MEDWIN PUBLISHERS
Committed to create value for Researchers

Open Access Journal of Pharmaceutical Research
ISSN: 2574-7797

# Biomass from *Fusarium Venenatum* and Preliminary Characterization of Bioactives: Exploration of Hypolipidemic Potential

**Patil SD[1], Shetane TD[1], Singha RG[2], Choudhary DM[1], Nanda RK[1], D'souza OR[3] and Thomas AB[1]***

[1]Department of Quality Assurance Techniques, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, India

[2]Glenmark Pharmaceuticals Limited, India

[3]Department of Pharmacology, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, India

***Corresponding author:** Asha B Thomas, Department of Quality Assurance Techniques, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, India; Tel: +91-9881236220; E-mail: dypharmachem@yahoo.co.in

**Research Article**

Volume 1 Issue 6

**Received Date**: September 15, 2017

**Published Date:** October 02, 2017

## Abstract

*Fusarium venenatum* derived mycoprotein (biomass) marketed under the trade name Quorn has been designated as a source of first class protein with low cholesterol. Our previous study, demonstrates the significant anti-hyperlipidemic potential of biomass in acute Triton X-100 induced hyperlipidemic model in rats and anti-oxidant activity by DPPH assay. The biomass was produced through cost effective fermentation process in Vogel's mineral medium using sucrose as the carbon source optimized using Central Composite Response Surface Design (CCRSD). In continuation to our previous reported work, in the current study the hypolipidemic potential of biomass was studied in the chronic high fat diet induced hyperlipidemic model in rats. The high fat diet fed rats showed significant increase in plasma lipid levels [total cholesterol (TC), triglycerides (TG), very low density lipoproteins (VLDL), low density lipoproteins (LDL)] with decreased high density lipoproteins (HDL) levels. Biomass (100, 200 and 400 mg/kg) and simvastatin (10 mg/kg) administered orally reduced the elevated serum lipids (TC, TG, VLDL, LDL), restored the decreased HDL and improved the atherogenic index ($p<0.01$). Also the treatment with biomass decreased the liver enzymes levels (SGOT, SGPT) comparable to standard treated group. The biomass treatment also improved histoarchitecture of hepatocytes in hyperlipidemic rats. The acute oral toxicity study carried out as per OECD guidelines demonstrated the safety of the biomass. LC-MS analysis of methanolic extract of biomass showed two major peaks (Compound 1: m/e 209.10 and Compound 2: m/e 329.25). Spectral matching with NIST libraries indicated that compound 2 may be structurally similar to pregnenolone, a naturally occurring steroid. Also, compound 1 may be 4- aminothiophenol, N,S,-diacety (m/e 209.10). These probable bioactives, exhibits varied biological activities including for the treatment of inflammation, and its related disorders as suggested by literature reports. In conclusion, the study proves the hypolipidemic potential of biomass derived from *Fusarium*

*venenatum* in preclinical acute and chronic animal models. Further investigations to isolate the bioactives and elucidate the probable mechanisms and role in triggering hypolipidemic activity need to be undertaken.

**Keywords:** *Fusarium venenatum*; LC-MS analysis; High Fat Diet; Antihyperlipidemic activity.

**Abbreviations:** CCRSD: Central Composite Response Surface Design; VLDL: Very Low Density Lipoproteins; HDL: High Density Lipoproteins; LDL: Low Density Lipoproteins; NTC: National Toxicological Centre; GRAS: Generally Recognized as Safe; HFD: High Fat Diet; TC: Total Cholesterol; TG: Triglycerides; CMC: Carboxy Methyl Cellulose; NIST: National Institute of Standards and Technology

## Introduction

The world is facing problems of malnutrition, mainly protein-energy malnutrition and micronutrient deficiencies, which continues to be a major health burden in developing countries [1]. It is globally the most important risk factor for illness and death, with hundreds of millions of pregnant women and young children particularly affected. Apart from marasmus and kwashiorkor (the two forms of protein- energy malnutrition), deficiencies in iron, iodine, vitamin A and zinc are the main manifestations of malnutrition. The Indian population is mainly dependent on cereals and pulses as the major sources of protein. The growing concern over the eventual food crisis triggered due to environmental and ecological reasons has laid a major research emphasis on use of single cell proteins [2,3].

Since many years *Fusarium venenatum* has been reported as an alternative protein source. It has been used for human consumption in England for over a decade under the trade name Quorn [4,5]. This protein product has high fibre content, carbohydrates essential amino acids, and is designated as a source of first class protein with low cholesterol. It is an excellent meat alternative. As it is rich in zinc, it is useful in vegetarian diet where zinc levels can be limited but is essential as an enzyme activator [6,7]. The mycoprotein has been reported to reduce blood lipid levels [8,9] with satiety and satiation properties [10,11].

