World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

REPURPOSING BIGUANIDE DRUG METFORMIN FOR TARGETING TNF-α INDUCED EPITHELIAL MESENCHYMAL TRANSITION IN HUMAN COLORECTAL CARCINOMA

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Article Received on 01/02/2025 **Article Revised on 21/02/2025** **Article Accepted on 11/03/2025**

ABSTRACT: According to Global cancer statistics by world region for the year 2024 based on IARS, Colorectal carcinoma (CRC) is the 3rd most prevalent cancer worldwide comprising approximately 9.6%. Various factors influence CRC progression, including genetic mutations in the microenvironment, angiogenesis, epithelial-mesenchymal transition (EMT), and lymphatic spread. EMT is particularly significantly regulated by signaling pathways including TNF-β. TNF-α, an inflammatory cytokine, has been shown to disrupt anticancer mechanisms by promoting EMT, which remains underexplored. This study aims to investigate the effects of TNF-α induction on EMT in CRC and to evaluate the potential of metformin, a biguanide drug traditionally used for type 2 diabetes, as a repurposed treatment with anticancer properties. The capacity of the HCT116 cell line, which is frequently employed in cancer studies to express characteristics of mature intestinal studies, hence HCT116 has been used for all our assays. Primarily, the antiproliferative effect of metformin on HCT 116 was studied by NRU assay. To examine the impact of metformin on metastasis, TNF-α was introduced to HCT116, and NRU assay was performed. Further, cell migration, cell aggregation, clonogenic, and ROS assays were carried out for validation. In addition to studying anti-inflammatory and antimetastatic activity, ELISA and to study the tumor pathway, qPCR was done. Metformin showed dose-dependent antiproliferative effects, inhibiting cell migration and upregulating E-cadherin. It also enhanced apoptosis Via ROS induction. Metformin also showed anti-inflammatory effects, reducing IL-1β levels and restoring SMAD4 expression, suppressing tumors.

1. INTRODUCTION

1.1 DRUG REPURPOSING

Drug repurposing, also known as drug repositioning or drug reprofiling, refers to the process of discovering new uses for existing drugs that are already approved for treating other diseases. Early to Mid-20th century, many drugs that are now widely used were originally discovered by accident or through observations of unexpected side effects. In the 20th century, the rise of computational methods and bioinformatics revolutionized drug repurposing. Now 21st Century Modern drug repurposing strategies increasingly rely on understanding the molecular mechanisms of diseases and drugs. This includes studying how drugs interact with biological networks and pathways to identify new therapeutic uses. It has several benefits in the medical field and pharmacology, such as the cost-effective, efficient, and innovative approach to drug discovery.

1.2 METFORMIN

It is an oral antidiabetic medication belonging to the biguanide class, with a chemical formula of $C_4H_{11}N_5$ and a molecular weight of 165g/mol. It appears as a white crystalline powder and is freely soluble in water. It primarily reduces hepatic glucose production and enhances insulin sensitivity in peripheral tissues. After oral administration, it reaches peak plasma concentrations in 2-3 hours and is eliminated unchanged in the urine, with a half-life of 4-8 hours. It is indicated for type 2 diabetes and is used off-label for polycystic ovary syndrome (Bailey, C. J. 2017).

1.3 THERAPEUTIC PROSPECTS OF METFORMIN

Metformin derived from galegine in Galega officinalis was recognized in the 1920s for lowering blood glucose but gained prominence in the 1950s when French

physician Jean Sterne introduced it as "Glucophage" for diabetes treatment. By the 1970s and 1980s, it became a leading medication for type 2 diabetes due to its effectiveness, safety, and low cost. Metformin has a lower risk of hypoglycemia and weight gain and it offers cardiovascular benefits. It shows promise for conditions like polycystic ovary syndrome and gestational diabetes. It has anti-inflammatory properties and potential cardiovascular benefits including improved lipid profiles and reduced atherosclerosis risk. Metformin has also been studied for potential benefits in psychiatric disorders like depression and schizophrenia (Barbora Waclawikova. 2018).

