes studied were normalized to that of GAPDH (internal control). The real time primers used in the present study are listed in Table 1.

Assays for determination of anti-proliferative activity

Trypan blue exclusion assay for Cell viability

Trypan blue exclusion assay was performed according to the method described previously.[13] All the viable and non-viable cells were counted individually and recorded. The percent of viable cells were calculated using the given formula:

$$\% \text{ Cell viability} = 100 \text{ X (Total number of viable cells/mL) / Total number of cells/mL}$$

Cytotoxicity assay

The cytotoxicity of MKE against U87MG cells was evaluated using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with minor modifications.[14]

Clonogenic assay

The effect of MKE on the colony forming ability of U87MG cells was evaluated using the method described previously with slight modifications.[15] The colonies were counted using ImageJ software and presented graphically as percentage of solvent control.

In vitro anti-proliferative and anti-EMT activity of phytoextract

U87MG cells were seeded at an optimum density of 5 X 10^4/well in a 24 well plate and cultured for 24 hr. Further, the cells were treated with MKE (10, and 200 µg/mL) and equivalent DMSO-dilution (solvent control) for 24 hr. After the treatment, the cells were collected in trizol for RNA isolation. After cDNA synthesis real-time PCR was performed for evaluation of any modulation in the expression of proliferative markers (*Ki-67* and *PCNA*), *TGF-β* and major epithelial/mesenchymal genes (*CDH1, LIX1, SNAIL, SLUG, VIM* and *ZEB1*).

Migration assay

Cell migration was evaluated using wound healing assay. In a 24-well cell culture plate, 5 X 10^4 cells/well were seeded and cultured for 24 hr to obtain confluency of cells forming monolayer. Further, a scar was created in the monolayer with a scratcher. Then the cells were treated with MKE (10, 100 and 200 µg/mL) and solvent control. After 24 hr of treatment, the wound in the monolayer was observed under inverted microscope for wound healing and images were recorded.

Statistical analysis

For statistical analysis ANOVA methods were used using GraphPad Prism 8.0.1. Statistical significance was set at $p<0.05$ and all the experiments were performed in triplicates.

RESULTS

In vitro anti-inflammatory activity of MKE

The anti-inflammatory effect of MKE was studied on the LPS stimulated THP-1 macrophages. After the treatment, the expression-modulation of major pro-inflammatory genes (TNF-α, IL-6 and IL-1β) were studied through RT-qPCR (Figure 1).

The morphology of THP-1 macrophages was affected by treatment with MKE at various doses, which resulted in reduction in the degree of differentiation and adherence (Figure 1A).

The qRT-PCR expression analysis showed significant ($p<0.0001$) downregulation in the mRNA expression of TNF-α, and IL-6 genes at both 20 and 200 µg/mL concentrations of MKE, but there was no significant change observed in the expression of IL-1β gene in comparison to the solvent control after 24 hr (Figure 1B).

Modulation of proliferative markers in THP-1 macrophages

The expression study of proliferative markers (Figure 1C), showed significant ($p<0.0001$) reduction in PCNA gene expression at both 20 and 200 µg/mL concentrations of MKE, while in case of Ki67, a significant ($p<0.0001$) reduction in the expression was observed at 200 µg/mL concentration of MKE in comparison to the DMSO solvent control.

Modulation of TGF-β gene in THP-1 macrophages

To evaluate the effect of MKE on the expression of TGF-β gene, we performed qRT-PCR and observed that MKE was effective in downregulating the expression significantly ($p<0.0001$) in comparison to the solvent control (Figure 1D).

