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EVALUATION OF ANTIOXIDANT AND ANTIDIABETIC POTENTIAL OF *MORTIERELLA ELONGATA* LIPIDS ISOLATED FROM WESTERN GHATS OF TAMIL NADU

IDA POORNIMA. S*[1] AND JUDIA HARRIET SUMATHY. V[2]

1: Research Scholar, PG & Research Department of Biotechnology, Women's Christian College, Chennai – 600 006

2: Assistant Professor, Department of Advanced Zoology and Biotechnology, Women's Christian College, Chennai – 600 006

***Corresponding Author: Dr. Ida Poornima. S: E Mail: idabiotech@gmail.com**

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ABSTRACT

Diabetes Mellitus (DM) stands as the most whacking chronic metabolic disorder which in turn damage kidney, heart, nerves, eyes and so on. Growing evidence connecting the oxidative stress with DM have authentically suggest antioxidant as a therapeutic agent against DM. Therefore, the present study has evaluated the anti-oxidant potency of lipids extracted from 7 different oleaginous fungi and the most potent antioxidant was further evaluated for its in vitro anti-diabetic potency against RIN5F cells. The free radicle scavenging potency of total lipids was evaluated by DPPH and NO assay. The lipids extracted from *Mortierella elongata* (Accession No. OK402027) isolated from the Western Ghats of Tamil Nadu was found to be more potent in scavenging free radicles. *In vitro* lipid peroxidation assay was evaluated against H9C2 cells. In vitro glucose utilization assay against RIN5F cells, Alpha-Amylase Inhibition Assay and Alpha- Glucosidase Inhibition Assay were evaluated to determine the anti-diabetic potency of *M. elongata* lipids. The present study indicated a significant uptake of glucose when compared with untreated control. Anti-diabetic potency of *M. elongata* lipids is directly proportional to its concentration.

Keywords: PUFA, Anti-diabetic, Antioxidant, NO, DPPH, Lipid Peroxidation

INTRODUCTION

Oxidative stress remains the plausible connecting root for the incidence of major diseases and metabolic disorders such as cancer, neurological disease, cardiovascular disease, atherosclerosis, diabetes, which in turn damage other organs such as eyes, kidney, nerves and heart **[1, 2]**. Antioxidants tend to delay or prevent the oxidation by removal of oxygen, scavenging or inhibiting the formation of reactive oxygen species and/or nitrogen species to name a few **[3, 4]**. Antioxidant potency of PUFAs were well evaluated using various assays that includes 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, nitric oxide (NO) scavenging activity to name a few leading to the inhibition of lipid peroxidation **[5]**.

Globally, incidence of Diabetes Mellitus (DM) keeps increasing and this whacking metabolic disease stands as 4th leading cause of mortality **[6]**. DM, the most prevailing one such is type 2 Diabetes Miletus (T2DM). Incidence of type 2 is caused either by insulin deficiency or insulin resistance or both. Modern sedentary lifestyle and obesity are often considered as major cause or risk factor for the development of T2DM **[7]**. Both the clinical and experimental research evidences have authentically convenience the role of oxidative stress in the pathogenesis of DM as well its contribution towards the development of its complications **[4, 8]**. Dietary supplements of omega 3 PUFA are reported to be protective against T2DM **[9]**. Numerous numbers of growing evidences have suggested antioxidant as therapeutic agent against DM **[6, 7, 10, 11]**.

Therefore, the present study as focused on evaluating the antioxidant potency of lipids extracted from 7 different oleaginous fungi isolated from Western Ghats of Tamil Nadu. Further, the most potent antioxidant was evaluated for lipid peroxidation against H9C2 cells and in vitro anti-diabetic potency using RIN5F cells.

MATERIALS AND METHODS

Oleaginous fungi

Seven oleaginous fungal isolates such as *Mortierella elongata* (accession no. 402027), *Acremonium sclerotigenum* (accession number OK427341), *Umbelopsis vinacea* (accession number OK458719), *Fusarium equiseti* (accession number OK462885), TN/NH/DB/04 isolate (query cover 65%) similar to the order Chaetophyriales, TN/NH/DB/06 (Accession number: ON677853) and TN/NH/DB/10 showed similarities with *Schizophyllum spp.* (with different Fatty acid profile) were isolated from the terrestrial soil of Nilgiris Hill, Western Ghats of Tamil Nadu at 11.4007 º N and 76.7358 º 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).