1.4 EFFECT AND EFFICACY OF EMT PATHWAY OF METFORMIN IN COLORECTAL CANCER

Mechanisms of Action: Metformin activates AMP-activated protein kinase (AMPK), which regulates energy homeostasis and can inhibit mTOR signaling promoting apoptosis and potentially slowing tumor growth. By improving insulin sensitivity and reducing insulin levels, metformin may also influence colorectal cancer progression, as high insulin levels are linked to increased cancer risk.

Investigating Metformin's Effect on EMT Pathway

Metformin has been shown to reduce epithelial-mesenchymal transition (EMT) markers such as N-cadherin and vimentin. It modulates signaling pathways, notably AMPK and mTOR, to inhibit EMT. Metformin may reduce the metastatic potential of colorectal cancer cells, potentially improving patient outcomes by limiting cancer spread. Further research is essential to fully understand these mechanisms and optimize metformin's role in treatment (Issam Ben Sahra *et.al*.,2010). Studies have explored combining metformin with other anti-cancer therapies, demonstrating synergistic effects in inhibiting EMT and enhancing treatment outcomes.

II. MATERIALS AND METHODS:

Colorectal cancer cell lines (HCC116) were initially procured from the National Centre for Cell Sciences (NCCS), Pune, India, and Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25cm 2 tissue culture flask with DMEM supplemented with 10% FBS, L-Glutamine, Sodium bicarbonate (Merck, Germany), and an antibiotic solution containing, Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml) Cultured cell lines were kept at 37°C in a humidified 5% CO_2, incubator (INBS Eppendorf, Germany).

1. Dulbecco's Modified Eagle's Medium (DMEM) Preparation

To prepare DMEM, 0.67g of the powder was dissolved in 25ml of tissue culture grade water without heating. Then, 0.187g of NaHCO3 was added and stirred until dissolved. The PH was adjusted to 7.1-7.2 using 1N HCl or NaOH, and the volume was made up to 50ml with water. The medium was sterilized using a sterile membrane filter (0.22µl) under positive pressure. Aspectically added supplements were dispensed into sterile containers and stored at 2-8°C in the dark.

2. Trypsinization and Subculturing:

Trypsinization involves using 500µl of trypsin to detach adherent cells after washing with phosphate-buffered saline. Following incubation at 37°C for 3 minutes, the cells were transferred to a new flask with fresh medium. For subculturing, a confluent plate was treated with 0.25% trypsin in 0.5 mM EDTA, mixed, and incubated for 3 minutes. Cell suspension (100µl) was then transferred to 24-well plats with fresh medium and incubated at 37°C with 5% CO_2.

3. Cell Seeding in 96-Well Plates

Two-day-old confluent cells were trypsinized, suspended in a 10% growth medium, and seeded at 100l (5×10^4 cells/well) in a 96-well plate. The plates were incubated at 37°C in a humidified 5% CO_2 environment.

4. Preparation of Stock Compound

a. Preparation of Metformin and TNF-α

The present work used HPLC-grade metformin as the test sample to determine its anticancer ability against HCT-116, Colorectal Cancer Cells. 1mg/ml stock was prepared in DMSO, sterilized under UV for 20 minutes, and used as the test stock in the whole study for various analyses. 10pg/ml of TNF-α was prepared by the addition of 10pg of TNF-α to 1ml of distilled water, was sterilized under UV for 20 minutes, and was used as the test shock in the whole study for various analyses.

2.1 In vitro antiproliferative effect of metformin on cultured HCT 116 cell lines using the Neutral red assay

Cell viability was evaluated by direct observation of cells using an Inverted phase contrast microscope, followed by a Neutral red assay. The assay was performed according to the method.