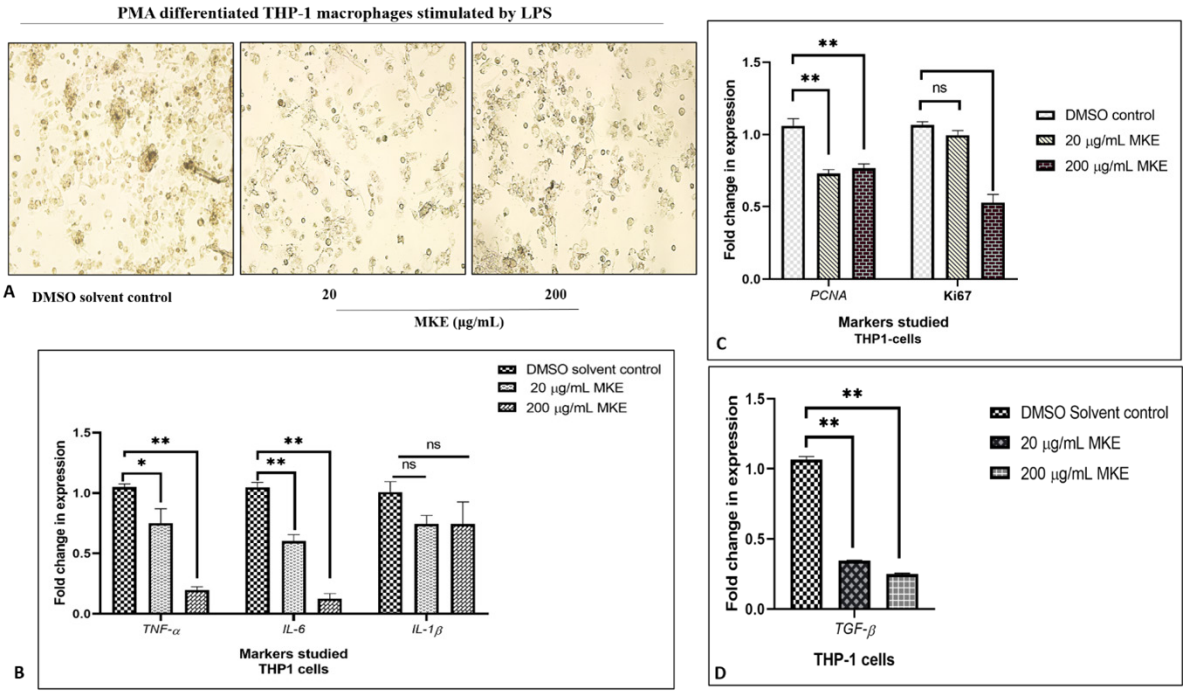

Figure 1: A. Effect of MKE (20 and 200 µg/mL) and DMSO-solvent control on THP-1 macrophages; B. Modulations in the expression of major pro-inflammatory genes (TNF-α, IL-6 and IL-1β); C. Modulation in the expression of *Ki67* and *PCNA* proliferative genes D. Modulation in the expression of TGF-β in THP-1 cells. **p*<0.001, ***p*<0.0001, ns: not significant.

