

## IN VITRO EVALUATION OF ANTI-INFLAMMATORY AND WOUND HEALING POTENCY OF LIPIDS EXTRACTED FROM *MORTIERELLA ELONGATA* ISOLATED FROM WESTERN GHATS OF TAMIL NADU

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### ABSTRACT

The present study has evaluated the *in vitro* anti-inflammatory and wound healing potency of lipids extracted from *Mortierella elongata* (Accession No. OK402027) isolated from the Western Ghats of Tamil Nadu. *In vitro* anti-inflammatory and wound healing potency was evaluated against mouse fibroblast 3T3 cells and the Human Embryonic Kidney cell lines (HEK cells), respectively. The TNF- $\alpha$  stimulated 3T3 cells were treated with various concentration (5, 10 and 25mg/mL) of *M. elongata* lipids to assess the expression of interleukin-6 (IL-6). Total RNA was extracted and cDNA was synthesized using 2 step quantification reverse transcription PCR. B-actin was used as an endogenous control. Data were analyzed with J6 densitometry. The scratched HEK cells were treated with 3 different concentration of *M. elongata* lipids for 24h. *M. elongata* lipids exhibited the anti-inflammatory activity by significantly decreasing the synthesis *M. elongata* of IL-6. Expression of IL-6 was down regulated with increased concentration of lipids. Similarly, *M. elongata* lipids have significantly induced the cell migration of HEK cells. Anti-inflammatory and wound healing activity of *M. elongata* lipids was directly proportional to its concentration.

**Key words:** PUFA, Anti-inflammation, Wound Healing, Omega 3, Omega 6

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### Abbreviations:

PUFA = Polyunsaturated Fatty Acid, HEK = Human Embryonic Kidney, EPA = Eicosapentanoic Acid, DHA = Docosahexanoic Acid, AEA = Arachidonylethanolamide, EPEA = Eicosapentaenoylethanolamide, DHEA = Docosahexaenoyl Ethanolamide, EET = Epoxyeicosatrienoic Acid, EEQ-EA = Epoxyeicosatetraenoic Acid, EDP-EA = Epoxydocosapentaenoic Acid ethanolamide, ARA = Arachidonic Acid, LA = Linolenic Acid, GLA = Gamma Linolenic Acid

### INTRODUCTION

Inflammation is an innate process of host's immunological defensive mechanism. It manifests upon various stimuli like microbial infection and invasion, surgical trauma, tissue injury and exposure of allergen or any foreign particles. Inflammation is well considered as indispensable as it protects and safe guard us infinitively from daily battles. Usually, inflammation is of short-lasting and it resolves immediately soon after the defence by a negative feedback mechanism (Calder, 2012). This indispensable process is considered to be protective only when it is acute and self-limited. If the inflammation persists in definitively (chronic) then it becomes the prominent cause for the development of many diseases and inflammation disorders. Auto immune disorder is a condition in which body's immune system mistaken attacks its own healthy cells and tissues. It approximately affects 5% of the US population.

Certain Polyunsaturated Fatty Acid (PUFA) are known to mediate both anti-inflammation and resolution of inflammation. Of which, Arachidonic Acid derived eicosanoids play a vital role in stimulating as well as terminating the inflammation process (Innes and Calder, 2018). Lipoxin is a potent and first identified anti-inflammatory and pro-resolving mediators. Lipoxins are metabolically synthesized from ARA, an omega 6 fatty acid as precursor via LOX pathway (Chandrasekharan and Sharma-Walia, 2015). The specialised pro-resolving mediators (SPM) such as resolvins, protectins and mersins metabolically derived from omega 3 fatty acids such as EPA and DHA are the most potent pro-resolving and anti-inflammatory mediators (Serhan *et al.*, 2008). Endocannabinoids such as AEA, EPEA and DHEA derived from ARA, EPA and DHA, respectively, are potent anti-inflammatory molecules. The epoxy derivatives of endocannabinoids such as EET-EA, EEQ-EA and EDP-EA possess anti-inflammatory properties (McDougle *et al.*, 2017). These endogenously synthesized anti-inflammatory molecules are considered as the salient molecules to maintain homeostasis. Thus, pro-resolution

mechanism is highly crucial for maintaining the homeostasis. Contradictorily, uncontrolled inflammation is deleterious to physiological conditions.