2.1.1 Without Induction of TNF-α

The cells treated with samples of different concentrations of metformin (100µg, 50µg, 25µg, 12.5µg, 6.25 µg in 500µl of 5% DMEM) were incubated for 24 hours before. 10 of the neutral red solution was added to culture plates treated with sample and incubated for 1 hour in a CO_2 incubator at 37°C. Cells were then fixed with 50µl of fixing solution (50% ethanol and 1% acetic acid. After 1 minute, the fixation solution was discarded and 100µg of extraction buffer was added and mixed properly. The absorbance was measured using a microplate reader at 540nm and the percentage viability was calculated.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

2.1.2 With Induction of TNF-α

The cells treated with samples of different concentrations of metformin (100µg, 50µg, 25µg, 12.5µg 6.25µg in 500µl of 5% DMEM) were incubated for 24 hours before the staining procedure started 10 of the neutral red solution was added to culture plates treated with sample and incubated for 1 hour in CO_2 incubator at 37°C. Cells were then fixed with 50µl of fixing solution (50% ethanol and 1% acetic acid). After 1 minute, the fixation solution was discarded and 100µl of extraction buffer was added and mixed properly. The absorbance was measured using a microplate reader at 540 nm and the percentage viability was calculated.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

2.2 SCRATCH MIGRATION ASSAY

The scratch wounds were made by a sterile 1ml pipette tip through a pre-marked line. After removal of resulting debris from 5 lineal scratches, the cell monolayer was subsequently rinsed 3 times with PBS followed by incubation with TNF and incubated for 48 hours further metformin of concentration of 3.1 for 0 hours, 24 hours, and 48 hours the wound areas were displayed by taking images just above the interchange between scratched wound areas and pre-marked lines and effect of a sample on wound closure was determined microscopically.

2.3 CLONOGENIC ASSAY

HCT116 cell line was cultured as per standard procedures described earlier and treated with TNF and incubated for 48 hours, then treated with LD 50 concentration from a stock of metformin 1mg/ml and incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator. Non-treated control cells were also maintained. The transfected cells were trypsinized. Clonogenic assay was performed in a 6-well plate. Finally, each assay was made in triplicate and only colonies containing at least 50 cells were counted.

2.4 AGGREGATION ASSAY

50µ L of the agar solution was transferred into each well of a 96-well microtiter plate and kept on a horizontal surface. The 1st cells were washed 2 times with 3 mL of Moscona solution. cell aggregates were evaluated after 24, 48, and 72 hours of incubation. The formation of large compact aggregates, after 72 hours, the cells were stained with crystal violet stain.

2.5 TO STUDY THE ACTIVITY OF CASPASE 7 BY INDIRECT ELISA

The trypsinized cells were then transferred to a 96-well plate where the cells were treated with the sample at a concentration of 1.5 µg/mL first incubated for 48hours after incubation the supernatant was collected and 100µL of each was added and a Concentration of protein was also estimated it was washed 2 times with PBS.50µl of primary antibody ILF BETA was added and left for 2 hours at room temperature. It was then washed with PBS TWEEN two times. The reaction stopped by adding 5N HCL (50ml). OD was read at 415 nm in an ELISA reader.

The activity of Antibody= OD Value / Protein Concentration

III. RESULTS

3.1 In vitro Anticancer Efficiency of Metformin on Cultured Colorectal Cancer Cells by Neutral Red Assay

The assay quantitatively measures viable cells and is read at OD 540nm. In this study, the HCT-116 cell line was used to evaluate the anticancer effects of metformin. Cells were treated with various concentrations of metformin, and the NRU assay was performed to assess its anticancer impact. The results indicated a 33% reduction in cell viability at a higher concentration of 100 g/ml using ED50Plus Vo.1 software. These findings confirm that metformin exhibits a strong anticancer effect against HCT-116 colon cancer cells. The percentage of viable cells is shown in the table.

Table 1: The Anticancer effect of metformin and its Percentage of viability on HCT-116 cells with Varied concentrations by using the *In-vitro* method.

