An In-vitro Pilot Study to Explore Therapeutic Adjuvant Potential of *Tinospora cordifolia* with Methotrexate in Rheumatoid Arthritis Management

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BY

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UNDER THE GUIDANCE OF

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

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DECLARATION BY THE CANDIDATE

I declare that this thesis "**An In-vitro Pilot Study to Explore Therapeutic Adjuvant Potential of *Tinospora cordifolia* with Methotrexate in Rheumatoid Arthritis Management"** submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bangalore, is my original work, conducted under the supervision of Dr. Girish Tillu (and co-supervision of Dr. Akash Saggam). I confirm that no part of the work reported herein has been submitted for a degree or examination at any other University. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.

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CERTIFICATE FROM THESIS SUPERVISOR/S

This is to certify that the work incorporated in this thesis **"An In-vitro Pilot Study to Explore Therapeutic Adjuvant Potential of *Tinospora cordifolia* with Methotrexate in Rheumatoid Arthritis Management "** submitted by Jyothish G Nair was carried out under my/our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

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SUMMARY

Rheumatoid arthritis (RA) is a chronic inflammatory disease with increasing global burden. It is characterized by pain, swelling and inflammation at the site of bone joints. The inflammatory response plays a crucial role in RA pathogenesis. The present therapeutic approaches include extensive use of disease-modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX) with known adverse effects. Ayurveda, an Indian traditional medicine system may offer cues to manage RA. *Tinospora cordifolia* (TC/Guduchi) is an extensively prescribed botanical in Ayurveda practice for management of chronic inflammatory conditions. The present study explored the adjuvant potential of aqueous extract of TC (TCA) in combination with MTX using SW982 cells.

The cytotoxicity of MTX and TCA on SW982 cells was tested using MTT assay. The SW982 cells were dosed with logarithmically increasing concentrations of MTX (0.01 – 1000nM) and TCA (0.01 – 1000 µg/ml) to identify IC25 value using dose-response curve. The anti-inflammatory potential of MTX and TCA alone and in combination was evaluated by cell migration assay. The scratches were made in confluent monolayer SW982 cells followed by induction of inflammation by 5 µg/ml of lipopolysaccharide (LPS). The monolayers with scratch were dosed with MTX (450 nM) and/or TCA (75, 88.5, and 100 µg/ml). The scratches were observed under 10X microscope and images were captured before and after 24 hrs. of dosing. The scratch images were analyzed using ImageJ software followed by statistical analysis and graphical representation using GraphPad Prism 9.1.1.

The cytotoxicity assay showed IC25 values of TCA ($p<0.0001$) and MTX ($p<0.0002$) as 88.511 ± 4.879 µg/ml and 442.588 nM respectively. The anti-migratory potential of MTX was enhanced by TCA (LMTC1-103.39± 2.44%, LMTC2-101.2839± 4.1304 %, and LMTC3-106.2± 3.3001 %) as compared to LPS group (130.8965% ± 2.8937).Overall, the present study showed adjuvant potential of TCA in combination with MTX to manage inflammation in RA.

PERSONAL REFLECTION

The past seven months have been amazing with numerous new academic and social skills learning. The new technical learning were mainly cell culture techniques, instrumentations; using new software like Graph pad prism, image j software etc. Also I learned how to plan and execute experiments within given time points and within our limited resources. I gained experience by ordering kits, the media needed for cells, and cell lines from NCCS Pune. Also I acquired knowledge about rheumatoid arthritis and related research works. In CCIH (Center for complementary and integrative health) I participated in various activities like Saturday clubs and some seminars. Along with lab procedures, I had the chance to present four speeches on varied topics to distinct audiences. I visited Tilak Ayurveda Mahavidyalaya to give a talk about Ayurveda biology as a guest speaker for a postgraduate orientation programme. I also had the chance to take part in a pilot pharmaco kinetics clinical experiment at the university health centre in Pune. These were great experiences. Living outside from Pune University has exposed me to a variety of individuals, cultures, and languages. Along with my mentor Dr. Girish Tillu and his team, I also participated in three traditional Maharashtra rituals. With the help of all these social connections, I was able to adjust with the ups and downs and less stressed overall.

Contents

LIST OF ABBREVIATIONS

Abbreviations	Definitions
1. TC	Tinospora cordifolia
2. TCA	Tinospora cordifolia aqueous extract
3. MTX	Methotrexate
4. LPS	Lipopolysaccharide
5. LM	Lipopolysacchaide + Methotrexate
6. LTC	Lipopolysaccharide + Tinospora cordifolia
7. LMTC	Lipopolysaccharide + Methotrexate + Tinospora cordifolia
8. PBS	Phosphate Buffer Saline
9. FBS	Fetal Bovine Serum
10. IL	Interleukin
11. TNF	Tumor Necrosis Factor
12. MAPKs	Mitogen-activated Protein Kinase
13. PGE	Prostaglandin
14. COX	Cyclooxygenase
15. NSAIDs	Nonsteroidal Anti-inflammatory Drugs
16. DMARDs	Disease Modifying Antirheumatic Drugs
17. FLS	Fibroblast LikeSynoviocytes
18. RA	Rheumatoid Arthritis
19. NF-KB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
20. RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
21. SW 982	Human Synovial Sarcoma Cells
22. DMSO	Dimethyl Sulfoxide

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1. INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory condition that can affect various body systems, including the joints, skin, eyes, lungs, heart, and blood vessels. It is classified as an autoimmune disorder, characterized by joint inflammation resulting in pain, stiffness, and reduced mobility. The immune system attacks the synovium, the lining of the joints, leading to swollen, warm, and painful joints. While the exact cause of RA remains unknown, it is believed to be influenced by a combination of genetic and environmental factors. According to a meta-analysis based on a systematic review, the global prevalence of RA between 1980 and 2019 was reported to be 460 cases per 100,000 populations(Almutairi et al., 2021).As inflammation plays a crucial role in RA, treatment options typically involve a combination of medications, such as non steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti rheumatic drugs (DMARDs), and biologic therapies, alongside physical therapy and lifestyle adjustments. The potential side effects of DMARDs and NSAIDs make scientists think about the possibilities for treatment from traditional medical systems. The available drugs against Rheumatoid arthritis from different systems of medicines are not experimented in combinations. Understanding the results of combining an allopathic medication with an ayurvedic medication is the goal of this study. In Ayurveda, an ancient Indian medicinal system, the herb Guduchi (*Tinospora cordifolia*) is commonly included in formulations to treat RA. *Tinospora cordifolia*, belonging to the Menispermaceae family, is a climbing shrub native to India and nearby regions. Its extract (TC) has been employed in various herbal remedies to address different inflammatory conditions. In numerous in vitro models, TC and its derived chemicals have exhibited immunomodulatory, anti-proliferative, and anti-angiogenic properties(Dhama et al., 2017). Ayurvedic texts mention Guduchi's ability to reduce Aama (unmetabolized or partially metabolized substances formed in the gut and cellular level) and promote metabolism and digestion through its pachana property.

In modern medicine, Methotrexate is a commonly used drug to manage Rheumatoid arthritis. It is coming under class of drugs called disease-modifying antirheumaticdrugs(DMARDS) and is considered as the first line of treatment. It works by suppressing the immune system, thereby reducing the production of inflammatory cells. It can reduce joint pain, swelling, stiffness and can slow down the progression of disease(Z. Zhao et al., 2022).