Skin serves as a defensive frontier in terms of body's defensive mechanism and therefore, wound healing stands as an indispensable process for the state of continued survival. Wound healing is a complex as well as more conserved physiological phenomenon characterised by a cascade of 4 overlapping phases such as inflammation, proliferation, tissue remodelling and scar maturation (Lu *et al.*, 2010; Campelo *et al.*, 2015). Lipids and lipid derived molecules are well known to play a vital role in co-ordinating and mediating the different phases of wound healing. Especially, ARA derived eicosanoids molecules serve as a key component in mediating platelet activation, inflammation and removing the wound debris. ARA and LA increases the proliferation of endothelial cell. LA is known to elevate the migration of fibroblasts. Thus, Omega 6 fatty acids are reported to involve in all the phases of wound repairment (Silva *et al.*, 2018). Omega 3 fatty acids such as DHA, EPA and their derivatives like 14,21-dihydroxy-docosahexaenoic acid mediate reepithelialisation, angiogenesis and tissue regrowth or tissue granulation (Lu *et al.*, 2010). Dietary balance of lipid plays an important role in wound healing as well. Supplement of  $\omega$ 9,  $\omega$ 6 and  $\omega$ 3 oil to thermal burnt rat have notably increased the cell proliferation by inhibiting NF- $\kappa$ B pathway (Campelo *et al.*, 2015).

Therefore, the present study has investigated the *in vitro* anti-inflammation and wound healing potency of *M. elongata* lipids against mouse fibroblast 3T3 cells and the Human Embryonic Kidney cell lines (HEK cells), respectively.

## MATERIALS AND METHODS

### Isolation of oleaginous fungus

The oleaginous fungal isolate, *Mortierella elongata* (accession no. 402027) was isolated from the terrestrial soil of Nilgiris hill, Western Ghats of Tamil Nadu at 11.4007° N and 76.7358° E. In our earlier study, the total lipids were extracted using low toxicity solvent system with 3:2 v/v of n-hexane: isopropyl alcohol. The extracted total lipids were esterified and fatty acids profiles were determined. The oleaginous fungus *M. elongata* was found to be a potential source of PUFA and was noted to produce the most essential fatty acids like omega 6 and omega 3 such as 0.79% of GLA, 1.24% of ARA, 1.24% of EPA and 6.83% of DHA. The *M. elongata* lipid was found to be biocompatible against the normal human embryonic cells (HEK).

### Anti-Inflammatory Activity

#### Cell Cultures

The 3T3 cells, the mouse embryonic fibroblast cells were procured from NCCS, Pune, India. The cancer cells were maintained in DMEM medium supplemented with 2mM l-glutamine and BSS altered to contain 1.5 g/L Na<sub>2</sub>CO<sub>3</sub>, 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 1.5 g/L glucose, 10mM HEPES and 10% FBS (GIBCO, USA). Antibiotic such as penicillin and streptomycin (100 IU and 100  $\mu$ g) were adjusted to 1 mL/L. The cultures were maintained at 37° C with 5% humidified CO<sub>2</sub> incubator.

#### Extraction of RNA

3T3 cells were seeded in a six well round bottom culture plate at a density of 5x10<sup>5</sup> cells/well. Subsequently, cells were stimulated with 10 mg/mL of Tumour Necrosis Factor  $\alpha$  (TNF  $\alpha$ ) for the inflammatory activity. Further, the cells were treated with different concentration of DB/01 sample (5, 10 and 25  $\mu$ g/mL) followed by 1 h incubation. The total RNA was then extracted from both treated and untreated cells (stimulated) using the RNA isolation and purification kit, Macherey-Nagel GmbH & Co, KG. The purity and the concentration of the extracted RNA was analysed by a Nano Drop ND – 2000 C spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). The absorbance was read at 260/280 nm.

#### Reverse Transcription of RNA by Real Time PCR

An aliquot of 1 $\mu$ g total RNA was taken for cDNA synthesis mixed with Light cycler 480 DNA SYBER Green I Master (Roche). The basal expression of IL-6 mRNA, the anti-inflammatory cytokine was quantified by real time PCR. Two step quantitative – reverse transcription PCR was carried out in duplicates using the Light Cycler 480 II real time PCR system (Roche). The IL-6 gene was amplified using the forward primers 5'-CCTGAACCTTCCAAAGATGGC-3' and the reverse primers 5'-CTGACCAGAAGAAGGAATGCC-3' The hot start enzyme was activated at 95° C for 5 min.