Conc. of metformin (µg/mL)	Percentage of viability (%)
Control	100
6.25	89.6
12.5	68.6
25	43.2
50	36.3
100	33

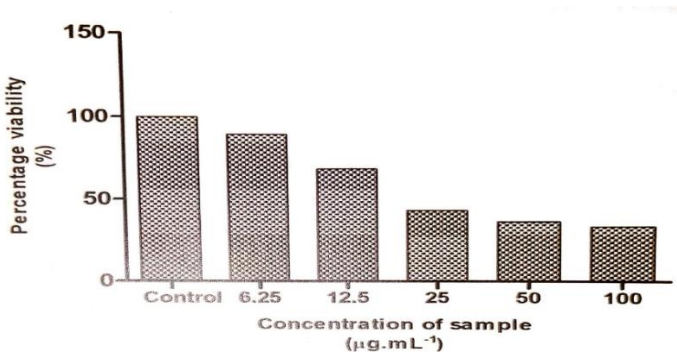

Figure 1: Graphical representation of the Anticancer effect of metformin and its Percentage of viability on HCT-116 Cells with Varied concentrations by using the *In-vitro* method.

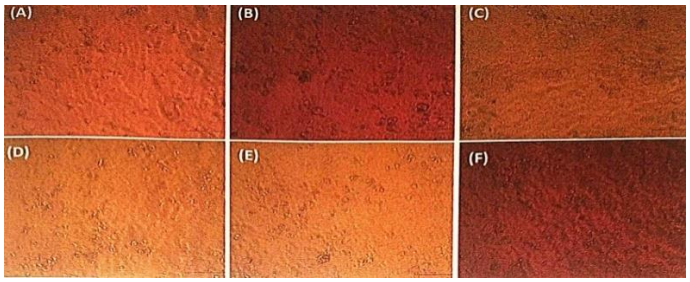

Figure 2: Photomicrographs illustrating the anticancer effect of metformin on cultured colorectal cancer cells (HCT-116) A. untreated control cells B. 6.25 C. 12.5 D. 25 E. 50 and F. 100µg/mL.

3.2 Determination of the anticancer effect of Metformin on the TNF-α Exposed HCT-116 Cells by NRU assay

In this study, HCT-116 colorectal cancer cells were treated with TNF (20pg/mL) for 48 hrs to induce metastasis. After this exposure, the cells were used to evaluate the anticancer effects of metformin. HCT-116 cells were treated with TNF- and co-administered with metformin, and the anticancer effect was assessed using the NRU assay. The significant reduction in viability confirms the anticancer effect of metformin. The percentage of viable cells is detailed in the table.

Table 2: The Anticancer Effect of Metformin and its Percentage of Viability on HCT-116 Cells exposed to TNF-α by using the *In-vitro* method.

Conc. of metformin (µg/mL)	Percentage of viability
Control	100
TNF-	54.9
6.25	39.9
12.5	33.8
25	32.6
50	29
100	26.2

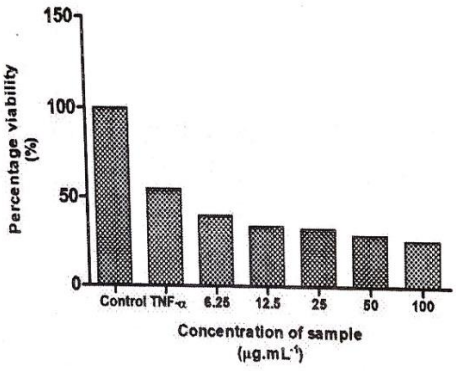

Figure 3: Graphical representation of the Anticancer effect of metformin and its Percentage of viability on HCT-116 Cells exposed with TNF-α by using the *In-vitro* method.