SW982- Human Synovial Sarcoma Cell line is a well-known model, isolated from human joint synovium. This cell line is utilized to investigate the anti-inflammatory potential of drugs like dexamethasone and fluvastatin through in vitro experiments(J. H. Chang et al., 2014). Inflammation-related cytokines like IL-1, IL-6, or TNF- alpha stimulate FLS (Fibroblast like synoviocytes) during the development of RA, converting their phenotype into that of cancer (RA FLS). As a result of cells losing their dependency on anchoring and contact inhibition and gaining strong proliferative and migratory capacities, followed by forming a structure resembling tumor called pannus. RA FLS not only grow quickly but also release a variety of chemicals that exacerbate the condition. Activated synoviocytes encourage the local inflow of immune cells aggravating autoinflammation by releasing large amounts of cytokines, chemokines, and adhesion molecules. The digestion of the cartilage and bone tissues is directly caused by the production of various metalloproteinases (MMPs) and cathepsin K. By creating M-CSF (macrophage colony-stimulating factor) and expressing RANKL (receptor activator of nuclear factor kappa-ligand), which promote osteoclastogenesis and further degrade the tissues, they also contribute to the activation of chondrocytes (Więcek et al., 2022).Due to the significant inflammation observed in RA, many traditional remedies have been explored for its management. Several botanicals, including turmeric, sesame, Ginkgo biloba, and the active compounds of zingibercassumunar, have been tested using the SW982 cell line(Prasad & Aggarwal, 2011; Rosillo et al., 2019; Y. Zhao et al., 2021)
In this study, properties of *Tinospora cordifolia* (TC) and Methotrexate were evaluated by measuring its effect on the cell toxicity and migration rate profile of SW982 cells. SW982 cells were treated with varying concentrations of *Tinospora Cordifolia* aqueous extract and Methotrexate(MTX) for 24 hours. Cell viability was determined using MTT assay. Also both *Tinospora cordifolia* and Methotrexate were used separately and in combinations to analyze the effect on cell migration of inflamed SW982 cells induced by LPS.

1.1. Pathogenesis of RA

Rheumatoid arthritis (RA) has a complicated aetiology that combines genetic, environmental, and immunological variables.Since rheumatoid arthritis (RA) has a higher occurrence among individuals with a familial background of the condition, it is evident that genetic factors contribute to its onset. A higher chance of having RA has been linked to specific genes, such as the HLA-DRB1 gene(Dedmon, 2020).Smoking and certain

infections have been linked to an increased risk of getting RA, among other environmental variables. One of the most well-known environmental risk factors for RA is exposure to cigarette smoke(K. Chang et al., 2014).An important process happening in pathogenesis of RA is Citrullination. It is the conversion of the amino acid arginine in a protein into the amino acid citrulline by peptidylargininedeiminase(PADs). During this conversion, the charge of amino acid is changing from positive to no net charge. This leads to change in protein folding and its structure. This citrulline acts as an autoantigen(Ciesielski et al., 2022). Antigen Presenting Cells (APC) recognize these autoantigen leads to activation of immune response. APCs then migrate to lymph nodes and lead to activation of T cells, B cells and produce autoantibodies. These autoantibodies then migrate to joints through blood vessels.Thesereactions stimulate macrophages for producing inflammatory molecules(TNF alpha, IL-1,IL-6) leading to inflammation(Kondo et al., 2021). Neutrophils present in synovial fluid produce reactive oxygen species and proteases lead to destruction of surrounding tissues.Inflammation and joint injury over time may result in the deterioration of bone and cartilage, which may result in deformities and disability. The lungs, heart, and blood vessels can all be impacted by the chronic inflammation linked to RA, increasing the risk of problems(Conforti et al., 2021).

1.2. Important Biomarkers in RA

Biomarkers like MMPs, IL-6, TNF Alpha, MAPKs, NF-kB pathways, RANKL, Prostaglandins and COX are playing an important role in pathogenesis of RA (Table 1).

Table 1 Biomarkers involved in RA with clinical features

SL NO	BIOMARKER	CLINICAL FEATURES	REFERENCES
1	MMPs	Pain, Enzymatic destruction,Bone erosion.	(Roomi et al., 2013)
2	IL-6 & TNF	Inflammation, Pain, Bone Erosion.	(Kondo et al., 2021)
3	MAPKs	Pain, Inflammation, Bone Destruction.	(Kondo et al., 2021)
4	NF-kB	Inflammation, Inhibition of apoptosis	(Ilchovska & Barrow,

		Immune cell proliferation, Bone resorption	2021)
5	RANKL	Bone erosion, Proliferation of T cells and B cells, Inflammation	(Tanaka & Tanaka, 2021)
6	PGE	Pain, Inflammation, Bone destruction	(Wang et al., 2011)
7	COX	Pain, Inflammation, Bone destruction	(Wang et al., 2011)

1.3. Ayurveda Biology view

In Ayurveda a disease called Amavata has symptomatic similarity with RA. Symptoms like stiffness, body pain, fatigue, fever and swelling of joints are common in RA and Amavata. So the hypothesis was whether the herbs mentioned to treat Amavata can be used for treating RA also. Biomarkers associated with RA can be used to assess efficacy of various Ayurveda herbs mentioned in Amavata. It has been noted that patients who have been given an ACR(American College of Rheumatology) or EULAR(The European Alliance of Associations for Rheumatology) diagnosis of RA who are also given an Ayurvedic diagnosis of amavata and are treated in accordance with these principles do well with Ayurvedic therapies. Systemic characteristics respond favorably and quickly in instances treated with Ayurveda(Pandey et al., 2023). RA cases who were undergoing Ayurvedic treatments report lower fatigue, stiffness, lack of interest, bettered appetite and increased weight (Basisht et al., 2012). Although the extent to which these systemic symptoms are related to the fundamental joint pathology in RA is unknown, the fact that they improve concurrently with a decrease in joint symptoms after ayurvedic treatment suggests that they are. These results provide a hint about the potential of Ayurveda formulations in RA. Based on Ayurveda Samhitas, samprapti(pathogenesis) of Amavata shows the involvement of Tridosa and formation of Ama from undigested food. Interactions of Ama with Tridoshas are called Samadosha(Sumantran& Tillu, 2012). Sama(involvement of Ama)state should be reversed to Nirama state. Herbs that can remove Ama as well as normalize doshas are usually selected to treat Amavata. Furthermore, Ayurvedic texts suggest that Ama can accumulate in specific areas of the body, such as the joints, where it can contribute to the development of Amavata. In modern biological terms, this Ama can be hypothesized as the accumulation of pro-inflammatory cytokines and other molecules in the joints, leading to

inflammation, pain, and joint damage.

1.4. Symptomatic similarities

Ayurvedic classical text Madhava Nidanam mentioned about Amavata in “Amavatanidana” (25th chapter). The symptoms mentioned in that chapter are compared with symptoms of RA (Table 2).