The synthesized cDNA was then amplified for about 40 cycles with denaturation steps at 94° C for 20 s and annealing and extension at 60° C for 20 s. The primer derived trimer and dimers were detected by performing a melt curve assay at 65° C for 1 min and the temperature was increased by 1.1° C every 10 s. Beta actin was used

as house endogenous control/ internal reference gene. The agarose gel electrophoresis was carried out for the experimental samples. The gene expression was measured by densitometric scanning. Data was determined by J7 densitometry. Quantification was evaluated by comparing the band intensity with the internal reference gene.

**Wound Healing Assay**

The Human Embryonic Kidney cell lines (HEK cells) were obtained from NCCS, Pune, India. HEK cells were seeded and grown at a density of  $1 \times 10^5$  cells/ 35x11 m dishes. The cultures were incubated for 24 h at 37° C. After attaining the confluence, the cells were treated according to the treatment schedules. The untreated cells were considered as control and the cells were treated with different concentration of DB/01 sample (5, 10 and 25 µg/mL). Then the culture layer of each plate was artificially scratched using a sterile plastic pipette tip. The migration of cells at the edge of the scratch were determined microscopically at 0 and 24 h, respectively. The observations were micro graphed at 0 and 24 h.

**Statistics**

All the *in vitro* cell culture studies were done in triplicates and the statistical analysis were performed by SPSS software version 17.0. The *p* value less than 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Anti-inflammatory Activity of *M. elongata* lipids**

The anti-inflammatory activity of *M. elongata* lipids were determined in 3T3 mouse fibroblast cells. The inhibitory effect *M. elongata* lipids on the expression of IL 6, one of the key inflammatory cytokines was evaluated. The 3T3 mouse fibroblast cells were stimulated by the pro-inflammatory cytokines TNF-α and the cells were treated with 5, 10 and 25 µg/mL of *M. elongata* lipids. The agarose gel electrophoresis of IL 6 gene expression by RT-PCR is represented in Fig. 1. The expression of β actin was measured as a control. The TNF α stimulation increases the production of IL-6 and the untreated stimulated cells exhibited the highest expression of IL 6. Calder (2017) reported that PUFAs had the ability to reduce the production of pro- inflammatory molecules. The present study is similar to that of *in vitro* studies by Serini *et al.* (2019) suggested that DHA have significantly inhibited the expression of IL6 in keratinocyte cell lines, Lee *et al.* (2014) revealed that PUFA have significantly inhibited the production of IL8 in inflammatory induced AGS cells and Saraswathi *et al.* (2021) confirmed the anti-inflammatory effects of *Sargassum* weed lipid RAW 264.7 macrophage cells.