DPPH Assay

The DPPH assay was carried out for the lipids extracted from all the 7 PUFA producers. 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a purple-coloured organic compound constitutes stable free radicals, it is widely used in spectrophotometric method to evaluate the free radical scavenging capacity of the test sample. The ability of the test sample to donate proton in order to reduce the DPPH is identified by the disappearance of violet colour and the absorbance of which was measured at 517 nm. The reaction mixture was prepared by the addition of 1ml of DPPH (0.02% in methanol) was mixed with 1ml of lipid fraction at various concentration ranging from 20, 40, 60, 80 and 100 mg/ml in methanol. Butylated hydroxy toluene (BHT), a lipophilic antioxidant at various concentration range between 1 to 20 µg/ml in methanol was used as a positive control. The methanolic solution without DPPH was used as blank. The reaction mixtures were vortexed well and incubated at RT in dark for 30 min. After the incubation period, the absorbance was determined spectro-photometrically at 517 nm using Shimazu UV-Visual spectrophotometer. The assay was performed in triplicates. The ability of the lipids to scavenge DPPH radicals were calculated using the mean values in the following equation.

$$\% \, Inhibition = \left\{\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right\} * 100$$

Where, $Abs_{control}$ is the absorbance of control sample; Abs_{sample} is the absorbance of the test sample

The graph was plotted with the concentration of sample along the X-axis and % inhibition along the Y-axis.

Nitric Oxide Radical Scavenging Capacity

Nitric Oxide (NO) scavenging assay was accomplished for lipids extracted from all the seven PUFA producing isolates. Sodium Nitroprusside is a widely used agent for the direct liberation of NO radicals in the aqueous solution at pH 7.2. The reaction mixtures were prepared with 1ml of sodium nitroprusside (10mM) and 1ml of lipid fraction containing 20, 40, 60, 80 and 100 µg/ml concentration in phosphate buffer. The reaction tubes were incubated at 28º C for 180 min. An aliquot of 1ml of reaction mixtures was mixed with equal volume of Griess reagent. The absorbance was

measured at 546 nm. The assay was performed in triplicates. The percentage of NO radicals scavenging potential for the lipid fractions was determined using the mean absorbance in the following equation.

$$\% \, Inhibition = \left\{\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right\} * 100$$

The graph was plotted with the concentration of sample along the X-axis and % inhibition along the Y-axis.

Lipid Peroxidation Assay

Lipid peroxidation assay was performed for *M. elongata* lipids. H9C2, rat cardiac myoblast cell lines were procured from the National Centre for Cell lines (NCCS), Pune, India. H9C2 cells of density $5x10^3$ were seeded in T-25 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) augmented with high glucose (4500mg/l), glutamine (4mM), sodium pyruvate (1mM) and sodium bicarbonate (1500mg/ml) Antibiotics such as penicillin and streptomycin of concentration (100IU/100 µg) adjusted to 1ml/l were used. Cells were incubated at 37º C in a 5% CO_2 humidified incubator.

H9C2 cells ($5x10^3$) were seeded in a six-well plate. The cells were then pre-treated with 10, 25 and 50 µl of *M. elongata* and then treated with H_2O_2 (250 µM). The treated cells were washed twice with PBS and then harvested. The harvested cells were homogenised with cell lysate buffer comprising of 20 mM Tris-HCl, 0.2% Triton X-100 and 0.5 mM PMSF. This homogenate was further sonicated for 30s at 4º C. The obtained complete cell lysate was centrifuged at 3000 rpm for 10 min at 4º C. The supernatant was collected and malondialdehyde (MDA) was estimated by TBARS method. Cells without treatment was served as control. ELIZA multi-well plate reader (ROBONIK, India) was utilized to determine the optical density at 532 nm.

Anti-Diabetic Activity

The anti-diabetic activity of *M. elongata* lipids was studied by using RIN5F cell lines. The Rat Pancreatic β cell lines were procured from NCCS, Pune, India. The RIN5F cells were seeded and maintained RPMI 1640 supplemented with FBS 10% (v/v), 100 µg/ml of antibiotics such as penicillin-G and streptomycin. The cells were maintained at 37º C at 5% CO_2 incubator.

Evaluation of Cytotoxicity

MTT assay was done to evaluate the inhibitory concentration (IC_{50}) of the *M. elongata* lipids against RIN5F cells. The cells were grown for 48h in a 96-well plate to attain the cell confluence of 80%. The spent medium was replaced with the fresh medium and supplemented with 0.5 mg/ml of MTT in complete medium. It is then incubated for 3 h at 37º C. After incubation, the formazan crystals formed were made solubilized by the addition of 200 µl of

DMSO in each well. The optical density was measured at 540 nm using a 96-well plate reader. The cytotoxicity of the *M. elongata* lipids was expressed as % and calculated using the following equation.

$$\%\ Cell\ Viability = \left[\frac{Absorbance\ of\ Test\ well}{Absorbance\ of\ the\ untreated\ well}\right] X\ 100$$

