

## Evaluating the cholesterol lowering and cytotoxicity of zaragozic acid, a novel therapeutic agent isolated from *Coprophilous fungi*

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**Abstract-** *Coprophilous fungi* were screened for the production of zaragozic acid. Out of the nine isolates obtained three isolates showed acid production during secondary screening followed by its purification using organic solvents. The presence of zaragozic acid was confirmed by antimony trichloride test (SbCl<sub>3</sub>) and its λ<sub>max</sub> at 190 and 249nm. Further the concentration of zaragozic acid was determined by titrimetric analysis, all the six isolates produced 75-80 mg / L of acid. Cell titer 96™ AQueous one solution reagent from Promega, USA was used to check the cytotoxicity of zaragozic acid. It was found that dosage up to 40 μl of cells did not prove toxic to the cells. The zaragozic acid was then checked for its cholesterol reduction activity. The cholesterol lowering effect of zaragozic acid on cholesterol synthesis was confirmed, 0.8 μg of cholesterol per gm of tissue was reduced by using 30 μl of zaragozic acid.

**Keywords-** Zaragozic acid, *Coprophilous fungi*, MTT assay.

### INTRODUCTION

A traditional approach to the discovery of new naturally occurring bioactive molecule utilizes screening. Screening is an assay procedure by which many compounds are tested for a particular activity. The most extensive use of screening is to search a compound with selective toxicity to bacteria, fungi or protozoa. Screening can be extended for searching a microorganism producing a bioactive substance which directly or indirectly enriches the human life. For instance the disorder *familial hypercholesterolemia* occurs in 1 individual per 500 of the population and results elevated plasma levels of cholesterol – bearing LDL. Male heterozygotes with dominant alleles of *familial hypercholesterolemia* have 85% chances of suffering heart attacks (myocardial infraction) before the age of 60. Homozygotes of either sex die of heart disease at early age. A much larger number of people who do not have *familial hypercholesterolemia*, have plasma levels of LDL at the upper limit of the normal range and are also at the risk for atherosclerosis. The goal of therapy in these subjects is to reduce the level of LDL without impairing cholesterol delivery to the cells this is achieved by partial inhibition of cholesterol biosynthesis. About 93% of the cholesterol in human body is located in cells where it performs indispensable structural and metabolic roles. 7% of it circulates in the plasma, where it contributes to atherosclerosis, formation of plaques on the walls of the arteries supplying heart, the brain, and the other vital organs. For delivery to tissues, plasma cholesterol is packaged in lipoprotein particles; two-thirds is associated with LDL and balance with HDL. Cholesterol is a product of the isoprenoid pathway in mammals as shown below-