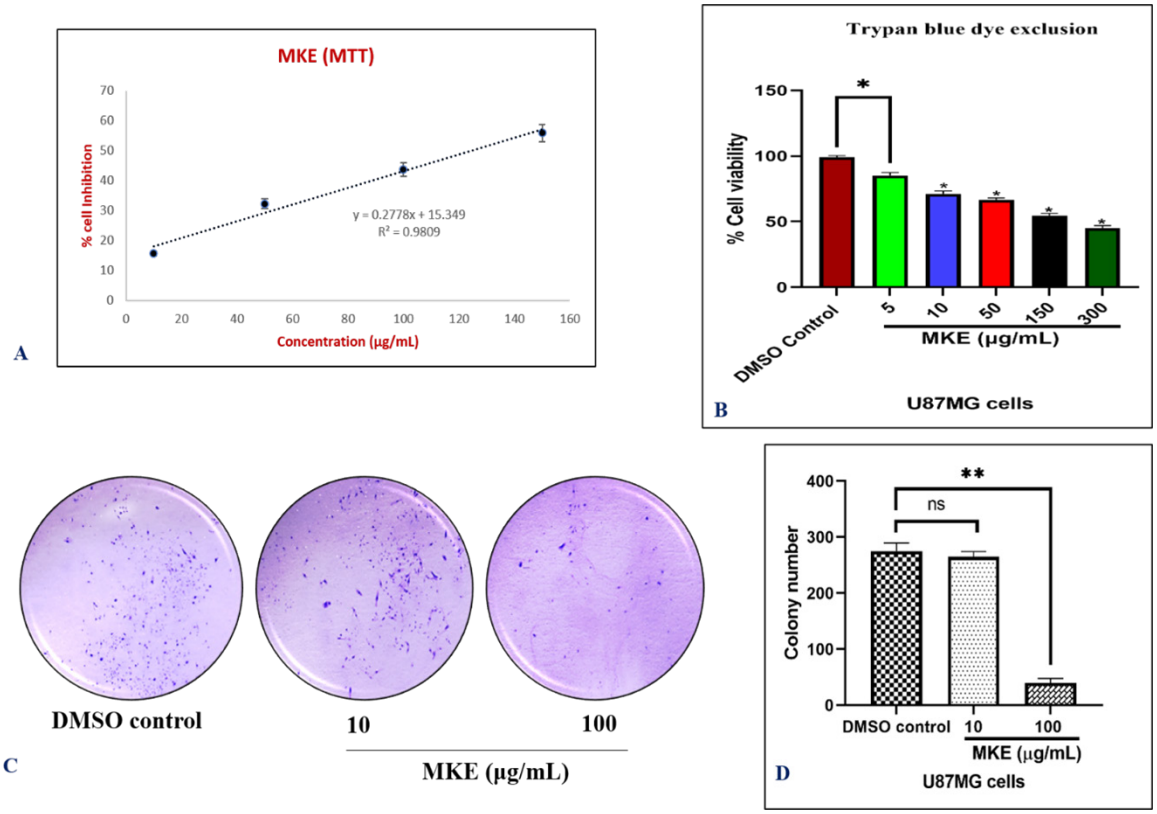

Figure 2: A. Cytotoxicity of MKE evaluated against U87MG cells using MTT; Graphical representation of percent inhibition of cells Vs concentration of MKE; B. Percent cell viability estimated through trypan blue dye exclusion method. C. Colony forming ability of U87MG cells with and without treatment with MKE (10 and 100 µg/mL). D. Graphical representation of number of colonies formed after treatment with MKE and DMSO solvent control. ***p*<0.0001, ns: not significant.