Glucose Utilization Assay

The RIN5F cells were seeded and grown at a density of $3x10^3$ cells/well in a 96-well culture plates. The cultures were incubated for 3 days at 37º C at 5% incubator. After incubation, different concentration of *M. elongata* lipids was added to the well. The cells were treated with Metformin (0.1 µg/ml) as a positive control and untreated cells as negative control. The cultures were further incubated for 48 h. The spent medium was removed and replaced with fresh RPMI supplemented with PBS, 0.01% BSA and glucose 8 mm. The cells were again incubated for 3 h at 37º C. An aliquot of 10 µl incubated medium was transferred to the fresh 96-well plate. Concentration of glucose present in the medium was evaluated by the addition of 200 µl of glucose oxidase reagent. The plate was finally incubated for 15 min at 37º C. After incubation, the absorbance was read at 492 nm using ELIZA multi well plate reader (ROBONIK, India). The percentage of glucose utilization was calculated using the following equation.

$$\%\ Glucose\ Utilization = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right] X100$$

Alpha-Amylase Inhibition Assay

The alpha-amylase inhibition assay was carried out for *M. elongata* lipids. The various concentration of *M. elongata* lipids such as 12.5, 25 and 50 µg/ml in phosphate buffer was loaded to the 96-well plate. To each well, 5µl of porcine pancreatic enzyme solution was added and incubated for 10 min at 37º C. After incubation, 20 µl of starch solution was added to initiate the reaction. It was further incubated for 30 min at 37º C. The reaction was ceased by the addition of 10 µl 1M HCl and 75 µl of Iodine to each well. The phosphate buffer of pH 6.9 without the test sample was evaluated as blank. Metformin was used as positive control. The α amylase inhibition was determined by evaluating the optical density at 580 nm using spectrophotometer. The percentage of α amylase inhibition activity was calculated by the following equation.

$$\%\ of\ \alpha\ amylase\ inhibition = \frac{\left[Abs_{control} - Abs_{sample}\right]}{Abs_{control}}\ X\ 100$$

Alpha- Glucosidase Inhibition Assay

The α-glucosidase inhibition assay was carried out for the *M. elongata* lipids. The various concentration of *M. elongata* lipids such as 12.5, 25 and 50 µg/ml were loaded in the 96 well plate. An aliquot of 20 µl of α-glucosidase of 50 µg/ml concentration and 60 µl of potassium

phosphate buffer (67 mM) of pH 6.8 were added. It was incubated for 5 min. After incubation, 10 µl of p-nitrophenyl-α-D-glucosidase (PNP-GLUC) of concentration 10 nM was added. It was further incubated for 20 min at 37º C. The reaction was ceased by the addition of 25 µl of sodium carbonate of concentration 100nM to each well. Metformin was used as a positive control. A blank was prepared without the test sample. The alpha glucosidase inhibition was determined by measuring the optical density at 405 nm using the spectrophotometer. The percentage inhibition of α-glucosidase was evaluated using the following equation.

$$\%\ of\ \alpha\ glucosidase\ inhibition = \frac{[Abs_{control} - Abs_{sample}]}{Abs_{control}} X\ 100$$

Statistics

All the invitro 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

DPPH radical scavenging activity

The DPPH scavenging assay is the most commonly used method to determine the antioxidant potential of the compound. The ability of total lipids of all the 7 PUFA producers to donate hydrogen ion to reduce DPPH was determined by disappearance of violet colour and the absorbance of which was measured at 517 nm. The DPPH scavenging activity was compared to the positive control, Butylated Hydroxy Toluene, BHT. The effect of total lipids on the reducing the free radicals in comparison with standard is represented in **Figure 1** and **Table 1**. All the 7 fungal lipids were found to possess the antioxidant activity in a dose dependant manner. Among 7, *M. elongata* has shown the highest free radical scavenging activity. This was followed by *F. equiseti,* DB/04*, A. sclerotigenum, U. vinacea, Schizophyllum spp.* (DB/06) and *Schizophyllum spp.* (DB/10) exhibited the DPPH scavenging activity. The scavenging percentage of 100 mg/ml of *M. elongata* was 81.88% and *F. equiseti* was 78.24%, which were significant in comparison with the standard. *Schizophyllum spp.* (DB/10) was found to less potential with 42.63% of DPPH scavenging activity at 100 mg/ml. Tepe *et al.,* (2005) reported that the DPPH test is used to measure the electron donating capacity of the essential oils to stabilize the DPPH radical **[12]**. Similarly, several researchers have evaluated the antioxidant activity of essential oils from several sources such *as Sargassum chordalis* **[13]**, *Cucumis melo cantalupensis*, *Cucumis melo reticulates* **[14]**, *Abrus precatorius* **[15]**, *Taraxacum officinale* **[16]** and *Ferulago campestris* **[17]** which revealed significant free radicles scavenging activities respectively.