In our previous study, we have reported the cost effective production of biomass from *Fusarium venenatum* using sucrose as the carbon source grown in Vogel's mineral medium. Optimization of selected carbon source and seed size using Central Composite Response Surface Design (CCRSD) indicated that that sucrose (1.64 g/100ml) and seed size (10% v/v) were optimal in maximizing biomass (0.5602 g/100 ml, $p<0.0001$) and protein yield (49.99%, $p<0.01$) of *Fusarium venenatum* [12]. The study also demonstrated the efficacy of the biomass to lower the elevated blood lipid levels in Triton X-100 induced hyperlipidemic model in rats. In continuation to our previous reported work, in the current study attempt has been made to further evaluate the anti-hyperlipidemic potential of biomass in animals using chronic model. Quantitation of the carbohydrate and steroid content in biomass was performed, as previously suggested by LC-MS study of the acetonitrile extract of the biomass.

## Materials and Methods

### Drugs and Chemicals

Cholesterol, cholic acid (Himedia Laboratories Pvt. Ltd., Mumbai, India), Simvastatin (Lupin Ltd., Mumbai, India) were used in the study. All other chemicals employed were of analytical grade and obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Diagnostic reagents kits (Bio Lab Diagnostics (I) Pvt. Ltd., Mumbai, India) were used for the biochemical assays.

### Fugal Strain and Other Materials

*Fusarium venenatum* (MTCC 9910; ATCC PTA-2684) as active culture slants, were obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The culture was sub-cultured and maintained on potato sucrose agar (200 g potatoes, 20 g sucrose, 20 g agar and distilled water 1000ml at pH 6.5).

### Inoculums, Medium Preparation and Production of Biomass

The inoculum, media preparation and production of biomass were carried out as per our previously reported procedure. The inoculum of *Fusarium venenatum* were prepared in Vogel mineral medium in Erlenmeyer flask, incubated with continuous shaking on a rotary shaker at 25-27°C at 200 rpm for 48 hrs to ferment, followed by RNA reduction treatment with subsequent separation by filtration and washings, and dried to obtain biomass [13].

### Preparation of Biomass Extracts

The biomass obtained was subjected to ultrasonication assisted solvent extraction. To approximately 5 g of wet biomass, methanol (30 ml) was added and then probe sonicated (approx. 11 - 13 cycles of 1 min duration each, with a rest time of 1 min between cycles, total sonication time of approximately 25 min) by applying 150 W. The extract was then filtered through five layers of muslin cloth, and then concentrated using rotary evaporator to obtain a concentrated extract. The concentrated extract was reconstituted in methanol to yield test sample solutions for further LC-MS studies.

### Qualitative Evaluation of Biomass

The preliminary phytochemical evaluation of the biomass was carried out using standard procedures [14].

### Quantification of Carbohydrate Content of Biomass Extracts Preparation of D-Glucose Standards and Calibration Curve

100 mg of glucose AR was weighed accurately and transferred to a 100ml volumetric flask. The volume was made up to the mark with 100ml by distilled water to obtain a standard of concentration 1mg/ml. Suitable aliquots (0.2-1.0 ml) of standard were withdrawn in test tubes, volume made up to 1.0 ml with distilled water. To each, 4.0 ml of anthrone reagent was added, heated for eight minutes in a boiling water bath, and cooled rapidly. The resulting green to dark green colour was read at 630 nm against a suitably prepared blank on a UV spectrophotometer (Shimadzu-1700). The standard graph was drawn by taking the concentration of glucose on X axis and spectrophotometer reading on Y axis.

### Preparation of Biomass Sample and Quantitation

An accurately weighed amount of biomass (100mg) was transferred to a boiling tube, hydrolysed with 5.0 ml of HCl (2.5 N) in a boiling water bath for three hours, then cooled to room temperature and neutralized with solid sodium carbonate until effervescence ceases. The volume was then made up to 100 ml with distilled water. The resulting solution was centrifuged (100 rpm) and the supernatant was collected. From it, 0.2 to 1.0 ml was taken for analysis. The sample solution was also treated with anthrone reagent, absorbance recorded at 630 nm. The concentration of carbohydrate equivalent to glucose in the sample was computed against the prepared calibration curve [15].