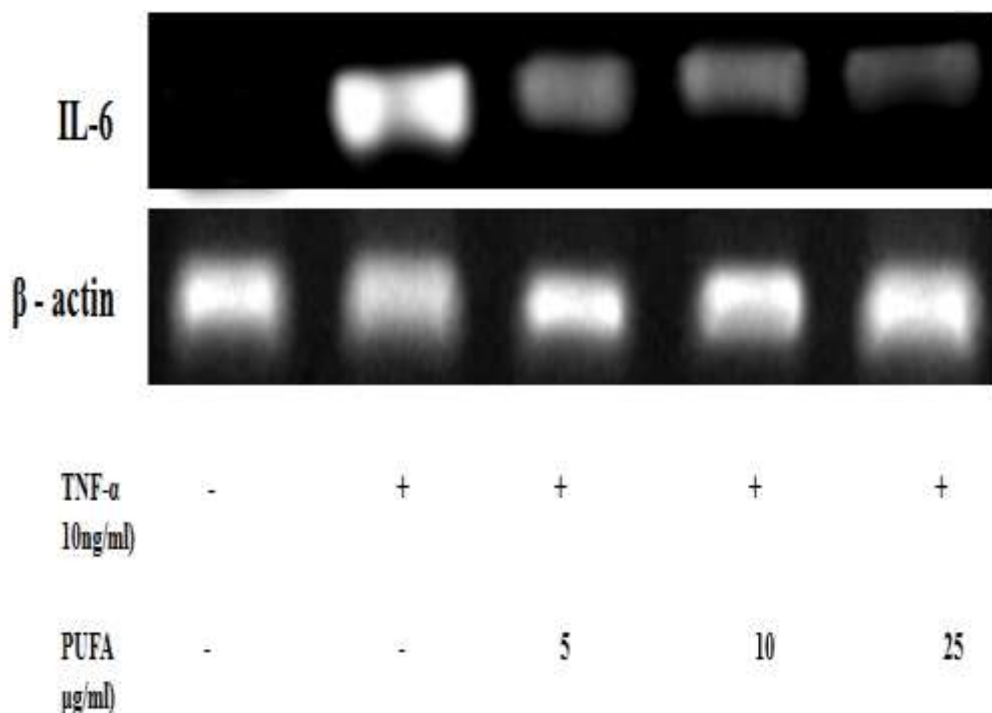


Fig. 1. Expression of IL 6 in 3T3 Mouse Fibroblast Cells treated with *M. elongata* Lipids.

The graphical illustration of densitometric analysis of IL 6 expression is represented in Fig. 2. The densitometric analysis indicated that *M. elongata* lipids were found to be significant in inhibiting the expression of IL-6 in a dose-dependent manner. The degree of inhibition was directly proportional to the concentration of samples. The concentration of 25  $\mu\text{g/mL}$  indicated a maximum inhibition of IL 6.

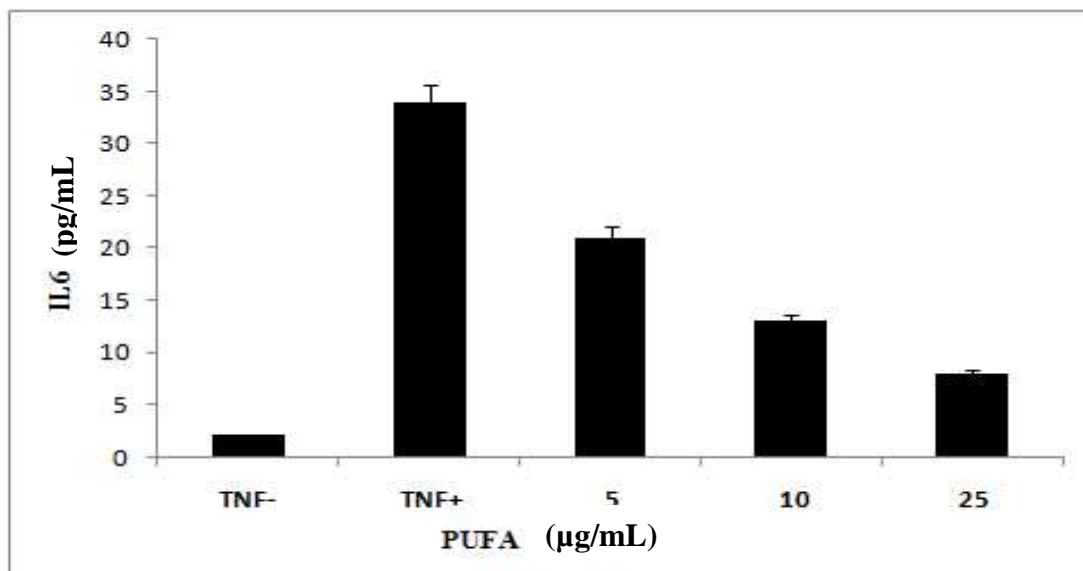


Fig.2. Inhibitory effect of *M. elongata* Lipids in 3T3 cells by Densitometry Analysis.