NO Scavenging Assay

Nitric oxide, a cell diffusible free radical, acts as an important mediator molecule in human physiological processes. When the same is produced in excess, it ends up in negative ill effects in human health. The NO scavenging potential of all the 7 fungal lipids were determined using Griess reagent. The NO scavenging activity was compared to the positive control, Butylated Hydroxy Toluene, BHT. The effect of total lipids on the reducing the NO radical in comparison with standard is represented in **Figure 2**. **Table 2** shows the mean values of NO scavenging % ± S.D of 7 fungal lipids and standard. Among 7, *M. elongata* has shown the highest NO scavenging activity. This was followed by *A. sclerotigenum, Schizophyllum spp.* (DB/06), *F. equiseti, U. vinacea,* DB/04*,* and *Schizophyllum spp.* (DB/10). The scavenging percentage of 100mg/ml of *M. elongata* was 69.45% and *A. sclerotigenum* was 63.6%, which were significant in comparison with the standard. *Schizophyllum spp.* (DB/10) was found to have less potential with 22.28% of NO scavenging activity at 100 mg/ml. similarly, Okoh *et al*., (2014) have reported the NO scavenging of lipids from *A. precatorius* **[15]**.

Lipid Peroxidation Assay

The lipid peroxidation assay was carried out for *M. elongata* lipids in rat cardiac myoblast cell lines (H9C2). Malondialdehyde (MDA) is one of the products formed majorly during lipid peroxidation. The MDA reacts with thiobarbituric acid and form TBA-abducts, which has a maximum absorbance at 532 nm. The cells without the treatment of both *M. elongata* lipids and H_2O_2 served as control. The MDA content of control, treated cells (both *M. elongata* lipids and H_2O_2) and H_2O_2 treated cells is represented in **Figure 3**. The *M. elongata* lipids had significantly reduced the MDA content at all the 3 concentrations when compared to the H_2O_2 treated cell. The MDA content in the untreated control was very less and in the H_2O_2 treated cell was very high. *M. elongata* lipids at a concentration of 50µg/ml showed a maximum reduction of MDA content of 2± 0.96 mM/ml.

According to Wijeratne *et al.,* (2005) and Ott (2007), lipid peroxidation is a crucial flag for the damage of membranous cellular structures **[18, 19]**. It has the ability to disturb the integrity of the plasma membrane thereby increasing the chance of cell death. Malondialdehyde (MDA), one of the products formed majorly during lipid peroxidation. The MDA reacts with thiobarbituric acid and form TBA-abducts, which has a maximum absorbance at 532nm. The cells without the treatment of both *M. elongata* lipids and H_2O_2 was served as control. In the current study, the *M. elongata* lipids have significantly reduced

the malondialdehyde at all the 3 concentrations when compared to the H_2O_2 treated cell. The malondialdehyde in the untreated control was very less and in the H_2O_2 treated cell was very high. *M. elongata* lipids at a concentration of 50µg/ml revealed a maximum reduction of malondialdehyde of 2± 0.96 mM/ml. Similarly, preventive nature of essential oils from cinnamon on lipid oxidation was well reported by Keshvari *et al.,* (2013) **[20]**.

Biocompatibility and Cytotoxic effect of *Mortierella elongata* lipid in RIN5F cells

The biocompatibility and cytotoxic effect of *M. elongata* lipids was investigated in RIN5F, Rat Pancreatic β cell lines. The RIN5Fcells were treated with various concentrations of *M. elongata* lipids such as 12.5, 50 and 100 µg/ml. The MTT assay indicated that lipids of *M. elongata* were not toxic at all concentration. The percentage of cell death was less than 5% which was observed to be concentration-dependant cytotoxic effect against RIN5F cells. Thus, lipids from *Mortierella elongata* was found to be compatible on pancreatic cell lines. The percentage of RIN5F cells viability on treatment with *M. elongata* lipids was compared with control and is graphically represented **(Figure 4)**.

Glucose Utilization Assay

The effect of *M. elongata* lipids on glucose utilization in RIN5F was investigated. The glucose utilization in RIN5F cells treated with 12.5, 25 and 50 µg/ml of *M. elongata* lipids is represented in **Figure 5**. The effect of *M. elongata* lipids on glucose utilization was compared with metformin, a positive control. *M. elongata* lipids indicated a significant uptake of glucose when compared with untreated control. Around 120% of glucose was utilised by RIN5F at 50 µg/ml concentration.