### Preparation of Cholesterol Standards and Calibration Curve

100 mg of cholesterol AR was weighed accurately and dissolved in 100ml glacial acetic acid transferred to a 100 ml volumetric flask. The volume was made up to the mark with glacial acetic acid to obtain a standard of concentration 1mg/ml. 0.1 ml and 0.2 ml of triple acid extract was taken and a set of standards (0.5 to 2.5 ml) were taken and made up to 5 ml with ferric chloride diluting reagent [15]. A blank was prepared simultaneously by taking 5.0 ml diluting reagent. Then add 4.0 ml of concentrated sulphuric acid to each tube. After 30 minutes incubation, intensity of the colour developed was read at 540 nm against a suitably prepared blank on a UV spectrophotometer (Shimadzu-1700). The standard graph was drawn by taking the concentration of cholesterol on X axis and spectrophotometer reading on Y axis.

### Preparation of Biomass Sample and Quantitation

An accurately weighed amount of biomass (100mg), add 50ml of glacial acetic acid was transferred to a 100ml volumetric flask Pipette out 0.01 to 0.2ml of extract. The volume was then made up to 5 ml with ferric chloride diluting reagent. 4ml concentrated sulphuric acid was added, incubated for 30 minutes and absorbance recorded at 630 nm. The concentration of steroids equivalent to cholesterol in the sample was computed against the prepared calibration curve [15].

### LC Separation and Preliminary Characterization of Compounds in Biomass Extract

Literature reports suggest that various fungal biomass may contain bioactive compounds exhibiting broad spectrum of biological activity [16]. Our previous study suggested the presence of a sterol glycoside in the acetonitrile extract of biomass established through LC-MS investigations. The study also demonstrated the anti-oxidant potential of both the acetonitrile and methanolic extracts through DPPH assay which suggest the possibility of presence of bioactives. In the current study, an attempt has been made to characterize the compounds in the methanolic extract of biomass by LC and LC-MS studies.

### HPLC Analysis

A Shimadzu HPLC system equipped with a manual injection valve (Rhenodyne injector) with a 20 µl loop, PDA detector and integrated software of LC-Solution was

used for the analysis. An isocratic elution was performed on a Neosphere C18 R column (250 mm×4.6 mm, 5µm particle size). The mobile phase consisted of two different solvents, solvent A and solvent B. Solvent A was acetonitrile and solvent B was methanol where the proportion of solvent A and B were in the ratio 50:50 % v/v. The mobile phase components were sonicated in water bath sonicator and filtered through 0.45 micron membrane filter under vacuum. Separation was effected by isocratic elution using acetonitrile: methanol (50:50 % v/v) as the mobile phase. The mobile phase flow rate was set to 1 mL/min and the injection volume was 20 µl. UV detection was performed at 295 nm. The total runtime was 20 min. The number of chromatographic peaks eluting out in the set run time were noted. Using these chromatographic conditions, the retention time of unknown compounds were determined.

### LC-MS Analysis

The LC-MS studies of the methanolic extract of biomass was performed on a Shimadzu LC- MS8040 system equipped with Quarternary pump HPLC with a PDA detector and coupled with Modes of analysis: Q3 Scan, Multiple reaction monitoring (MRM), Mass Spectrometer System was used for the analysis. The LC conditions were similar to the previously optimized chromatographic conditions, only the column used here was Acclaim TM 120 (C18 5µm 120 A° 4.6 X 250 mm) Mass Conditions: Ionization mode: Positive Electrospray Ionization Ion source voltage: 2500V, Source Temperature: 200˚C, Nebulizer: 0.3 Bar, Dry Gas Flow: 4.0 l/min. A scanning range of 50-1200 m/z was used for the mass acquisition.

### Acute Oral Toxicity Study Animals

Acute oral toxicity study of biomass produced under the modified experimental conditions was performed on 05 healthy Swiss albino mice (males and females) , 25 - 30g body weight, aged 8 to 10 weeks obtained from National Toxicological Centre (NTC), Pune, India. The mice were distributed into two groups. The experimental protocol was approved by the Institutional Animal Ethical Committee as per protocol number DYPIPSR/IACE/16-17/P-21 and conducted according to the Indian National Science Academy Guidelines (INSA) for the use and care of experimental animals [17].