Table 2 Common symptoms seen in Amavata and RA

AMAVATA	**RHEUMATOID ARTHRITIS**
Sthabdam	Stiffness
Angamarda	Body Pain
Alasyam	Fatigue
Jwara	Fever
Soonataanganam	Swelling of joints
Apaka	Indigestion, Citrullination(cellular level).
Dhamanis Involved	Vasculitis, hematologic abnormalities

1.5. *Tinospora cordifolia* and Rheumatoid arthritis

Tinospora cordifolia is a herb that has been traditionally used in Ayurveda to reduce Aamavishas (toxic metabolic waste). Research has shown that *Tinospora cordifolia* can enhance the activity of macrophages and natural killer cells, which play a key role in removing foreign particles and toxins from the body(Dhama et al., 2017). Furthermore, *Tinospora cordifolia* has been shown to possess anti-inflammatory properties. Research has demonstrated that it can inhibit the production of pro-inflammatory cytokines, such as TNF-alpha and IL-1 beta, which are known to contribute to joint inflammation and pain(Pharmacol, 2021). As RA is associated with the production of pro-inflammatory cytokines like TNF Alpha, *Tinospora cordifolia* can be a best choice to check its efficacy against RA. Based on the Rasapanchaka and karmas of Guduchi, it is expected to reduce

Tridosa(mainly pitta) as well as Ama. Guduchi is proven for its anti-rheumatoid arthritis potential by showing positive impact on NF-kB ligand (RANKL),M-CSF,MMP-9,IL-1β,IL-6,IL-23,TNF-αandMMP-9(Pharmacol, 2021).

As the symptoms of RA and Amavata are nearly identical, *Tinospora cordifolia* can control RA if it is successful in treating Amavata. TNF Alpha and other RA-relevant biomarkers can be used to study this. In this study, properties of *Tinospora cordifolia* were evaluated by measuring its effects on the inflammatory profile of SW982 cells, induced by stimulation with LPS. To assess the effects of *Tinospora cordifolia* on the chosen cell line, a series of experiments were conducted including MTT survivability assays and analysis of migration using Scratch wound healing assay.

1.6. SW 982 cell line and Rheumatoid arthritis

SW 982- Human Synovial Sarcoma Cell line is the best-known model used to study synovitis in RA. SW982 cells are isolated from synovium of human joints. Dulbecco's Modified Eagle Medium can be used to culture these cells.

The SW982 cell line is a human synovial sarcoma cell line that has been used in research studies related to rheumatoid arthritis (RA). Synovial tissue, which lines the joints, is a major site of inflammation in RA, and the SW982 cell line has been used as a model to study synovial fibroblasts, which are involved in the development and progression of RA. Synovial fibroblasts, which are the primary cells in the synovial lining of joints and play a key role in the pathogenesis of RA, can be isolated from synovial tissue from patients with RA and cultured as the SW982 cell line. This provides a relevant human cell model for studying the behavior and response of synovial fibroblasts in the context of RA.This cell line is used to study the anti-inflammatory potential of drugs such as dexamethasone and fluvastatin in vitro experiments(J. H. Chang et al., 2014). Several studies have used the SW982 cell line to investigate the effects of various treatments on synovial fibroblasts, including anti-inflammatory agents and disease-modifying anti rheumatic drugs (DMARDs). In addition, the SW982 cell line has been used to investigate the molecular mechanisms underlying the pathogenesis of RA, such as the role of cytokines and transcription factors in the activation of synovial fibroblasts. In addition, the SW982 cell line has been extensively characterized and is a well-established tool for studying synovial fibroblast biology in vitro. Its use in RA research has resulted in many important findings, including the identification

of key signaling pathways and molecular targets involved in synovial fibroblast activation and the development of RA(Wada et al., 2005).

As RA is a disease having high levels of inflammation, lots of traditional medicines were used for its management. Some of the botanicals tested in SW 982 are *turmeric, sesame*, *Ginkgo biloba*, active compound of *zingiber cassumunar* etc. SW982 cells are isolated from synovium of human joints(Y. Zhao et al., 2021).

2. MATERIALS AND METHODS

2.1. Botanical extracts

The standardized and authenticated botanical extract of TC was (Annexure 1) obtained from Pharmanza Herbal Pvt. Ltd. (Table 3). The raw material i.e., stems of TC was received from authentic sources.

Table 3 Details of botanical extracts

Extracts	**Batch No.**
Tinospora cordifolia aqueous extract	LT02EG2203J06

2.2. Cell culture

The SW 982cells were cultured in T-25 or T-75 cell culture flasks in complete growth media containing DMEM medium with 2mM L-glutamine, 10% FBS and 1% antibiotics. Culture reagents are from HIMEDIA. The cells were incubated at 37° C with 5% CO2to achieve desired growth and proliferation. 48th passage cells were employed for the tests. The used medium was renewed 2 to 3 times per week. The cells were subcultured after trypsinization using trypsin-EDTA solution once reaching approximately 80-90% confluency in aseptic condition under laminar air flow. The cell count was done by trypan blue exclusion method.

2.3. Trypan blue exclusion assay

Principle-

Trypan blue dye only enters into the cells with ruptured membranes because it is cell membrane impermeable. When trypan blue enters the cells, it binds with intracellular proteins thereby giving the cells a blue color. This assay allows us direct identification of live and dead cells in a given sample.

Protocol-

With 0.5 ml of trypsin, cells are trypsinized, then incubated for 4 minutes. After incubation, the cells got detached from the floor of flask and could be seen under an inverted microscope. Then addition of the entire media and proper mixing of the cells with the media is done. Then, 20 µl of the cell suspension and 20 µl of trypan blue are collected and mixed properly. After injecting a little amount of the sample into the hemocytometer, the cells are counted under an inverted microscope. With the use of a hemocytometer, the numbers of live and dead cells per millilitre were calculated. The percentage of viable cells was calculated by dividing the number of alive cells by the total number of cells, then multiplying that figure by 100.

2.4. Extract preparation

Tinospora Cordifolia (TC) Preparation

100mg *Tinospora cordifolia* aqueous extract was taken in a 15ml falcon tube.
Then 10ml 1x PBS is added into it. The necessary mixing was then carried out using a vortex machine, sonicator, and rotospin.

Methotrexate (MTX) Preparation

100 µl of DMSO and 45.444 mg of MTX are combined to create the main stock. Then, 1 micro liter of main stock MTX is combined with 999 micro liters of distilled water to create intermediate stock. Then, 10 micro liters of intermediate stock and 990 micro liters of distilled water are combined to create working stock.

2.5. Solubility studies of *Tinospora cordifolia* aqueous extracts

Principle

A substance's solubility is the greatest amount of that substance that will dissolve in a given volume of solvent at a given temperature. A substance's solubility is influenced by the physical and chemical characteristics of the solute and solvent, as well as by temperature, pressure, and the existence of other chemicals in the solution. To identify a compound's ability to dissolve in a solvent, solubility studies are carried out. As per Indian pharmacopeia

approximate volume of solvent required to dissolve per gram of solute is mentioned in table 4.

Table 4 Solubility as per Indian pharmacopoeia 1996

Descriptive term	**Approximate volume of solvent in milliliters per gram of solute.**
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Insoluble or practically insoluble	More than 10000

Instruments needed**:** Weighing balance, Vortex.

Miscellaneous**:** Butter paper, spatula and 15 ml tubes.