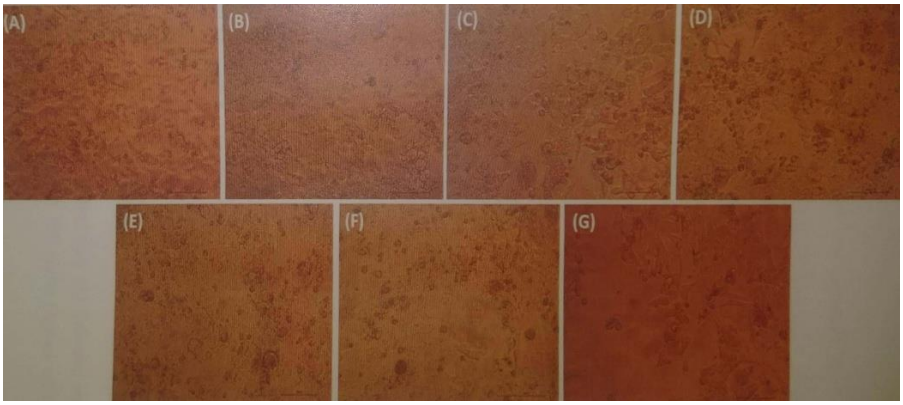

Figure 4: Photomicrographs depicting the effect of metformin co-administration cultured HCT-116 cells exposed to TNF-α. A. untreated control cells B. TNF-exposed cells with varied concentrations of metformin C. 6.25 D. 12.5 E. 25 F. 50 and G. 100µg/mL.

3.3 MIGRATION ASSAY

In this study, we understand how metformin affects TNF-α induced invasion and metastasis. During the migration assay, images are taken at 0, 24, and 48 hours. It was observed that TNF-α exposed HCT-116 cells migrated into the scratch area by 48 hours and quickly covered the scratch's center line, in contrast to the untreated control cells. The results indicate that metformin treatment reduced the number of TNF-α exposed cells migrating to the scratch.

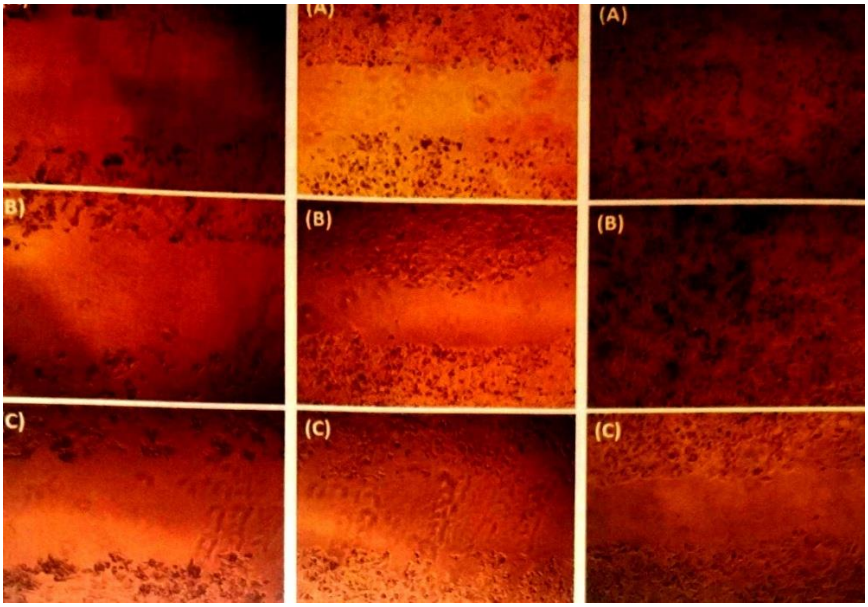

Figure 5
0th hour

Figure 6
24th hour

Figure 7
48th hour

Figure 5: Phase contrast analysis of Migration assay A. Untreated control at 0th hr B. TNF-α exposed cells at 0th hr C. TNF-α exposed cells co-treated with metformin at 0th hr.

Figure 6: Phase contrast analysis of Migration assay A. Untreated control at 0th hr B. TNF-α exposed cells at 24th hr C. TNF-α exposed cells co-treated with metformin at 24th hr.

Figure 7: Phase contrast analysis of Migration assay A. Untreated control at 0th hr B. TNF-α exposed cells at 48th hr C. TNF-α exposed cells co-treated with metformin at 48th hr.

Table 3: Migration Assay.