### Acute Toxicity Assay

The mice were housed individually in polypropylene cages and marked on the tail for individual identification. Animals were maintained on a 12-h light/dark cycle, at room temperature (25 ± 2°C) with constant humidity. They were acclimatized to laboratory conditions for a week prior to experimentation. Animals had access to drinking water and food provided ad libitum throughout the experiment. The GRAS (generally recognized as safe) notifications (GRAS) of Quorn product suggest that the safety assessment of the mycoprotein has been carried out extensively in experimental animals and in human clinical subjects [18]. As per the expert panel recommendations, the mycoprotein is safe and suitable for human consumption. AS per the GRAS notification and according to the procedures outlined in the OECD guidelines (OECD), the acute oral toxicity of biomass was evaluated in mice [19]. A single dose of biomass (2000 mg/kg) was administered to test mice by the oral route as suspension in 1% w/v carboxy methyl cellulose (CMC). The mice were closely monitored for any toxicity indications within the first six hours after the treatment period, and further daily for a period of 14 days. During the study period, the weight of the animals were recorded and visually observed for signs of mortality, convulsion, muscle spasm, lacrimation, diarrhoea, salivation, respiration, skin colour, visual placing response, mucus membrane changes in behavioral pattern, physical appearance, injury, pain and signs of illness. At the end of the treatment period, the mice were sacrificed. Vital organs such as heart, kidneys, liver, lung and spleen were isolated and examined for any lesions.

### Evaluation of Antihyperlipidemic Potential of Biomass in Chronic Animal Model

Male Wistar rats (body weight 150-200 g), aged 3.0-4.0 months, were used in the study. Animals were obtained from the NTC, Pune, India, and were housed in animal place at room temperature being maintained at 25 ± 2°C. Animals were fed on a standard pellet diet and kept under normal light/dark cycle. Animals were given free access to food and water. The experimental protocol (protocol number DYPIPSR/IACE/16-17/P-21) approved by the Institutional Animal Ethical Committee was conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

### High Fat Diet Induced Hyperlipidemia in Rats

Hyperlipidemia was induced in male Wister rats (150-200 g) by maintaining them on a high fat diet HFD (Table 1) for a period of 45 days. The rats were randomly divided into 5 groups (6 rats/group) and received the following treatment: Group I- Normal control (NC) maintained on normal diet throughout 45 days + 1% w/v CMC (1 mL/100 g, body weight, po) for 15 days (from 31$^{st}$ - 45$^{th}$ day of treatment period). Group II- HFD control group maintained on high fat diet throughout 45 days + 1% w/v CMC po for 15 days (from 31$^{st}$ - 45$^{th}$ day of treatment period). Group III- Standard group, maintained

on HFD throughout 45 days + Simvastatin (10 mg/kg, po.) for 15 days (from 31st – 45th day of treatment period); Group IV, V and VI- Test 1, Test 2 and Test 3, maintained on HFD throughout 45 days + biomass 100 mg/kg, po, 200 mg/kg, po and 400 mg/kg, po respectively for 15 days (from 31st - 45th day of treatment period). At the end of the treatment period, animals were fasted for 12 h and the blood was collected by cardiac puncture under light ether anesthesia and then sacrificed. The liver was excised promptly, washed in cold normal saline and the liver tissue was preserved in 10% formalin for further histopathological studies. Serum was separated by centrifugation and further analysis was done by using Elitech Selectra automated biochemistry analyzer. The serum lipid and enzyme levels were estimated using biomedical kits (Total cholesterol, HDL, SGOT and SGPT: Pathozyme Diagnostics, Mumbai, India; Triglycerides: GPO-PAP-kit, Biolab Diagnostics Pvt. Ltd., Mumbai, India; ALP: Merk Diagnostics, Pune, India) [20-22].

| S. No | Content | Per 100 g of food pellet |
|---|---|---|
| 1 | Sodium chloride | 0.5 |
| 2 | Cholic acid | 0.5 |
| 3 | Cholesterol | 1 |
| 4 | Egg yolk | 80 |
| 5 | Gram flour | 20 |
| 6 | Coconut oil | 20 |
| 7 | Corn flour | 20 |
| 8 | Wheat flour | 30 |

Table1: Composition (%) of high fat diet used for inducing hyperlipidemia in rats.

### Measurement of Body Weight

The body weights of rats were recorded at every 15 days interval during the experimental period.

### Determination of Serum Lipid Profile and Biomarkers

TC, TG and HDL in serum were assayed using the readymade diagnostic kits. VLDL-TC and LDL-TC values were calculated using formula, (VLDL-TC = TG/5), LDL-TC = TC - (HDL-TC+ VLDL-TC). Atherogenic index was calculated using formula, Atherogenic index = TC / HDL-TC). The serum aspartate amino transferase (AST) and alanine amino transferase (ALT) were estimated and expressed as IU/L [22].