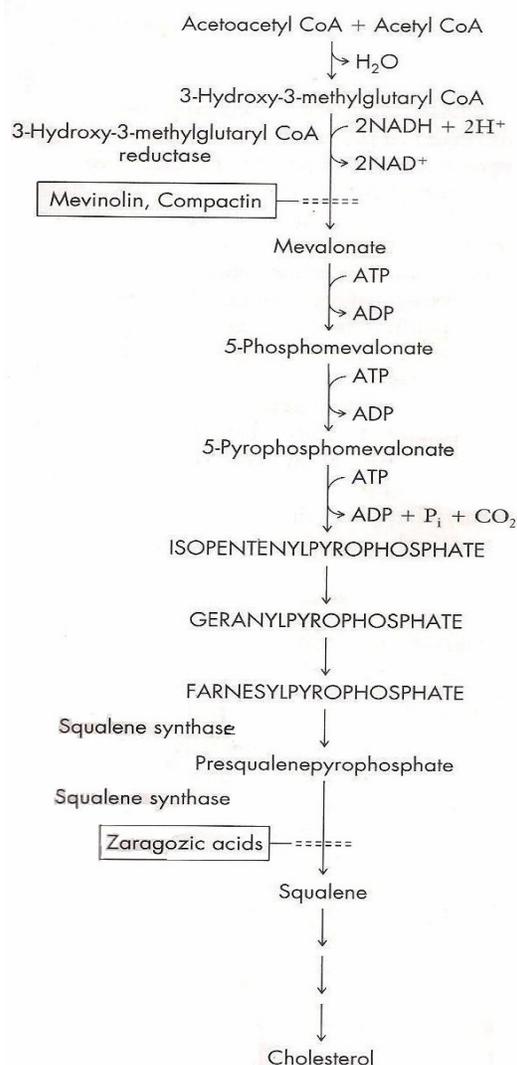


Fig. 1- Pathway leading to biosynthesis of cholesterol in humans. Isopenteny-, geranyl-, farnesyl-, are precursors of cholesterol and

other vital isoprenoid derivative. Mevinolin [*Aspergillus terreus*] Compactin [*Penicillium spp*] are two other popular cholesterol reducing drugs; they inhibit 3HMG CoA reductase, thereby affecting production of all the products in the pathway as against Zaragozic acid which inhibits squalene synthesis, first committed step in cholesterol synthesis whereas the essential products of isoprenoids are produced normally.

In addition to cholesterol and other steroids produces several key metabolites essential to cells namely: dolicol, ubiquinone, the farnesyl and geranyl moieties of pentenylylated proteins and the isopentenyl side chain of isopentenylated adenine. The pathways for the synthesis of these compounds diverge from synthesis of cholesterol either at or before the farnesyl diphosphate branch point. The first committed step in cholesterol biosynthesis is squalene synthase catalyzed conversion of 2 moles of farnesyl pyrophosphate to 1 mole of squalene. Therefore squalene synthase is an attractive target for selective inhibition of cholesterol biosynthesis. The compound "zaragozic acid"; produced by *Coprophilous fungi* [fungi inhabiting dung of herbivore animals] has been found to be a potent inhibitor of this enzyme. In comparison to presently used cholesterol lowering drug viz: lovastatin, mevacor; "Zaragozic acid" has proven to be a better drug for its ease in production, low cost and low cell and genotoxicity. Significance of zaragozic acid over Mevinolin and Compactin has already discussed in the fig 1.



Fig. 2- Structure of zaragozic acid inhibitor of squalene synthase

Two fungi known to produce zaragozic acids are *Sporomiella intermedia* and *Leptodontium elatius*.

#### MATERIAL AND METHODS

96 well cell culture flat bottom plates with lid [Corning Incorporated 3569, corning, NY14831 US]; Organic solvents were obtained from Sisco Research Labs, India [AR grade]; TLC silica gel 60 F254 plates [Merck] Cholesterol determination was done by using CHOD – PAP method; Pathozyme Diagnostics, India, Cytotoxicity was performed by G358C 24125103 Cell Titer titer 96™ AQueous one solution reagent, kindly provided by Promega, USA.

#### SCREENING OF FUNGI

Cow dung was obtained from local cowshed in sterile buffered saline. Primary screening of dung samples was done by soaking 10g of cow dung in 100ml sterile phosphate buffered saline [pH 7.2].

On subsequent day the 10% dung solution was serially diluted to 10<sup>-5</sup> to 10<sup>-7</sup> dilutions. All three dilutions were plated on CMC medium and DPY medium in triplicate. The plates were incubated at 37°C till the colonies appeared. All the isolates of primary screening were subjected to secondary screening. Secondary screening was performed by using buffered media containing methyl orange indicator. Only three isolates i.e. colonies giving sufficient red color around the colonies were picked for further investigation. The morphology of fungus was determined by taking a fraction of fungus on the glass slide, teasing with back of the forceps, followed by staining it with lactophenol cotton blue and observing it under 45X objective of compound microscope. Identification of fungus genus and species is under process. All the three isolates were further cultured in 100ml CMC and DPY broth in 500ml conical flask, on Scigenics make orbital shaker with agitation of 70rpm for 10 days. On eleventh day the broth was centrifuged at 5000g for 10min. the supernatant was used for extraction of acid.