Alpha-Amylase Inhibition Assay

The inhibitory activity of *M. elongata* lipids on α amylase was investigated at three different concentrations such as 12.5, 25 and 50 µg/ml. The effect of *M. elongata* lipids on α amylase is represented in **Figure 6**. Metformin was used as a positive control. *M. elongata* lipids exhibited a significant effect on α amylase at all the concentration when compared to the untreated control. Around 40% of amylase inhibition was reported at 50 µg/ml of *M. elongata* lipids. The inhibitory effect of *M. elongata* lipids was observed to be a dose-dependent.

Alpha- Glucosidase Inhibition Assay

The inhibitory activity of *M. elongata* lipids on α glucosidase was investigated at three different concentrations such as 12.5, 25 and 50 µg/ml. The effect of *M. elongata* lipids on α glucosidase is represented in **Figure 7**. Metformin was used as a positive control. *M. elongata* lipids exhibited a significant effect on α

glucosidase at all the concentrations when compared to the untreated control. The highest inhibition percent of – was reported at 50 µg/ml of *M. elongata* lipids. The inhibitory effect of *M. elongata* lipids was observed to be a dose-dependent.

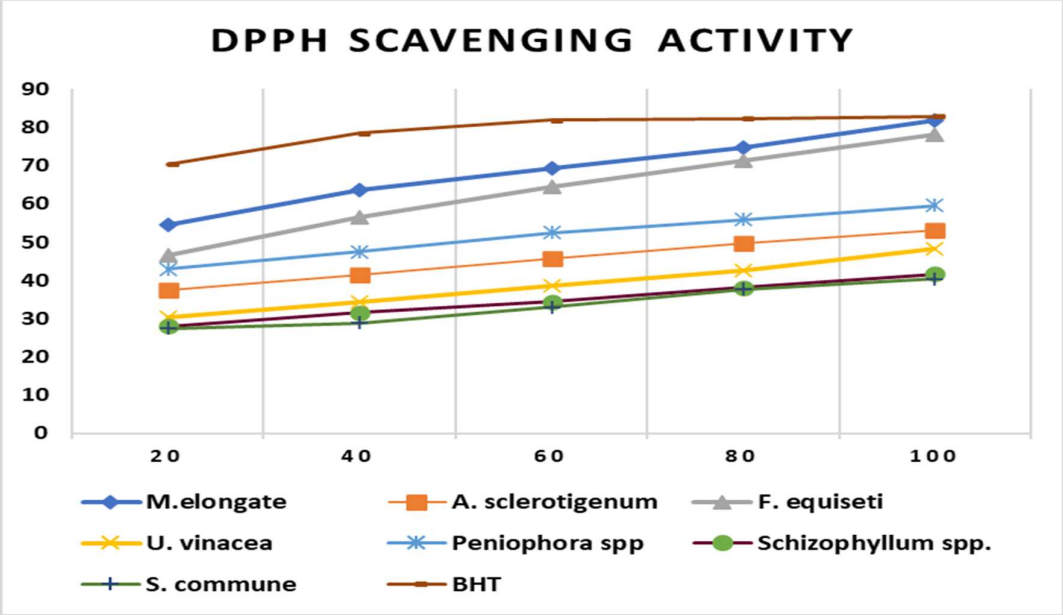

Figure 1: DPPH Radical Scavenging Activity

Table 1: DPPH Radical Scavenging Activity

S. No	Concentration mg/ml	DPPH Radical Scavenging %							
		M.elongata	DB/04	*Schizophyllum spp.* (DB/06)	*A. sclerotigenum*	*Schizophyllum spp.* (DB/10)	*F. equiseti*	*U. vinacea*	BHT
1.	20	54.43±0.22	42.88±0.26	28.05±0.53	37.56±0.42	27.45±0.22	46.62±1.22	30.45±0.07	70.34±0.18
2.	40	63±0.13	47.46±0.11	31.63±0.17	41.45±0.14	28.92±0.15	56.48±0.32	34.24±0.15	78.56±0.07
3.	60	69.44±0.33	52.44±0.25	34.45±0.23	45.61±0.29	32.95±0.42	64.38±0.28	38.65±0.21	81.9±0.15
4.	80	74.82±0.18	55.86±0.9	38.14±0.21	49.71±0.02	37.73±0.25	71.21±0.25	42.63±0.12	82.3±0.06
5.	100	81.88±0.12	59.53±0.25	41.66±0.19	53.11±0.09	40.41±0.22	78.24±0.21	48.43±0.16	82.88±0.01