### Histopathological Studies

All the animals were sacrificed by using $CO_2$ asphyxiation, cut open and observed grossly for abnormality, if any Liver from all the animals were collected and fixed in neutral buffered formalin (10%v/v). The tissues were trimmed longitudinally and processed in ascending grades of alcohol, xylene and finally embedded in paraffin wax. Paraffin wax embedded tissue blocks were cut into sections at 3-6 µm thickness with rotary microtome (Make-Leica, Model no-RM 2125). All the slides of liver tissues were stained with Hematoxylin & Eosin (H & E) stain. The prepared slides were examined under microscope to note histopathological lesions, if any. Severity of the observed lesions were recorded as minimal (<1%), mild (1-25%), moderate (26-50%), moderately severe (51-75%), severe (76-100%) and distribution was recorded as focal, multifocal and diffuse.

## Statistical Analysis

The results were expressed as mean ± S.E.M. values using statistical analysis software, GraphPad InStat, version 3.01. Statistical evaluation was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. P value <0.05 was considered statistically significant and were expressed as *p<0.05, **p<0.01.

## Results

### Production of Biomass from *Fusarium venenatum*

The biomass was produced from *Fusarium venenatum* on Vogel's mineral media using sucrose as carbon source (1.64 g/100 mL) and seed size (10% v/v). Under the previously optimized conditions, optimal biomass yield of 0.50 g/100 ml with 50.32% protein yield was obtained.

### Quantitative Evaluation of Biomass Quantification of Carbohydrate Content of Biomass Extract

The total carbohydrate content of biomass extract was estimated by the method of Hedge and Hofreiter, 1962. In this procedure, carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid (glucose is dehydrated to hydroxmethyl furfural). These compounds react with anthrone to obtain a green coloured product with absorption maximum at 630 nm. The concentration of carbohydrate present in the biomass sample was found to be 0.50% w/w as obtained against the standard calibration graph constructed by measuring the absorbance of D-glucose standards at 630 nm (Figure 1).

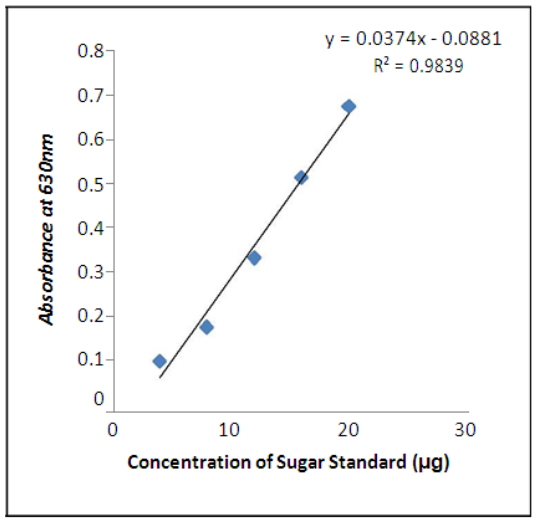


Figure 1: Calibration curve of standard D- glucose.

## Quantification of Steroid Content of Biomass Extract

The steroid content in biomass was determined by Zak's method. Here steroids, in the presence of concentrated sulphuric acid react with ferric chloride to give a pink colour read at 540 nm on a UV-visible spectrophotometer. The intensity of the color developed is directly proportional to the amount of steroids present in sample. The concentration of steroid in biomass sample was obtained from the calibration curve of standard cholesterol as 6.973µg/ml (0.60% w/w, Figure 2)

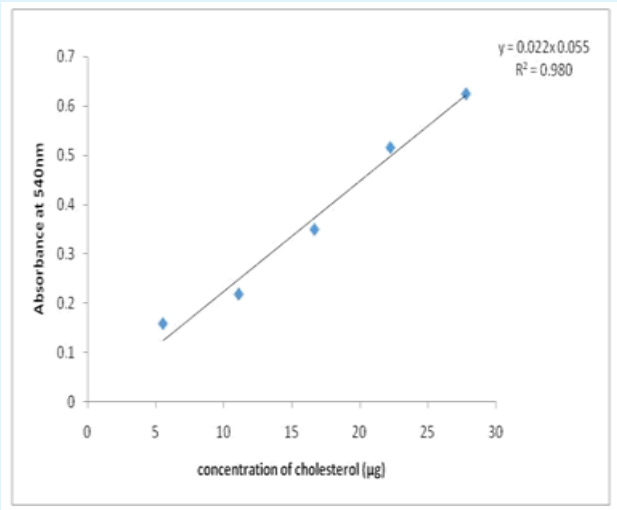


Figure 2: Calibration curve of standard cholesterol.