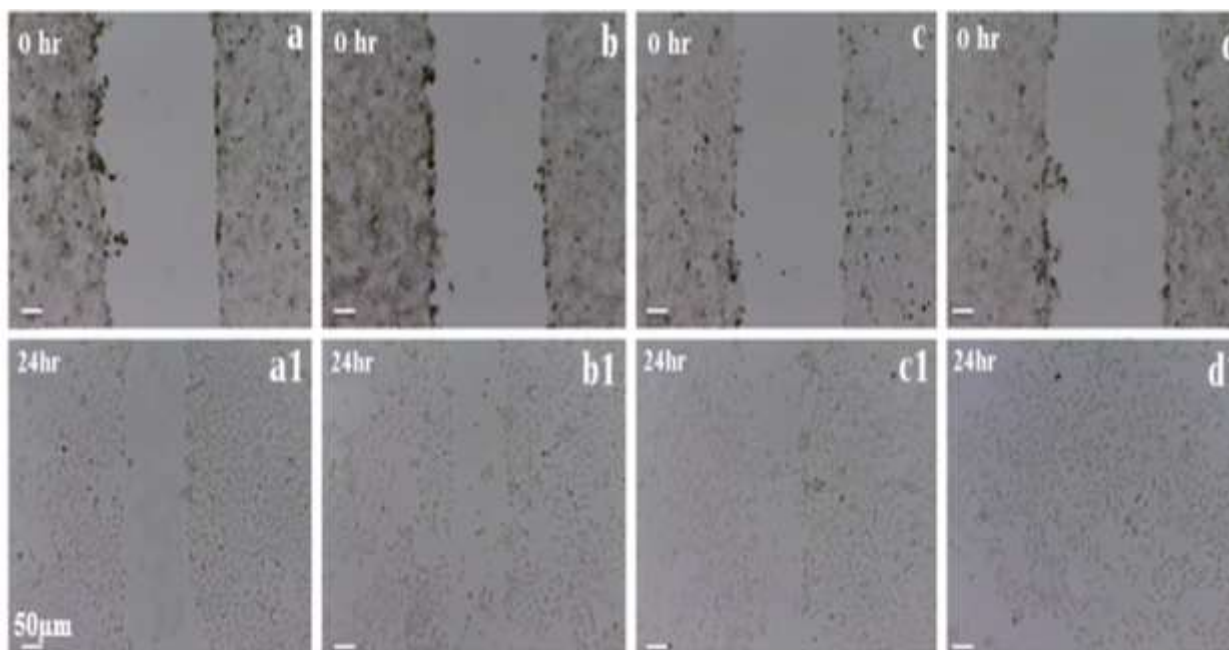


Fig. 3. Wound healing effect of *M. elongata* Lipids on HEK cells. a- untreated cells at 0 h; a1- untreated cells at 24 h; b- cells treated with 25  $\mu\text{g/mL}$  of *M. elongata* lipids at 0 h; b1- cells treated with 25  $\mu\text{g/mL}$  of *M. elongata* lipids at 24 h; c- cell treated cells with 50  $\mu\text{g/mL}$  at 0 h; c1- cell treated cells with 50  $\mu\text{g/mL}$  at 24 h; d- cells treated with 100  $\mu\text{g/mL}$  of *M. elongata* lipids at 0 h; d2- cells treated with 100  $\mu\text{g/mL}$  of *M. elongata* lipids at 24 h.

#### Wound Healing Activity of *M. elongata* lipids

The wound healing activity of *M. elongata* lipids were determined using Human Embryonic Kidney cell lines (HEK). The HEK cells were treated with 25, 50 and 100  $\mu\text{g/mL}$  of *M. elongata* lipids for 24 h. The treated cells have indicated the significant proliferation and migration of HEK cells when compared to untreated cells.

The cell migration at 0 h and 24 h were micro graphed. The wound healing nature of *M. elongata* lipids is represented in Fig. 3. *M. elongata* lipids have significantly induced the cell migration of HEK cells resulting in the closure of wound. The gap of the wound was almost closed in the concentration of 100 µg/mL. Tallima and El Ridi (2018) reported the wound healing capacity of arachidonic acid by regulating the endothelial cell functions and production of angiogenic factors. The present study revealed that PUFA rich lipid was found to be more significant in closure of wound. Santos *et al.* (2020) reported the effective migration of L929 fibroblast cells by the activity of medium chain PUFA of Babassu oil.

## CONCLUSION

*M. elongata* lipids was found to possess the anti-inflammatory and wound healing potency against 3T3 mouse fibroblast cells and HEK cells, respectively. The densitometric analysis indicated that *M. elongata* lipids were found to be significant in inhibiting the expression of IL-6 in a dose-dependent manner. The degree of inhibition was directly proportional to the concentration of samples. The concentration of 25 µg/mL indicated a maximum inhibition of IL 6. *M. elongata* lipids have significantly induced the cell migration of HEK cells resulting in the closure of wound. The gap of the wound was almost closed in the concentration of 100 µg/mL. This *in vitro* anti-inflammation and wound healing studies could be further taken for *in vivo* studies in future.

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