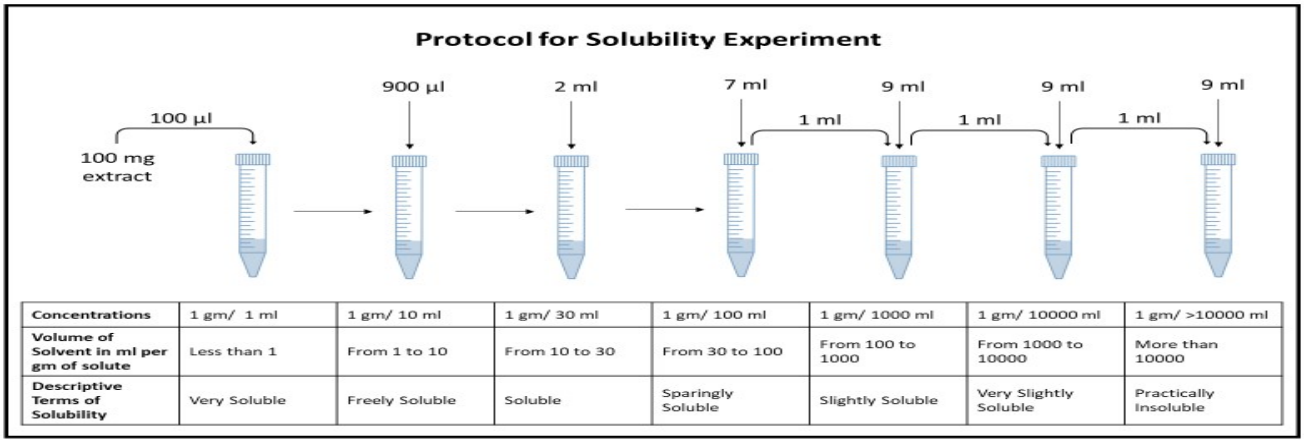
Solvents**:** Distilled water, 70 % ethanol, DMSO

Extract**:** The following extract was used to perform solubility studies

Table 5 Details of botanical extracts

SL No	**Name of extract**	**Acronym**	**Batch No.**
1	*Tinospora cordifolia* aqueous extract	TCA	LT02EG2203J06

Methodology:



Concentrations	1 gm/ 1 ml	1 gm/ 10 ml	1 gm/ 30 ml	1 gm/ 100 ml	1 gm/ 1000 ml	1 gm/ 10000 ml	1 gm/ >10000 ml
Volume of Solvent in ml per gm of solute	Less than 1	From 1 to 10	From 10 to 30	From 30 to 100	From 100 to 1000	From 1000 to 10000	More than 10000
Descriptive Terms of Solubility	Very Soluble	Freely Soluble	Soluble	Sparingly Soluble	Slightly Soluble	Very Slightly Soluble	Practically Insoluble

Figure 1 Protocol for solubility experiment

Solubility of extracts was performed as depicted in Figure 1. The extracts were dissolved in mentioned volumes of respective solvents. However, further steps of dilutions were skipped when found soluble. Briefly, the steps of dilutions were as follows:

1. 100 mg of desired extract was dissolved in 100 µl of desired solvent in a 15 ml tube. A vortex was applied to the tube.
2. To above tube 900 µl of desired solvent was added and subjected to vortex.
3. To above tube 2 ml of desired solvent was added and subjected to vortex.
4. To the above tube 7 ml of desired solvent was added and subjected to vortex solvent.
5. From above tube 1 ml of solution was aspirated and 9 ml solvent was added.
6. From above tube 1 ml of solution was aspirated and 9 ml solvent was added.
7. From above tube 1 ml of solution was aspirated and 9 ml solvent was added.

A substance's solubility is the greatest amount of that substance that will dissolve in a given volume of solvent at a given temperature.

2.6. Cell cytotoxicity studies

Principle

The MTT assay is a widely used colorimetric test employed to assess cellular metabolic activity, providing valuable information about cell viability, proliferation, and cytotoxicity. In this assay, active cells metabolize a yellow tetrazolium salt called MTT, converting it into

purple formazan crystals that are soluble in DMSO. This transformation occurs due to the presence of mitochondrial dehydrogenase enzymes in living cells.

Protocol

To perform the assay, cells were initially seeded at a density of 10^5 cells per well in 96-well culture plates and allowed to culture for one day. Afterward, the cells were exposed to varying concentrations of *Tinospora cordifolia* aqueous extract for 24 hours. The SW982 cells were dosed with logarithmically increasing concentrations of MTX (0.01 – 1000 nM) and TCA (0.01 – 1000 µg/ml) to identify IC25 value using dose-response curve. Subsequently, the MTT solution (5 mg/ml) was added to each well and incubated for 4 hours in a CO_2 incubator.

Following the 4-hour incubation, the 96-well plates were centrifuged at 2000 RPM for 10 minutes at a temperature of 20 degrees Celsius. The supernatant was then carefully removed, and 100 µl of DMSO was added to each well to solubilize the formazan crystals. The plate was further incubated for 10 minutes, allowing the crystals to dissolve. To ensure thorough mixing, the plate was shaken on a multiwell shaker for 10 minutes.

Finally, the absorbance of the samples was measured at a wavelength of 570 nm using a Multiskan PC spectrophotometer. This measurement provides an indication of the amount of formazan product generated, which correlates with the metabolic activity of the cells.

2.7. Scratch wound healing assay

Principle

In vitro cell migration is frequently studied using the migration test, usually referred to as the scratch assay or wound healing assay. It entails making a scratch or cell-free space in a confluent monolayer of cells and watching how cells flow into the scratched area as time passes.

Protocol

SW982 cells were seeded at a density of 1×10^5 cells per well on a 24-well plate and allowed to form a confluent monolayer. Subsequently, a scratch wound was created in each experimental group using a 1000-µl pipette tip, and the non-adherent cells were removed by washing with PBS. The resulting scratch wounds were documented using a microscope and recorded photographically. Following the creation of the scratch wounds, the cells were

subjected to different treatment conditions for duration of 24 hours.The cells with scratch were dosed with MTX (450 nM) and/or TCA (75, 88.5, and 100 µg/ml). The treatments included LPS (5 µg/ml) alone, LPS+MTX (450 nM), LPS+TC1 (75 µg/ml), LPS+TC2 (87.5 µg/ml), LPS+TC3 (100 µg/ml), LPS+MTX+TC1, LPS+MTX+TC2, and LPS+MTX+TC3. The scratches were observed under 10X microscope and images were captured before and after 24 hrs of dosing. The scratch images were analyzed using ImageJ software followed by statistical analysis and graphical representation using GraphPad Prism 9.1.1.

2.8. Image j software standardization

The image J software is a tool used for quantitative image analysis. Cell migration and cell division can be examined in cell tracking. Therefore, we can use image j software to compare the size of the scratch before and after dosing in the scratch wound healing assay. When analyzing the scratch, the ability of image j software to change the variance radius and threshold is crucial. The measurement is more precise when higher the threshold. However, more thresholds will occupy part of the radius cells for minor scratches. Standardization of threshold is therefore crucial before moving forward with the analysis. Four pictures with significant scratches and four pictures with very little scratches were taken and three different thresholds were used in the analysis. For each image, the thresholds used were 100, 150, and 200. Then, by comparing area and successful edge detection, we arrived at the analysis threshold.