## LC and LC-MS Analysis of Methanolic Biomass Extract

The RP-HPLC separation on C-18 column employing acetonitrile (pH adjusted to 6.0 with triethylamine) and methanol (50:50 v/v) with spectral scanning at 295 nm showed partial separation and presence of two chromatographic peaks at retention time of 2.30 min and 3.24 minutes respectively (Figure 3a). The LC-MS recording was performed under similar chromatographic conditions using positive ESI mode.

The mass spectral recording of compound 1 showed a peak at m/z 209.10amu, which might be the molecular ion peak indicating molecular mass of 209.22 (Figure 3b). The spectral matching with library of compounds available in National Institute of Standards and Technology (NIST) database indicated that it may be S-(3-acetamidophenyl) ethanethioate which is widely used as an intermediate to synthesize various classes of compounds [23]. The mass spectra of compound 2 (Figure 3c), showed a peak at m/z 329.25, which on spectral matching with NIST database suggest the presence of pregna-5,16-diene-20-one oxime, 3beta-hydroxy). This is supported by the quantitative testing that suggests presence of 0.6% w/w of steroid in the biomass. In our previous study, the LC-MS analysis of the ACN extract also showed presence of a steroidal glycoside at m/z 701.49. It is postulated that the glycoside linked to the steroidal moiety through the glycosidic bond may have undergone hydrolysis in the alcoholic medium to yield the steroidal structure (Figure 4).

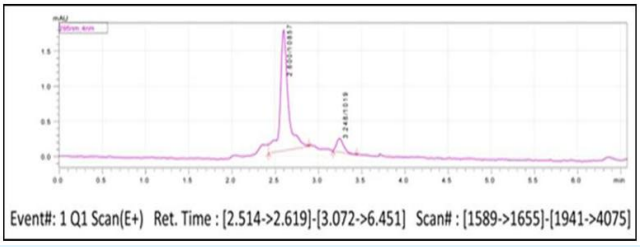


Figure 3a: Mass spectra peak eluting at 2.303 min.

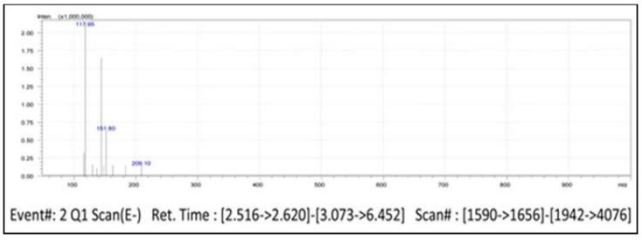


Figure 3b: Mass spectra of compound 1 showing peak at m/e 209.10.

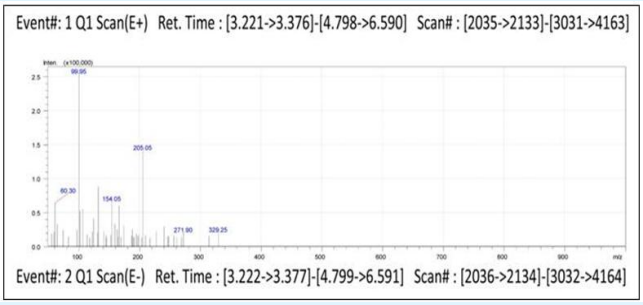


Figure 3c: Mass spectra of compound 2 showing peak at m/e 329.25.

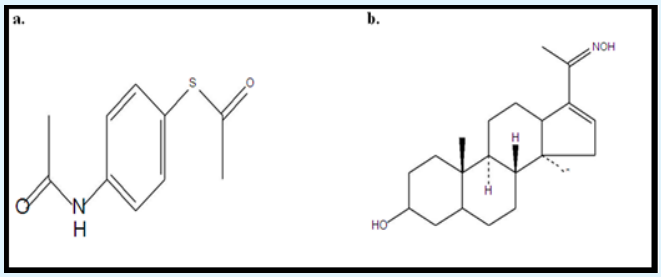


Figure 4: a) S-(3-acetamidophenyl) ethanethioate: Mol. Wt. 209.10; b) Pregna-5, 16-diene-20-one oxime, 3beta-hydroxy): Mol. wt. 329.25

## Acute Oral Toxicity Study

The toxic effect of methanolic extract of *biomass* on appearance and general behavioural pattern of mice were recorded. No toxic clinical symptoms or mortality were observed in any of the test animals, up to 14 days after the administration of methanolic extract at single dose level of 2000 mg/kg, body weight po. The behavioural pattern of animals was observed upto 14 h after administration of biomass. The animals did not display any significant changes in behavior, skin effects, breathing, impairment in food intake and water consumption, postural abnormalities and hair loss, lacrimation, salivation, skin colour, or muscle spasm. The animals did not exhibit any significant changes in body weight post treatment with biomass. At the end of the experiment period, histopathology analysis of isolated organs revealed no apparent changes in the liver, kidney and heart in both control and treated mice. The microscopic structures of the organs (Figure 5) showed no observable differences between the control and test group. No major alteration in cell structure or any unfavorable effects were observed when compared with the control organs.