#### EXTRACTION OF ACID

The supernatant was acidified with 1% H<sub>2</sub>SO<sub>4</sub> in water. The acidified supernatant was dissolved in equal amount of chloroform, and was allowed to stand in separating funnel for 24 hours, Aqueous phase was discarded and organic phase containing zaragozic acid was basified with equal amount of 2% NH<sub>4</sub>OH which converted zaragozic acid to its corresponding salt, therefore rendering it soluble in water phase. Water phase was obtained by using separating funnel. The sample was concentrated by using rotary evaporator [Superfit make] at 85°C for 2 and ½ hours. The concentrated sample was plated on the TLC plate and sprayed with 2% antimony trichloride (SbCl<sub>3</sub>) in H<sub>2</sub>SO<sub>4</sub>. The plate was incubated in hot air oven for ten minutes at 110°C, development of pink color was indicator of zaragozic acid. Further confirmation of the zaragozic acid was done by determination of its λ<sub>max</sub> using Jasco 530 make UV – VIS spectrophotometer. Quantification of zaragozic acid was done by titrimetric method

#### QUANTIFICATION OF ZARAGOZIC ACID

1ml of concentrated sample was diluted to 10 ml with water and 2 drops of phenolphthalein indicator and titrated against 0.5 N NaOH. Endpoint determination was colorless to pink. By using formula-

$$C_1 \times V_1 = C_2 \times V_2$$

Normality of zaragozic acid was obtained; subsequently g/l of zaragozic acid was calculated by multiplying equivalent weight into normality.

#### CHOLESTEROL SYNTHESIS INHIBITION ASSAY

Goat liver was procured from the local slaughter house aseptically in 250ml sterile chilled phosphate buffered saline [pH 7.4]. It was chopped to fine pieces with the help of sterile scissors in vertical laminar air flow. The chopped pieces were incubated with equal amount of TPVG for 45min in Orbitech rotary shaker at 37°C at 105 rpm. 10% Serum was added to inhibit the

action of trypsin after the completion of incubation. The cells were centrifuged at 3250 rpm for 10min; the supernatant was subjected to Neubaur's Chamber for cell counting.

Fifteen to twenty thousand cells were seeded in 96 well plates in 40µl of PBS, incubated on rotary shaker for 2 hours at 37°C at 95 rpm. 1 ml of the cholesterol reagent was added to each well followed by addition of 3µl, 10µl and 30µl of zaragozic acid. The plate was incubated at 37 °C for 10 min at RT. Absorbance of the standard and test samples were measured at 505 nm.

**Cholesterol in mg/dl = At/As\*200**

#### CELL TITER 96™ AQUEOUS ONE SOLUTION REAGENT CYTOTOXICITY ASSAY

The Cell Titer 96™ AQueous One Solution cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The Cell Titer 96™ Aqueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. This convenient "One Solution" format is an improvement over the first version of the Cell Titer 96™ AQueous Assay, where phenazine methosulfate (PMS) is used as the electron coupling reagent, and PMS Solution and MTS Solution are supplied separately. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding a small amount of the Cell Titer 96™ AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording absorbance at 490nm with a 96 well plate reader.

The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. Because the MTS formazan product is soluble in tissue culture medium, the Cell Titer 96™ AQueous One Solution Assay requires fewer steps than procedures that use tetrazolium compounds such as MTT or INT. The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570nm. For the assay same goat liver cells were used. 20000cells /100µl of DMEM were introduced in each well followed by addition of 20 µl of the Cell Titer 96™ AQueous One Solution. The plates were incubated for 4 hours at 37°C in a humidified, 5% CO2 atmosphere. The absorbance was read at 490 nm using Eliza plate reader. The cell viability was determined using following formula –

$$\% \text{ viability} = \frac{\text{Mean absorbance of sample}}{\text{Mean absorbance of control}} \times 100$$

## OBSERVATIONS AND RESULTS

### SCREENING

The fungi were found to be very slow growing and showed colony after incubation of 5-7 days. Initially 9 isolates were obtained out of which 6 were seen on CMC medium and 3 on DPY medium. On growing these isolates on buffered medium containing methyl orange, only 3 colonies labeled as A, B and C respectively out of the 9 showed profuse red coloration around their colony indicating acid production, .