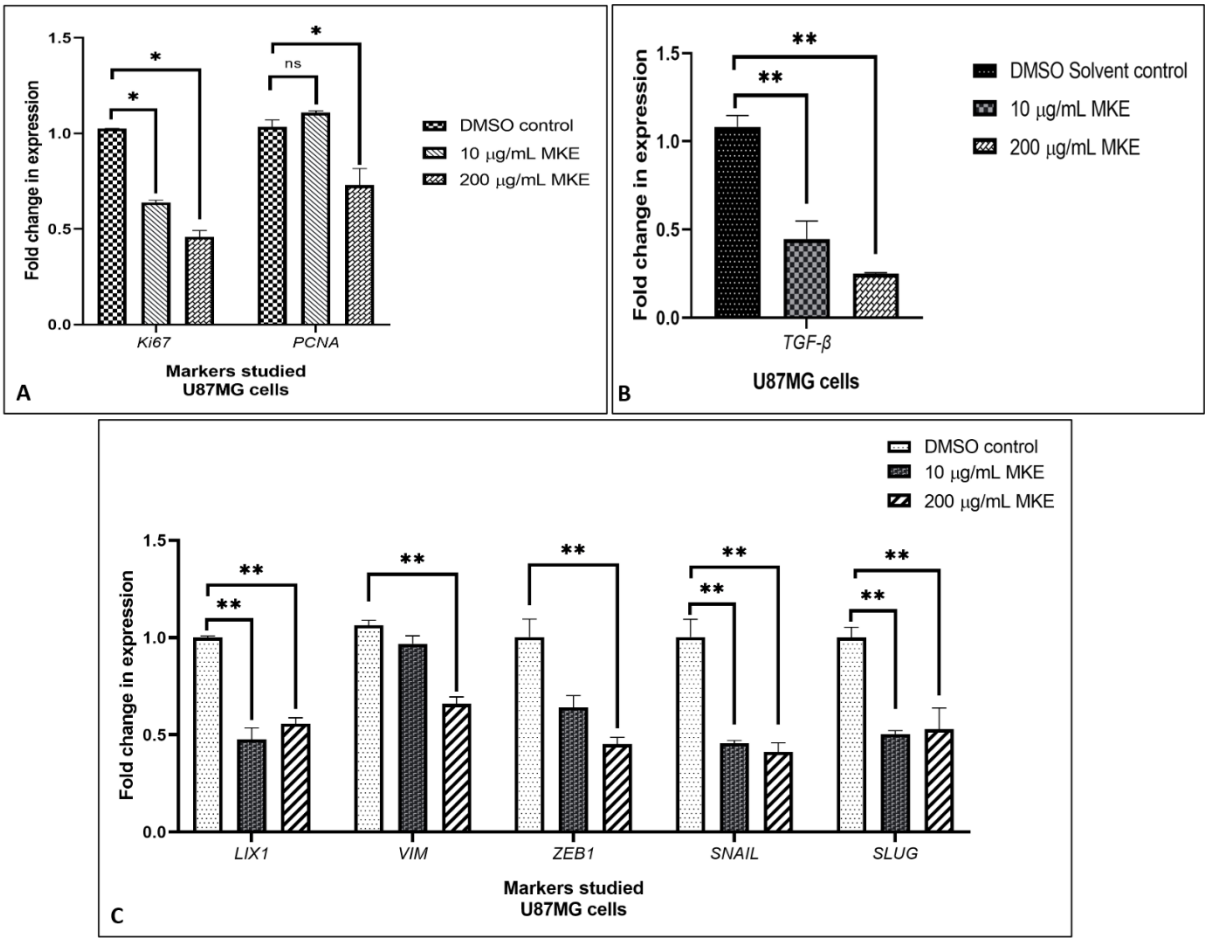

Figure 3: Expression modulation of A. Proliferative genes (*Ki67* and *PCNA*); B. *TGF-β* in U87MG glioblastoma cells in response to treatment with MKE (10 and 200 µg/mL) and solvent control. C. Expression modulation of major mesenchymal genes in MKE treated and untreated U87MG cells. *p<0.001, **p<0.0001, ns: not-significant.

Overall, it was observed that MKE was significantly effective in controlling the proliferation of THP-1 cells, downregulating pro-inflammatory gene, and TGF-β gene expressions in THP-1 macrophages, indicating its efficacy to act as a potential anti-inflammatory agent.

Effect of MKE on U87MG cells estimated (MTT and trypan blue exclusion)

The cytotoxicity of MKE on U87MG glioblastoma cells was evaluated through MTT assay. MKE was found highly effective in controlling the proliferation of glioma cells with an IC_{50} value of 124.73 µg/mL as estimated through MTT assay (Figure 2A).

The effect of MKE on the viability of U87MG cells was also evaluated using trypan blue exclusion assay. The results showed substantial reduction in the viability of cells in a dose-dependent manner in comparison to the solvent control (Figure 2B).

Clonogenic assay

The results of colony forming assay confirmed the inhibitory effect of MKE on U87MG cells with significantly pronounced attenuation of cell viability at a concentration of 100 µg/mL (Figure 2C, D).

The inhibition of proliferation of U87MG glioma cells by MKE was validated by all the three functional assays (anti-proliferative/MTT, cell survival, and colony forming assays), which independently demonstrated the significant anti-proliferative activity of MKE on U87MG cells. From the results obtained it is evident that MKE is highly effective in inhibiting proliferation of both THP-1 macrophages and U87MG glioblastoma cells.

Expression modulation of proliferative genes in U87MG cells

In MKE treated U87MG cells it was observed that 10 and 200 µg/mL concentrations were significantly (p<0.001) downregulating Ki67 gene expression. However, 200 µg/mL concentration of