3. RESULTS AND DISCUSSION

3.1. SW 982 cells images at different time points

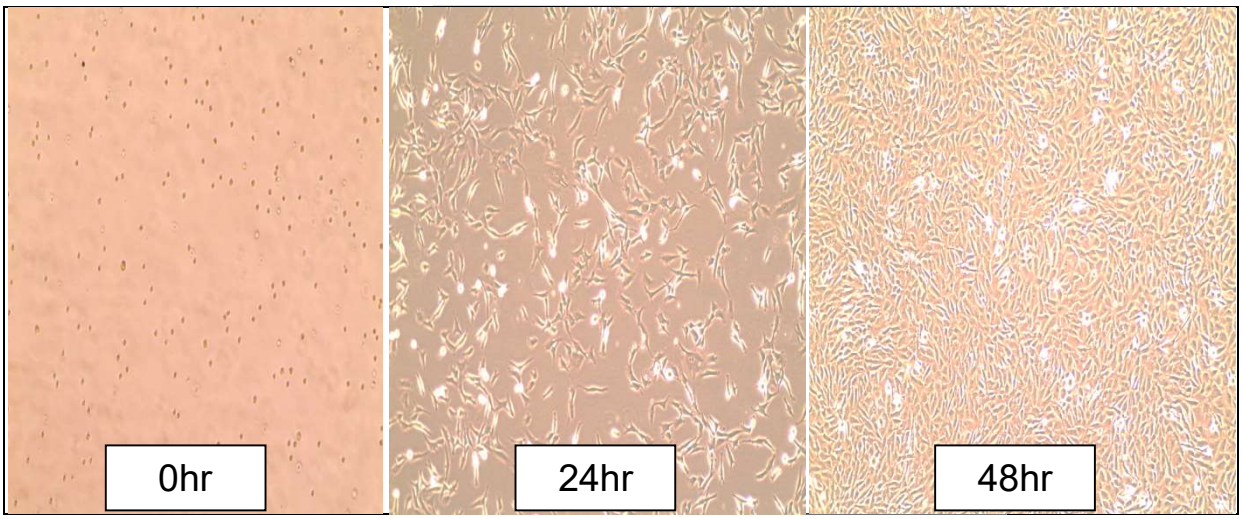


Figure 2 SW 982 cells at different time points after seeding the cells in T 75 flask.

3.2. Trypan blue exclusion assay

Total numbers of live cells were 14.2×10^5 cells/ml.

Total numbers of dead cells were 3.75×10^5 cells/ml

Cell viability percentage= (Total number of live cells/ Total number of cells) × 100.

Cell viability percentage= 79.1%.

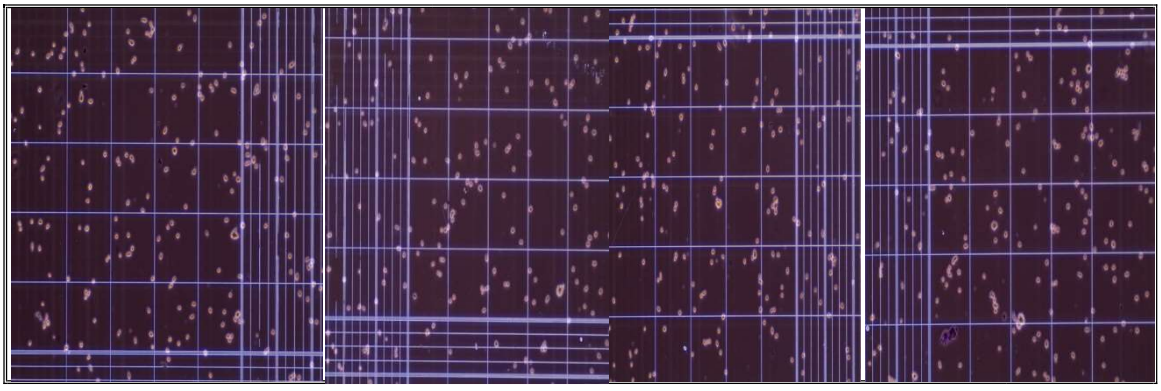


Figure 3 Cells stained with trypan blue observed under microscope using hemocytometer.

3.3. Solubility study

Aqueous extract of *Tinospora cordifolia* is very slightly soluble in distilled water and it is practically insoluble in both 70% ethanol and DMSO as shown in Table 6 and Figure 4.

Table 6 Aqueous extract of: *Tinospora cordifolia*

SL. No.	Solvents	Observations
1	Distilled water	VERY SLIGHTLY SOLUBLE
2	70%Ethanol	PRACTICALLY INSOLUBLE
3	DMSO	PRACTICALLY INSOLUBLE

Figure 4 Solubility test of *Tinospora cordifolia* in different solvents

.

3.4. MTT cytotoxicity studies

The dose-response curves (Figure 5) of cytotoxicity Methotrexate (MTX) and botanical extract (TCA) to Human Synovial Sarcoma cell line (SW 982) identified the IC25 values (Table 7). These IC25 value represent the concentration of test material at which 25% of cells are killed.The cytotoxicity assay showed IC25 values of TCA($p<0.0001$) and MTX($p<0.0002$) as 88.511 ± 4.879 µg/ml and 442.588 nM respectively.

Table 7 Values of MTX and TC in SW 982 Cell lines

Extract or Drug	IC 25 Value
MTX	442.588 nM
TCA	88.511 ± 12.5 µg/ml

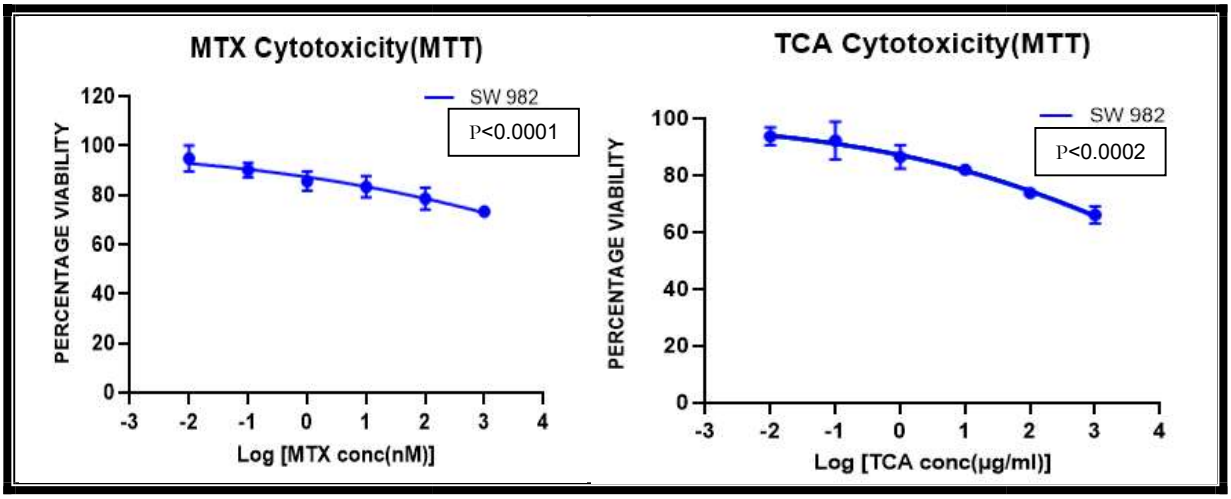


Figure 5 Methotrexate and Tinospora cordifolia MTT Cytotoxicity results in SW 982 Cell lines

3.5. Image j software standardization

After comparing area and successful edge detection, we finalized 150 as the threshold for further analysis. With highest threshold (200) in minor scratch images, some cells were occupied inside the radius. So by comparing all the images with different thresholds, we come to a conclusion to select 150 as the threshold (Figure 6).