Sample treated	**Time Interval (in Hours)**		
	0th	24th	48th
	Wound area (px)		
Control	2381740	1218942	25681
TNF-α	2489547	98578	0
TNF-α+ metformin	2437550	1265296	659712

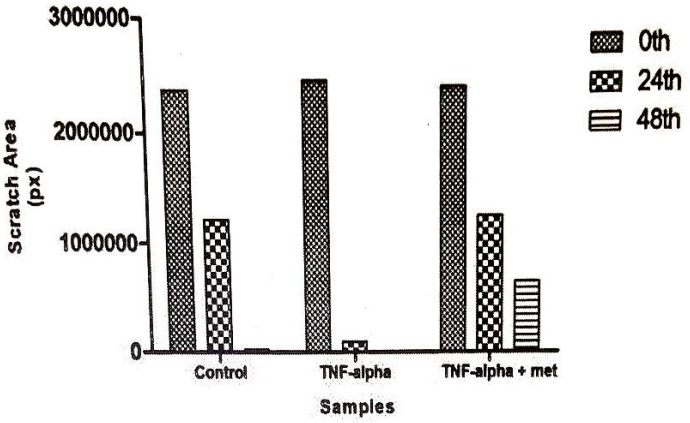

Figure 8: Graphical representation of the migration assay.

3.4 CLONOGENIC ASSAY

In this assay study, the untreated control HCT-116 cells continuously divided and formed a substantial number of colonies (224 colonies). These results indicate that metformin effectively reduces the uncontrolled division and clone formation of cancer cells.

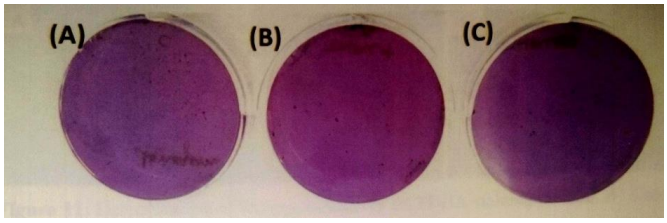

Figure 9: Determination of colony formation; Phase contrast image of colonogenic assay A. untreated control cells B. TNF-α exposed cells co-treated with metformin C. TNF-α exposed cells.

3.5 CELL AGGREGATION ASSAY

In this assay study, tumor cells formed more aggregates after exposure to TNF-α compared to untreated controls.

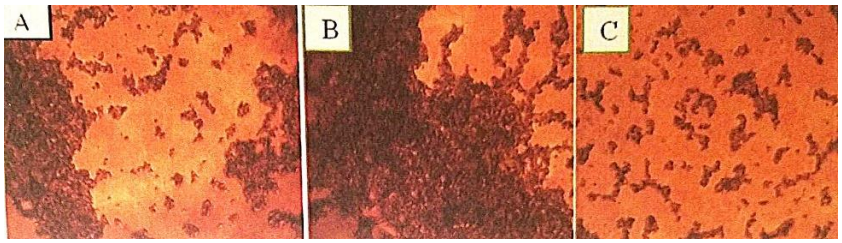

Figure 10: Determination of cell aggregation; Phase contrast image of cell aggregation, A) untreated control cells at 48th hr, B) TNF-α exposed HCT-116 cells at 48th hr, C) TNF-α exposed HCT-116 cells co-treated with metformin at 48th hr.

3.6 DETERMINATION OF IL1- β ACTIVITY BY INDIRECT ELISA

Interleukin-1β is a pro-inflammatory cytokine that plays a critical role in the interactions between cancer cells. It is involved in tumor growth and inflammation regulation within the tumor. In colorectal cancer cells, IL-1β expression and activity can be altered, showing upregulation in affected tissues. The results show that IL-1β levels were significantly higher in TNF-α exposed HCT-116 cells compared to untreated controls, whereas metformin treatment resulted in a reduction of IL-1β levels.

Table 4: IL 1 Beta Activity.