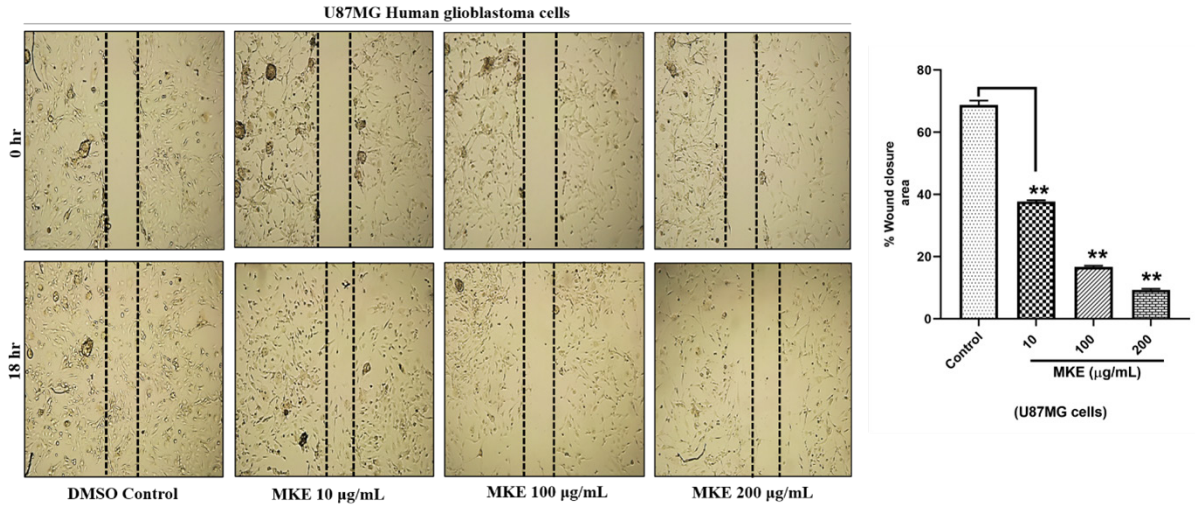

Figure 4: Migration/wound healing: Effect of MKE and DMSO solvent control on U87MG cells at 0 hr and 18 hr and representative percent wound closure at different concentrations of MKE. (*p<0.0001).

MKE was effective (p<0.001) in downregulating PCNA gene expression (Figure 3A) 200 µg/mL concentration of MKE was effective (p<0.001) in downregulating PCNA gene expression (Figure 3A).

Expression modulation of *TGF-β* gene in U87MG cells

The expression study showed the effectiveness of MKE in significantly (p<0.0001) downregulating the expression of *TGF-β* at 10 and 200 µg/mL (Figure 3B).

Expression modulation of EMT genes

The effect of MKE (10 and 200 µg/mL) on major mesenchymal genes (LIX1, ZEB1, SNAIL, SLUG, VIM) in U87MG cells was evaluated through qRT-PCR. The expression-based study showed significant downregulation in the expression of these mesenchymal genes which are responsible for regulating the process of EMT in various cancers. Both, 10 and 200 µg/mL concentrations of MKE was significant in downregulating the expression of LIX1, VIM, ZEB1, SNAIL, and SLUG genes (Figure 3C). Similarly, we also attempted to investigate the modulation in the expression of an epithelial marker (E-cadherin; CDH1) but despite using two different primer sets, CDH1 could not be amplified.

Cell-Migration assay

Wound healing assay was performed to evaluate the effect of MKE on the migration of U87MG cells. 10 µg/mL concentration of MKE showed moderate inhibition of U87MG cells, the wound was covered with migratory U87MG cells in both DMSO solvent control and 10 µg/mL MKE. While, both 100 and 200 µg/mL concentration of MKE was highly effective in preventing the migration of U87MG cells. Thereby, preventing wound closure (Figure 4). Overall, the results of the study clearly showed the anti-EMT potential of MKE which was also validated through the migration assay.

DISCUSSION

In the present study, we evaluated the anti-inflammatory, anti-proliferative and anti-EMT activities of methanol extract of *C. intybus* leaves through various functional assays and real time expression-based study. We found excellent anti-inflammatory activity of MKE which was validated by the significant downregulation of major proinflammatory genes (TNF-α, and IL-6) expressions in LPS-induced THP-1 macrophages. Also, there was significant reduction observed in the expression of proliferative genes (PCNA, Ki67) in THP-1 macrophages which was validated by the decrease in the degree of adherence and differentiation of THP-1 macrophages on treatment with MKE.