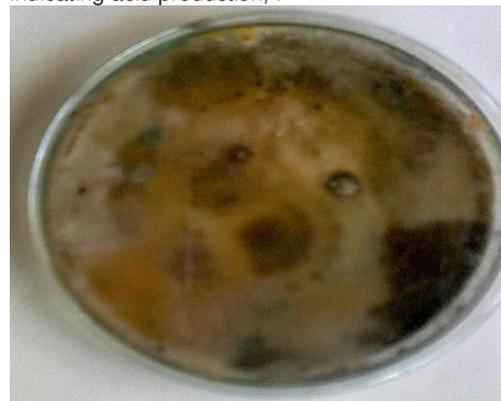


Fig. 3- Plate showing fungal colonies on CMC medium

### COLONY CHARACTERS

**Table 1-** Colony characters of 3 isolates used for further investigations followed by secondary screening

Colony	Size	Color	Elevatio	Margin
A	1.0cm	Gray	Raised	Undulated
B	1.0cm	Greenish yellow	Flat	Entire
C	0.3cm	Yellow	Flat	Undulated

### MORPHOLOGY

On staining with lactophenol cotton blue the isolates showed following characteristics:

All the colonies exhibited common characteristics like the presence of globose to sub-globose fruiting body. Numerous spores were produced and arranged in the form of chains on the fruiting body in the form of basipetal succession.

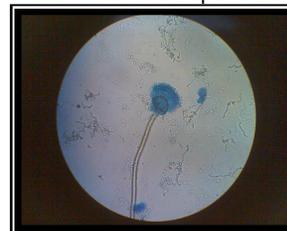


Fig. 4- Fungi as seen under Microscope at 45X

### TITROMETRIC ANALYSIS

All the titration was performed induplicate and the mean readings were as follows:

**Table 2- Quantification of zaragozic acid by titrometric assay**

Isolate	Initial volume	Final volume	Difference	g/L of acid produced
A	0	0.8	0.8	0.08
B	0	0.8	0.75	0.075
C	0	0.7	0.7	0.07

The titrometric analysis showed that about 75-80 mg of acid / L of broth was produced by the fungi.

### CHOLESTEROL SYNTHESIS INHIBITION

The extracted zaragozic acid when plated in increasing order showed the following results.

**Table 3- Zaragozic acid showing inhibition of cholesterol synthesis by goat liver cells**

Zaragozic acid (in $\mu$ l)	Cholesterol ( $\mu$ g/gm of tissue)
[stock :0.80mg/L]	
0	2.1
3	1.9
10	1.7
30	1.3

There was a significant lowering of cholesterol synthesis 0.8  $\mu$ g of cholesterol per gm of tissue was reduced by using 30  $\mu$ l of zaragozic acid. Negative control used for the above reaction was cells plus BSA; there was good lowering in biosynthesis of cholesterol when 30 $\mu$ l of zaragozic acid was used, then after there was no significant lowering of cholesterol.

### CELL VIABILITY ASSAY

The cell viability count at 60 $\mu$ l sample [maximum] was 100%, 93.46%, and 91.66% for isolate A, B and C respectively. The Cell Titer 96™ Aqueous One Solution cell Proliferation Assay gave following results

**Table 4- The cell viability count at 60 $\mu$ l sample [maximum] was 100%, 93.46%, and 91.66% for isolate A, B and C respectively**

Isolate	O.D of control	O.D of sample
A	0.069	0.069
B	0.043	0.046
C	0.011	0.012

### CONCLUSION

From the above results, it can be concluded that zaragozic acid, a secondary metabolite produced by fungi has a good therapeutic potential not only because of its ability of lowering the biosynthesis of cholesterol but also of its non cytotoxicity as observed in *in vitro* assays. Its genotoxicity is yet to be investigated furthermore its potential as a future therapeutic agent can only be firmly concluded after its usage in bioassays.

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