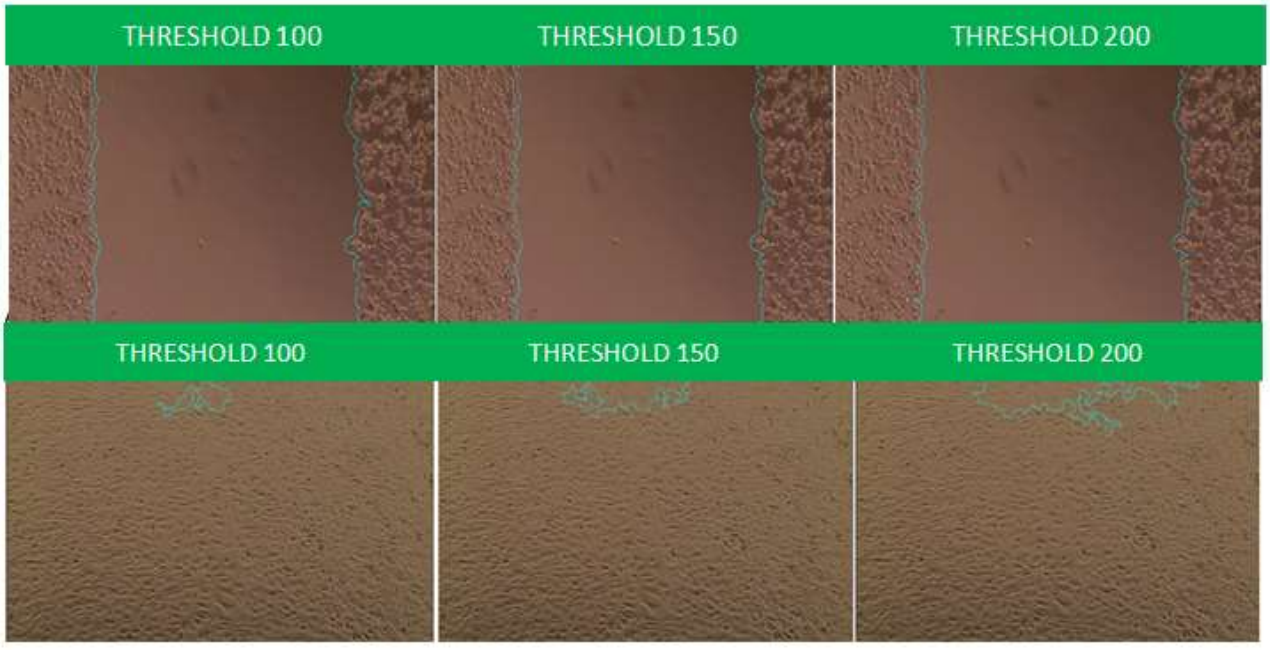
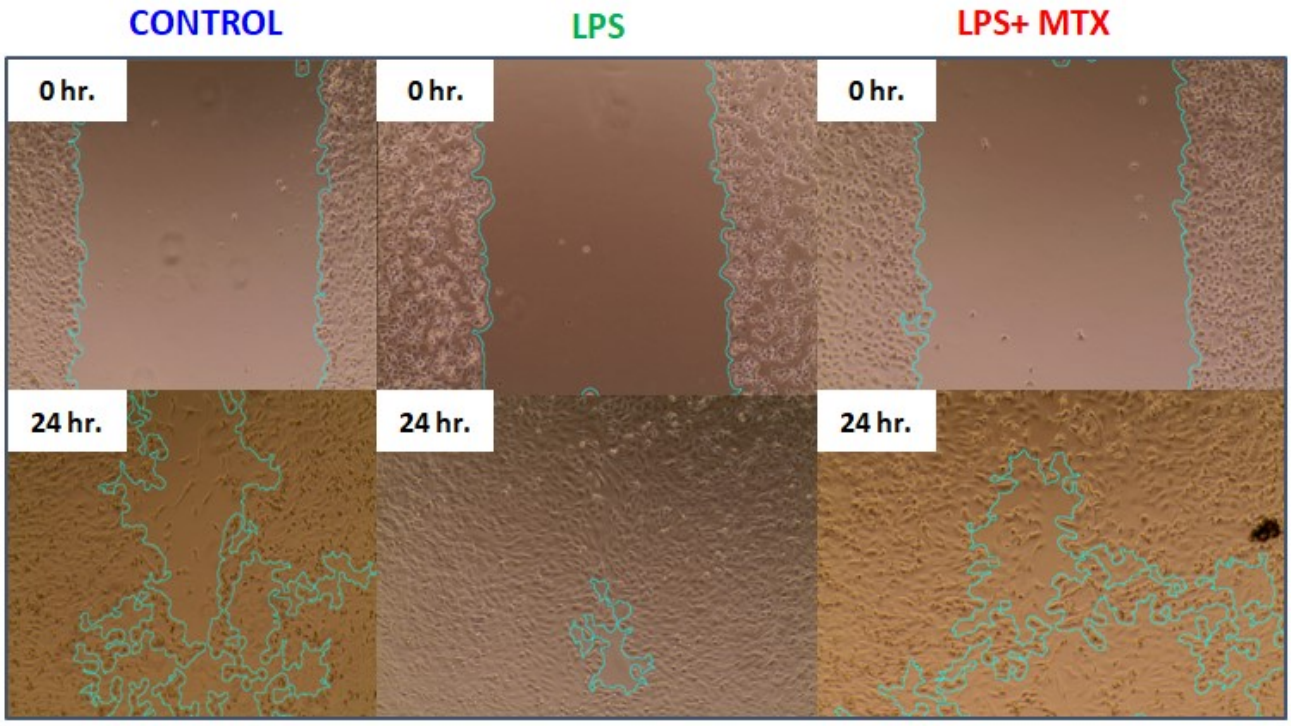


Figure 6 Standardizing the Image j software with different thresholds

.

3.6. Scratch wound healing assay

The migration rate (%) in the LPS-treated group exceeded 130% while it was close to 100% in the LPS-free control group. The cell migration assay revealed significant (p=0.0237) anti-migratory potential of MTX and TCA when dosed in combination(LMTC1-103.39± 2.44%, LMTC2-101.28± 4.13 %, and LMTC3-106.2± 3.3 %) as compared to LPS group (130.8965% ± 2.89).The combination therapy (MTX+TC groups) showed promising results to reduce the migration rate of cells close to the control group, despite the fact that single drug therapies like MTX group and TC group were unable to do so for SW 982 cells(Figure7&8)