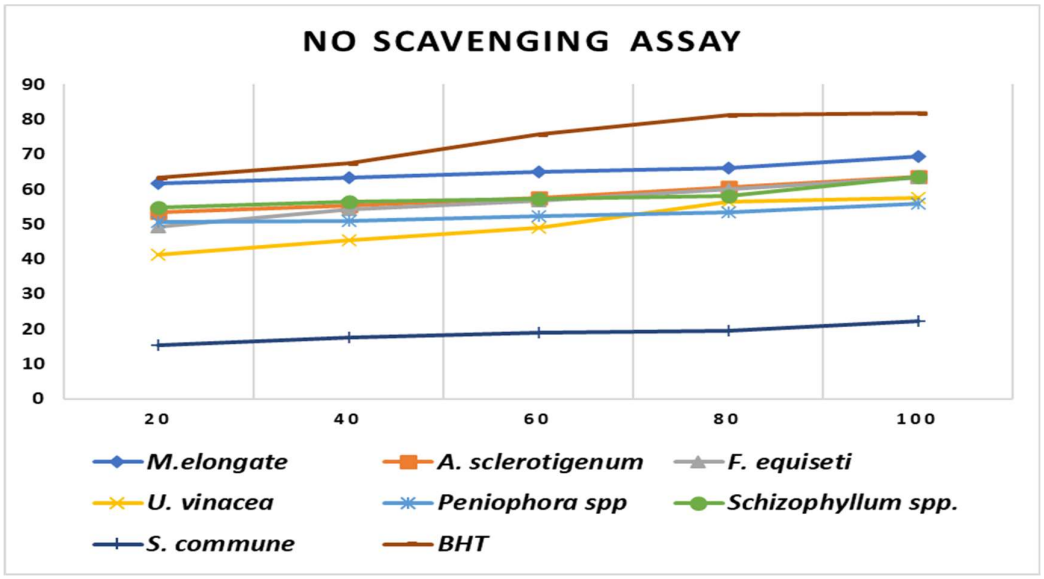

Figure 2: Nitric Oxide Scavenging Assay

Table 2: Nitric Oxide Scavenging Assay

S. No	Concentration mg/ml	NO Radical Scavenging %							
		M.elongata lipid	*DB/04*	*Schizophyllum spp.* (DB/06)	*A. sclerotigenum*	*Schizophyllum spp.* (DB/10)	*F. equiseti*	*U. vinacea*	BHT
1	20	61.72±0.05	50.65±0.07	54.88±0.26	53.39±0.31	15.39±0.26	49.32±0.21	41.26±0.4	63.45±0.26
2	40	63.20±0.32	50.86±0.04	56.38±0.23	55.45±0.14	17.63±0.23	54.36±0.29	45.43±0.18	67.85±0.25
3	60	64.95±0.1	52.2±0.26	57.13±0.09	57.52±0.31	18.77±0.23	56.8±0.02	48.9±0.06	75.96±0.17
4	80	66.09±0.21	53.48±0.1	58.2±0.31	60.58±0.06	19.42±0.29	60.07±0.41	56.41±0.25	81.56±0.13
5	100	69.45±0.46	55.99±0.16	63.59±0.08	63.6±0.33	22.28±0.2	63.29±0.16	57.56±0.18	81.78±0.07