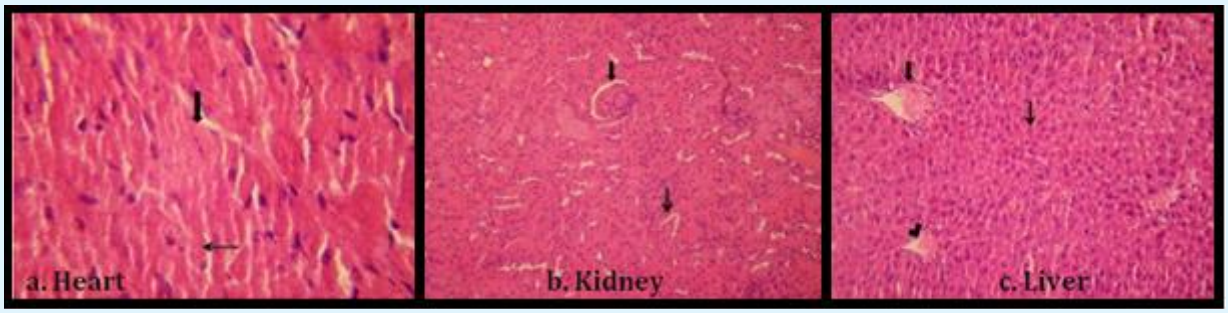


Figure 5: a. Liver: Showing normal histology, Portal triad (large arrow), hepatocyte (small arrow), central vein (arrow head)
b. Kidney: Showing normal histology, Glomerulus (large arrow), tubule (small arrow)
c. Heart: Showing normal histology, myofibre (small arrow), muscle bundle (large arrow).

## High Fat Diet Induced Hyperlipidemia in Rats

The rats maintained on HFD showed significant increase in body weight as compared to the normal group fed with standard diet (Table 2). Hyperlipidemic rats treated with biomass and standard simvastatin showed significant lowering of body weight. At the end of the 45 days treatment period, the plasma total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels of standard simvastatin and test biomass treated groups of animals is as shown in (figure 6a). The results confirmed that high fat diet successfully induced hyperlipidemia among experimental rats. The lipid profile of hyperlipidemic control rats was significantly higher (**$p<0.01$) than corresponding normal control rats. The HFD rats receiving biomass 100, 200 and 400 mg/kg/day respectively for 15 days, showed highly significant reduction in TC, TG, LDL-TC, VLDL-TC, artherogenic index with increased plasma HDL-TC levels (**$p<0.01$) (Figures 6a-6c).