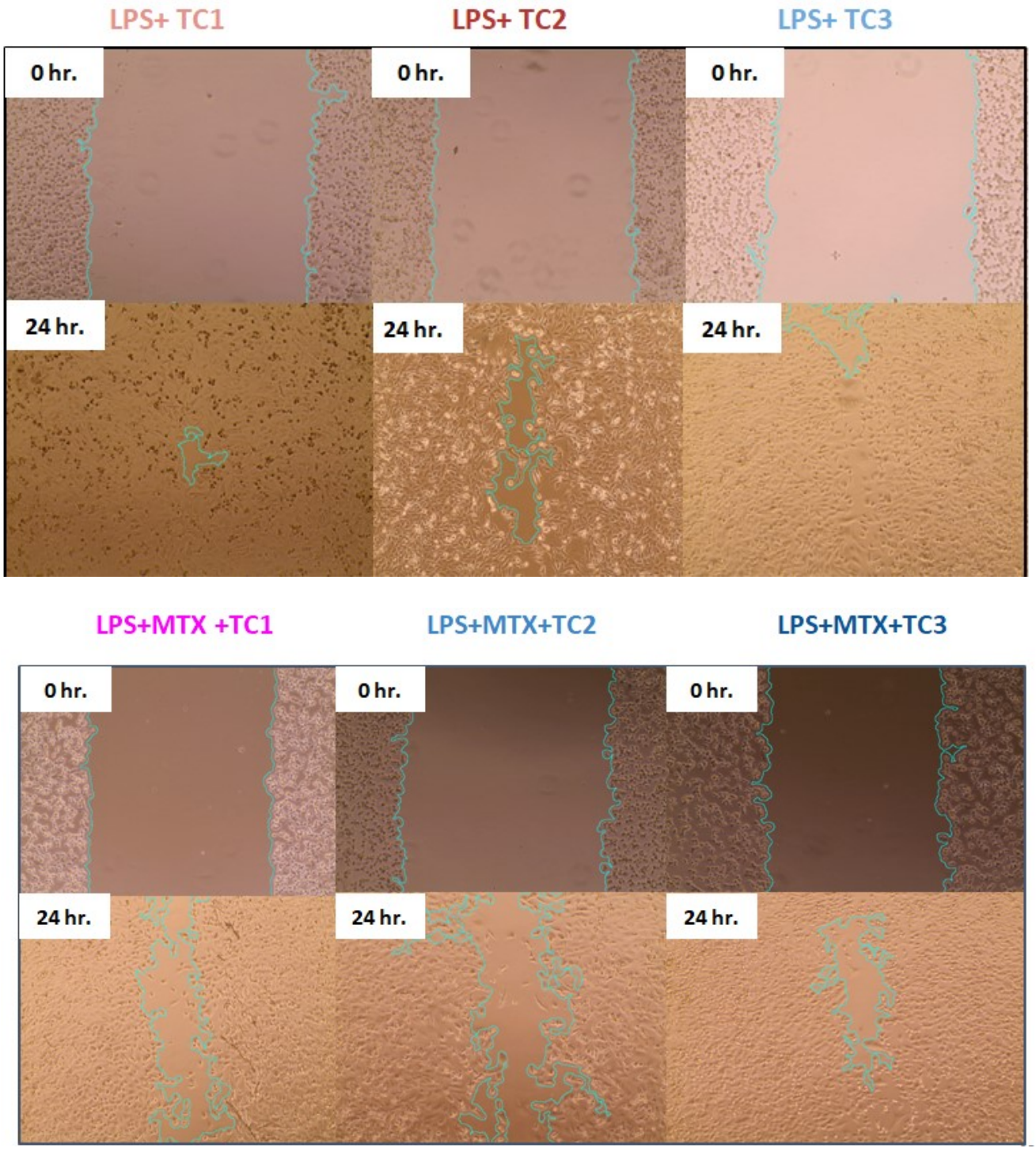


Figure 7(a): The cell migration rate of SW 982 cells when treated with TC and MTX alone and in combinations in Image J software.

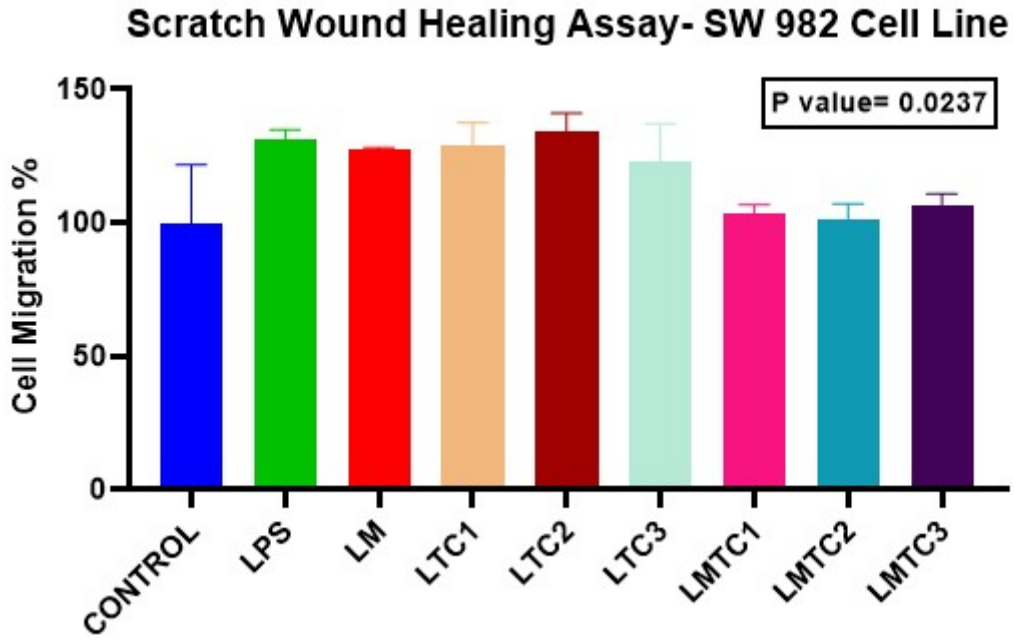


Figure 7(b). The cell migration rate of SW 982 cells when treated with TC and MTX alone and in combinations.

3.7. DISCUSSION

The results of our study demonstrate the potential of combination therapy in the treatment of rheumatoid arthritis (RA). Our findings indicate that the combination of TCA and MTX exhibited favorable outcomes in terms of cell cytotoxicity and scratch wound healing assay compared to single drug therapies.The cytotoxicity assay showed IC25 values of TCA($p<0.0001$) and MTX($p<0.0002$) as 88.511 ± 4.879 µg/ml and 442.588 nM respectively. Despite the fact that single drug therapies like MTX group and TC group were unable to do so for SW 982 cells, the combination therapy (MTX+TC groups) demonstrated promising outcomes to reduce the migration rate of cells near to the control group. In RA, the inflammatory cytokines, chemokines and some enzymes like MMPs can induce cell migration to reach other joints. Through minimizing the migration rate, it can prevent further spreading of diseases to other parts of the body. In one of the study, they observed the anti-migratory potential of a Chinese herb (Baicalein) on SW 982 cells through a scratch wound healing assay. They observed a significant reduction in lateral migration of SW 982 cells in Baicalein treated groups((25 µM and 50 µM) at 24, 48, 72, and 96 hours)(Zhang et al., 2022).This further supports the notion that targeting cell migration can be a valuable approach in managing RA. Our study adds to the existing knowledge by highlighting the potential of combination therapy involving TCA and MTX in reducing cell migration and potentially limiting the progression of RA.