The process of EMT is regulated by a variety of factors in which proinflammatory cytokines (TNF-α, IL-6 etc.,) play important role in induction of TGF-β, via NF-κB signalling. Pathologically, the activated TGF-β, in both inflammatory and tumor microenvironment act as a master inducer in conjunction with other transcription factors (SNAIL, SLUG, ZEB1) for the activation of EMT.[16] According to Ye *et al.* (2012), inflammatory processes in gliomas are predicted to cause the recruitment of TGF-β producing macrophages or microglia into the tumour stroma, where they are known to influence transcription factors associated with EMT in cancer cells to activate the process of EMT.[17] TGF-β, regulates invasion and metastasis of cancer cells by upregulating mesenchymal markers while downregulating epithelial markers and is also an important factor in the controlling the invasiveness and metastasis of tumours through EMT.[16] Thus, the significant downregulation in the expression of TGF-β by methanol *C. intybus* leaves extract in tumor micro-environment

of both THP-1 macrophages and U87MG glioma cells showed its plausible ability to abate the TGF-β mediated EMT and further progression of cancer.

Ki-67 and PCNA are used as prognostic markers in gliomas and are related to the histological grade of the disease. The Ki-67 protein stimulates numerous carcinogenic pathways in different cancer types, necessitating substantial changes to transcriptional programs.[18] In this study, the methanol *C. intybus* leaves extract showed significant reduction in the expression of proliferative genes (PCNA, Ki67) in THP-1 macrophages and U87MG gliomas. The anti-proliferative and cytotoxic effect of the extract was validated by MTT and cell viability assays.

Glioma cells have high levels of ZEB1, which increases the tumours aggressiveness.[19] Similarly, expression of VIM is associated with the histopathological grade for glioma. LIX1L is also highly upregulated in numerous cancer tissues.[20] Additionally, high ZEB1 and VIM gene expressions in gliomas contributes to drug resistance.[19,21] The two main master regulators of EMT, *SNAIL* and *SLUG*, function by suppressing E-cadherin. These proteins are highly expressed in gliomas during the process of EMT.[22] The anti-EMT effect of MKE was validated by the ability of MKE in inhibiting the migration of U87MG cells in wound healing assay (migration assay). The real time expression study showed significant downregulation of major mesenchymal genes (LIX1, SNAIL, SLUG, ZEB1, VIM) in U87MG glioma cells. Thus, indicating the anti-EMT potential of methanolic extract of *C. intybus* (MKE). The various EMT markers explored in the present work are associated with the enhanced stemness, mesenchymal phenotypes, invasion, metastasis, and resistance to chemotherapy and radiation therapy. Thus, owing to the significant ability of methanolic extract of *C. intybus* in downregulating the major drivers of EMT, inflammation, and proliferation, it could be concluded that the phytoextract may contribute in chemoprevention by targeting inflammation, cellular-proliferation and EMT.

For further assessing the chemo-preventive or therapeutic potential of MKE or their purified phytoconstituents, experiments on animal xenograft models of human cancers can be carried out. Using the appropriate model system(s), their potential for pharmacological sensitization (perhaps through EMT-inhibition) could also be investigated. Overall, this study lays the groundwork for future research on *C. intybus* extracts and/or their isolated phytochemicals in the production of drugs to treat various malignancies and other chronic inflammation-driven disorders.

CONCLUSION

The findings of the current study highlight the significant potential of the methanolic extract derived from *C. intybus* leaves. This extract has demonstrated substantial potential in preventing inflammation, inhibiting cell proliferation, and epithelial-mesenchymal transition (EMT). These effects were confirmed through experiments conducted on THP-1 human leukemia cell lines and U87MG human glioblastoma cell lines. Overall, it could be concluded that *C. intybus* may have a valuable role in various pharmaceutical preparations aimed at treating conditions such as inflammation, cancer, and various other human diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; **IL-6:** Interleukin 6; **IL-1β:** Interleukin 1 beta; **TNF-α:** Tumor necrotic factor alpha; **TGF-β:** Transforming growth factor beta; **ZEB1:** Zinc finger E-box 1; **VIM:** Vimentin; **LIX1L:** Limb and CNS expressed 1 like; **LPS:** Lipopolysaccharide; **PCNA:** Proliferating cell nuclear antigen.

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