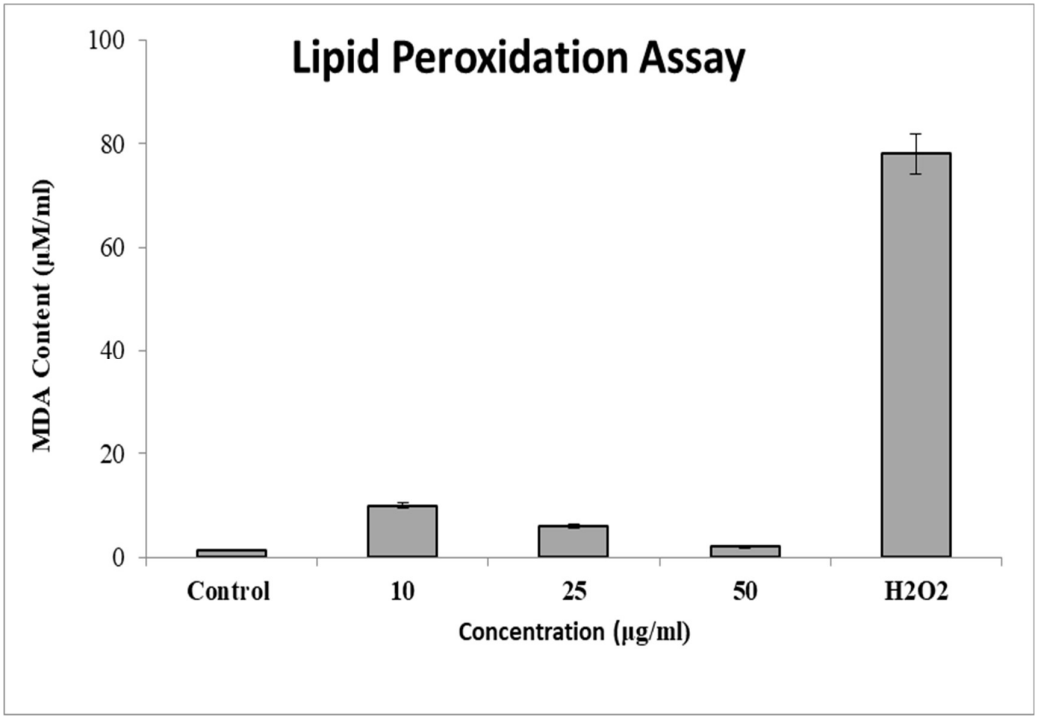

Figure 3: Lipid Peroxidation Assay - *M. elongata* Lipids

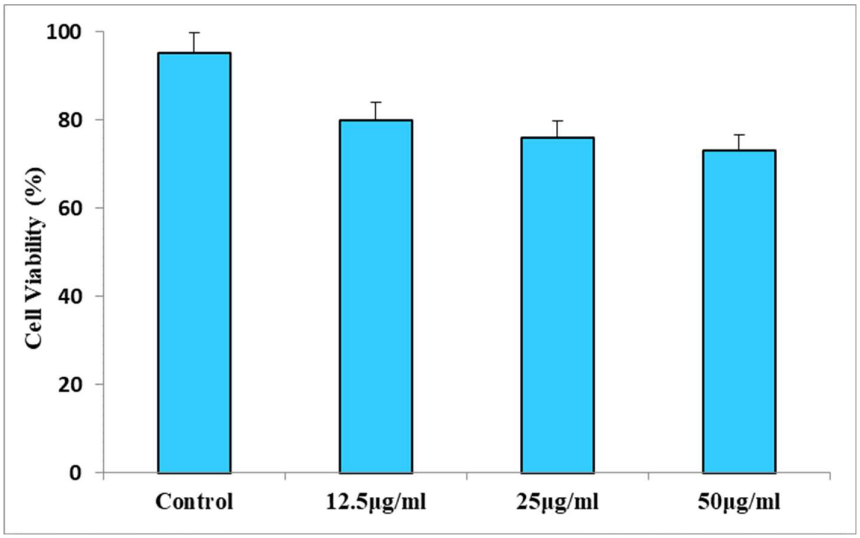

Figure 4: Biocompatibility and Cytotoxic effect of *M. elongata* Lipids in RIN5F cells