In clinical scenario, the patients are mostly suffering with pain, swelling, redness, stiffness and joint deformity. Even though we could analyze the anti inflammatory potential of combinations (TC+MTX) through migration assays, it is not complete without assessing the biomarkers involved in RA. The biomarkers like IL-6, TNF-alpha, PGE-2, COX, MMPs, MAPKs and RANKL plays major role in the pathogenesis of RA for producing the symptoms like pain, swelling etc(Kondo et al., 2021).The above mentioned biomarkers were studied in SW 982 cell line model with various herbs. More than 20 research papers are available in Pub med related with herbs other than TC against RA in SW 982 cell line model. Our findings suggest that combination therapy has the potential to be an effective therapeutic approach for managing RA and warrants further investigation in preclinical and clinical settings.

4. CONCLUSION

In conclusion, this research aimed to understand the potential of *Tinospora cordifolia* (TC), an Ayurveda-based botanical, as a combination medicine with Methotrexate (MTX) for the treatment of Rheumatoid arthritis (RA). The study was done in the SW 982 cell line and conducted some assays to assess the efficacy of the combination therapy.The MTT assay results demonstrated that TC at concentrations lower than 10,000 µg/ml did not exhibit significant toxicity to SW 982 cells within 24 hours. The IC25 values, which represent the concentration required to inhibit 25% of cell growth, were determined to be 88.511µg/ml for TC aqueous extract and 442.588 nM for MTX.In the scratch wound healing assay, the LPS-free control group exhibited a migration rate close to 100%. The migration rate of cells in the LPS-treated group exceeded 130%, indicating enhanced migration. Notably, the combination therapy (MTX+TC groups) demonstrated promising results by reducing the migration rate of cells, bringing it closer to the control group. In contrast, single drug therapies with MTX or TC alone were unable to achieve the same effect on SW 982 cells.

Furthermore, through the use of ImageJ standardization, a threshold value of 150 was determined. This finding provides a standardized parameter for analyzing subsequent data and comparing results across experiments.

Based on these findings, it can be concluded that the combination therapy of TC and MTX holds potential for Rheumatoid arthritis treatment. The synergistic effect of the two drugs was evident in their ability to reduce the migration rate of SW 982 cells, a key characteristic of RA. These results support further investigation and development of the TC-MTX combination as a potential therapeutic approach for RA.

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6. ANNEXURE-1

Pharmanza Herbal Pvt. Ltd.

Plot No. 214, Near Vadadla Patiya, Borsad - Tarapur Road, At. Kaniya - 388 435. India.
(C: (02697) 245450, 244051, Fax : (02997) 245550 Email : info@pharmanzaherbals.com

CERTIFICATE OF ANALYSIS

Format NO:	Revision NO:	Effective Date:	Page NO:
SF/QC/CHI/026-04.03	00	07/05/2021	Page 1 of 1
NAME OF PRODUCT	Tinospora cordifolia Extract Granules		
PRODUCT CODE	06000EGTCP	HARVEST SEASON	March – May
COMMON NAME	Guduchi, Gallo	BATCH / LOT NUMBER	LTO2EG2203J26
PLANT PART USED	Stem	BATCH / LOT SIZE	500Kg
ORIGIN	India	MFG. MONTH (Month & Year)	26th September 2022
HARVEST METHOD	Wild Crafted	PO REF. NO / DATE	Preshipment Sample– 05/10/2022

PHYSICAL CHARACTERISTIC	SPECIFICATION	TEST METHOD	RESULTS
Identification	Characteristic to species	QC/TEC/065-TLC-(Cur. Ver.)	Confirms
Appearance	Granular	QC/TEC/153-Visual (Cur. Ver.)	Confirms
Color	Light to Dark Brown*	QC/TEC/153-Visual (Cur. Ver.)	Confirms
Taste	Bitter	QC/TEC/153-Organoleptic (Cur. Ver.)	Confirms
Particle Size	NLT 95.00% thru 40mesh	QC/TEC/088-USP <786>-(Cur. Ver.)	96.64%
	NMT 30.00% thru 100mesh	QC/TEC/088-USP <786>-(Cur. Ver.)	28.49%
Bulk Density	NLT 0.50g/mL	QC/TEC/089-USP <616>-(Cur. Ver.)	0.63g/mL
Tap Density	NLT 0.60g/mL	QC/TEC/089-USP <616>-(Cur. Ver.)	0.74g/mL
CHEMICAL ANALYSIS	**SPECIFICATION**	**TEST METHOD**	**RESULTS**
Assay for Actives:			
a] Total Bitter	NLT 5.00%	QC/TEC/090-Gravimetric (Cur. Ver.)	6.79%
b] Total Polysaccharide	NLT 20.00%	QC/TEC/088-UV-VIS (Cur. Ver.)	37.52%
Loss on Drying	NMT 6.00%	QC/TEC/087-USP <731>-(Cur. Ver.)	3.66%
IMPURITIES	**SPECIFICATION**	**TEST METHOD**	**RESULTS**
Lead	≤1.0 ppm	USP <233>-ICP-MS- (Cur.Ver.)	0.258 ppm
Mercury	≤3.00ppm	USP <233>-ICP-MS- (Cur.Ver.)	<0.05 ppm
Cadmium	≤1.00ppm	USP <233>-ICP-MS- (Cur.Ver.)	<0.05 ppm
Arsenic	≤1.00 ppm	USP <233>-ICP-MS- (Cur.Ver.)	0.211 ppm
Solvent Residue	USP <467>/ Screen	QC/CHI/045-USP <467>-(Cur. Ver.)	Complies
Pesticides Residue	USP <561> Screen	USP <561>- (Cur. Ver.)	Complies**
MICROBIOLOGY	**SPECIFICATION**	**TEST METHOD**	**RESULTS**
Total plate count	≤100000cfu/g	QC/MIC/007-USP <2021>-(Cur. Ver.)	100 cfu/g
Yeast & Mold	≥300cfu/g	QC/MIC/007-USP <2021>-(Cur. Ver.)	Absent
Coliforms	<3.0mpn/g	QC/MIC/012- FDA BAM Chapter 4	Absent
Enterobacteriaceae	<100cfu/g	QC/MIC/007-USP <2021>- (Cur. Ver.)	Absent
Escherichia coli	Absent/25g	QC/MIC/007-USP <2022>- (Cur. Ver.)	Absent
Salmonella	Absent/25g	QC/MIC/007-USP <2022>- (Cur. Ver.)	Absent
Staphylococcus aureus	Absent/25g	QC/MIC/007-USP <2022>- (Cur. Ver.)	Absent
INGREDIENTS DISCLOSURE:			
Tinospora cordifolia extract of Stem	NLT 100.00%		
Extraction Solvent	Water		
Herb Extract ratio	10~15:1		
PACKAGING DETAILS:			
Packaging:	25 Kg net double bagged in HDPE Drum.		
Shelf Life:	48 months from date of manufacture.		
Storage:	Store in ambient temperature away from excessive heat.		
Certification of the Facility:	NSF-GMP, FSSAI, ISO9001:2015 (QMS), ISO22000:2018 (FSMS), HALAL, Kosher.		
Declaration:	This product has not been irradiated or ETO treated		

REMARK / COMMENTS IF ANY:
*Colour of Botanical products is subject to seasonal condition and may vary from lot to lot.
**Pesticides results mentioned in COA based on historical data.

Limit of Quantification for Lead, Mercury, Arsenic & cadmium: 0.05 mg/kg.

VS Product Code # TNP005EGPH

Reviewed by:
Date: 05/10/2022

Approved by:
Date: 05/10/2022