Samples treated	IL1 Beta Activity (Enzyme units/mg protein)
Control	0.0825
TNF-α	0.3583
TNF-α+ Metformin	0.2809

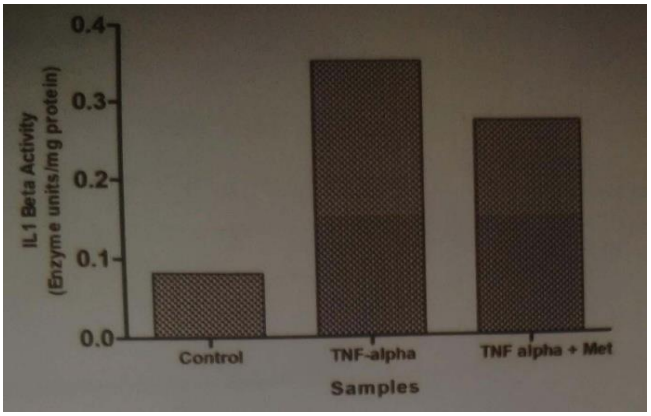

Figure 11: Graphical representation illustrating the IL 1 Beta activity upon the exposure of Metformin by indirect ELISA.

IV. DISCUSSION

Metformin, a widely used anti-diabetic drug, has emerged as a promising for cancer therapy due to its multi-faceted mechanisms of action. It has shown potential in various cancers, such as breast, colon, and pancreatic cancer, demonstrating its ability to inhibit tumor growth and enhance the efficacy of traditional therapies. Clinical trials are investigating metformin's role as an adjuvant or monotherapy in oncology, emphasizing its potential to improve cancer treatment outcomes while minimizing adverse effects. Drug repurposing offers cost-effectiveness (Mohamad Alijofan *et.al*., 2019). This study focuses on the role of TGF- as a key regulator of EMT. One key regulator is TNF- a cytokine with roles in apoptosis, angiogenesis, inflammation, and immunity. TNF- can exert both pro- and antitumor effects, mediated through two major receptors: The 55kDa TNFR1 and the 75kDa, TNFR2, each with distinct and overlapping functions. This study aimed to examine the impact of metformin on metastasis by utilizing TNF-activated human colorectal cells (HCT-116) as an in vitro model. The well-established anticancer effects of metformin were further validated following a 24-hour treatment of HCT-116 cells stimulated with TNF- the NRU assay showed that metformin demonstrated strong anticancer efficacy by lowering cell viability and demonstrating its capacity to target activated cancer cells. It was observed that HCT-116 cells stimulated by TNF- were able to seal the wound in a matter of 24 to 48 hours. Treatment with metformin shows significantly reduced cell migration, demonstrating its capacity to obstruct EMT-related mechanisms.

The development of colorectal cancer and cytokines such as interleukin-1 contribute to and support tumor growth. Specifically, IL-1 is a strong inflammatory mediator in the tumor. Indirect ELISA analysis revealed a notable elevation of IL-1 in TNF-activated HCT-116 cells in this investigation, while metformin co-treatment decreased IL-1 levels. The TGF signaling pathway was suppressed by TNF treatment in HCT-116 cells, contributing to enhanced metastasis. This supports the hypothesis that metformin not only inhibits metastatic potential but also reactivates critical tumor suppressive mechanisms in colorectal cancer.

V. CONCLUSION

The present study investigates the inhibitory effects of metformin on EMT in TNF-activated HCT-116 colon cancer cells. Metformin demonstrated a dose-dependent anticancer effect with an IC50 value of 21.6µg/mL, as determined by the NRU assay, accompanied by notable morphological changes. Cells were activated with 20µ µg/mL of TNF, and metformin treatment significantly showed the anticancer activity. Metformin shows significant potential as an anti-metastatic agent in colorectal cancer by inhibiting EMT, reducing pro-inflammatory cytokines, and restoring tumor suppressor pathways such as SMAD4. These findings suggest that metformin could be a promising way to prevent metastasis and enhance the therapeutic efficacy of colorectal cancer treatments (Muhamad Noor Alfarizal Kamarudin *et.al*., 2019).

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