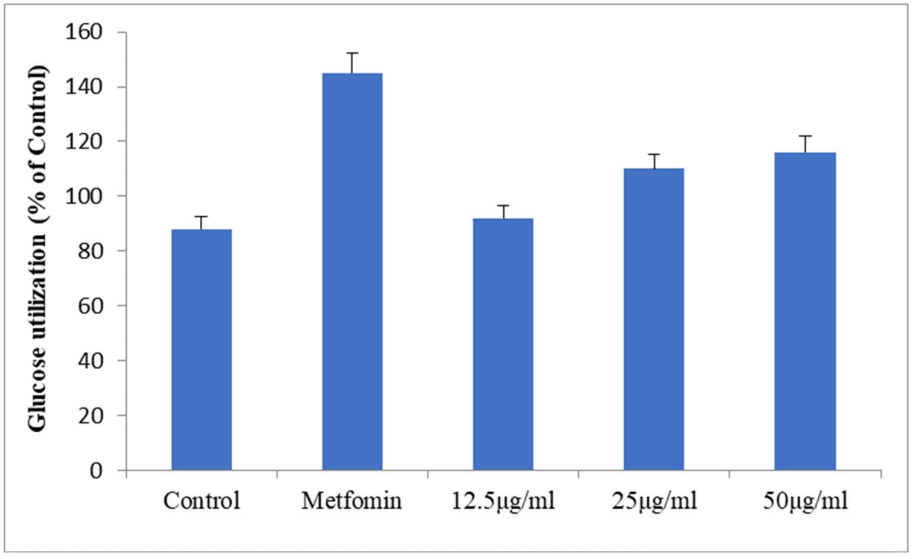

Figure 5: The effect of *M. elongata* Lipids on Glucose Utilization in RIN5F

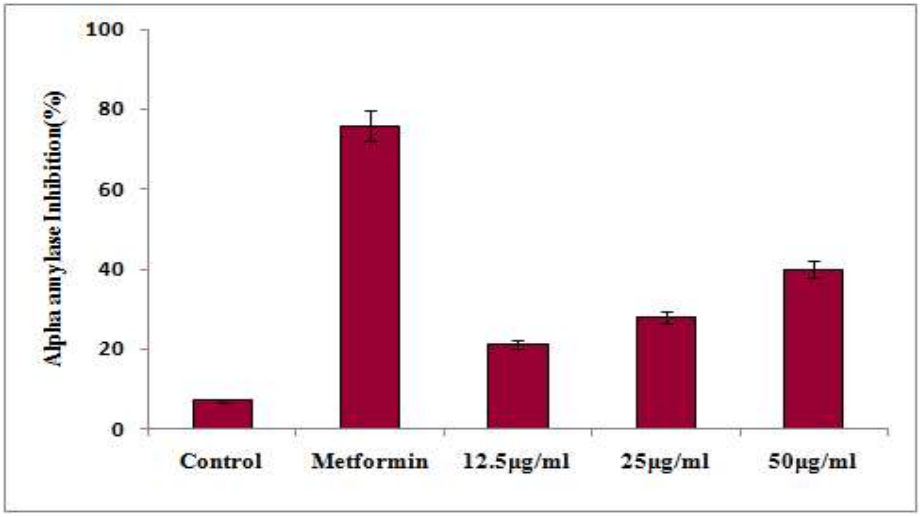

Figure 6: The Alpha-Amylase Inhibition Activity of *M. elongata* Lipids

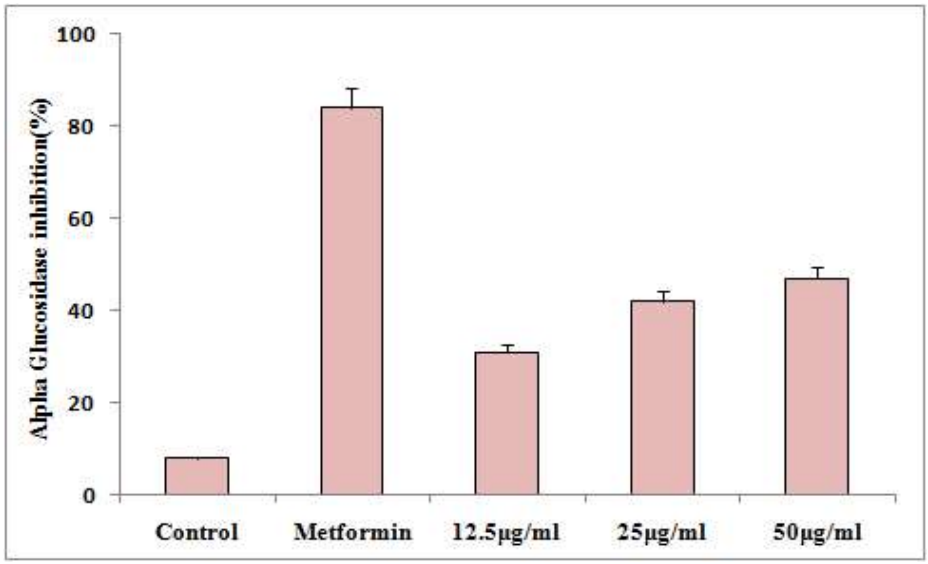

Figure 7: Alpha- Glucosidase Inhibition Activity of *M. elongata* Lipids

Neigo *et al.,* (2021) reported that a variety of macrofungal bioactive compounds possessed anti-diabetic properties **[21]**. A meta-analysis comprising of 20 randomised trials have concluded that omega 3 supplement have greatly reduced triglycerides and increased EPA/DHA ratio have significantly reduced HbAc1 and total cholesterol (Chen *et al.,* 2015) **[22]**. Thus, omega 3 supplements were reported to manage glucose and fatty acid levels. The anti-inflammatory properties of omega 3 PUFA plays an important role in improving the insulin sensitivity and protect against development of DM, which was well reported by several invitro studies and clinical trials. Omega 3 supplements are reported to improve the insulin secretion as well as sensitization (Guadarrama-Lopez *et al.,* 2014) **[23]**.

4. CONCLUSION

Among the 7 PUFA producers, *Mortierella elongata* was found to be more potential PUFA producer as well as possess more antioxidant potential. The biocompatibility and cytotoxic effect of *M. elongata* lipids was safe in RIN5F (Rat Pancreatic β cell lines). The *M. elongata* lipids showed a significant anti-diabetic activity. It has potential in inhibiting the Alpha-Amylase and Alpha- Glucosidase. The inhibitory effect of *M. elongata* lipids on both the enzyme was observed to be a dose-dependent. This in vitro anti-cancer studies could be further taken for in vivo studies in future.

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