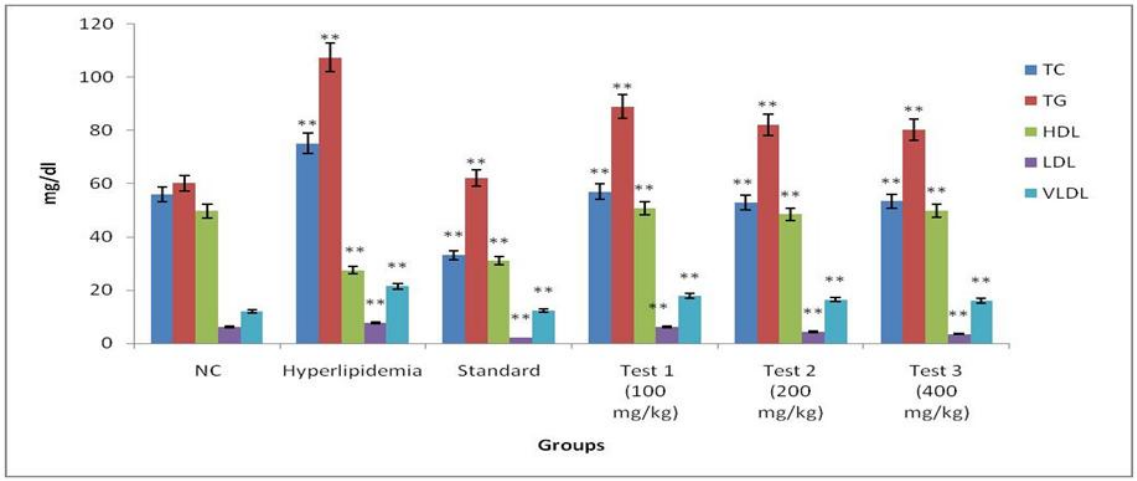


Figure 6a: Effect of mycoprotein on lipid levels

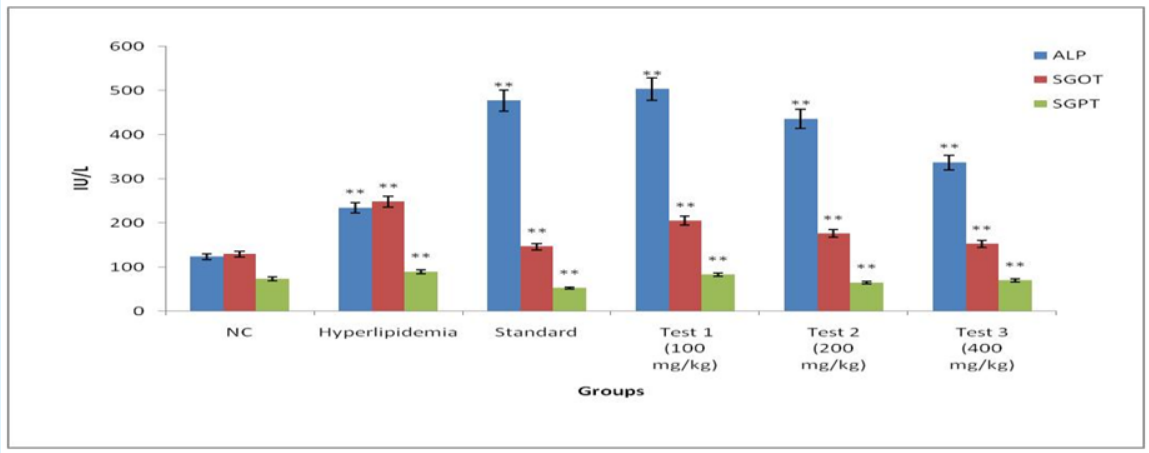


Figure 6b: Effect of mycoprotein on SGPT, SGOT and ALP

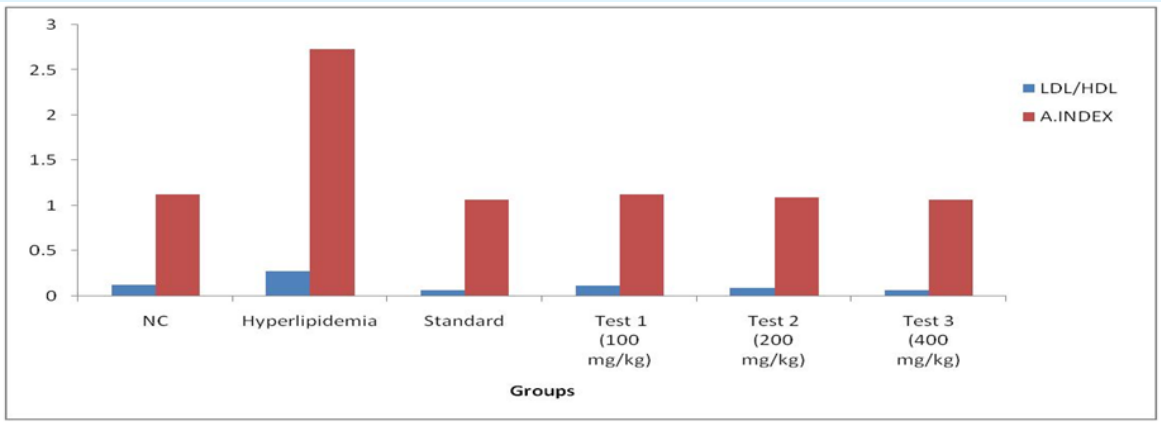


Figure 6c: Effect of mycoprotein on LDL/HDL and atherogenic index

| S. No. | Treatment & Dose (mg/kg po) | Body weight (g) | | | |
|---|---|---|---|---|---|
| | | Day 0 | Day 15th | Day 30th | Day 45th |
| 1 | Normal Control | 210 ± 7.3598 | 217 ± 4.4907 | 230 ± 5.9581 | 248 ± 3.8987 |
| 2 | HFD | 200 ± 3.0110** | 230 ± 5.9581** | 235 ± 5.6361** | 256 ± 5.2025** |
| 3 | HFD + statin | 225 ± 6.2822** | 237 ± 5.8906** | 246 ± 5.8878** | 224 ± 5.4680** |
| 4 | HFD + MP 100 mg/kg | 227 ± 4.9261** | 239 ± 4.2739** | 248 ± 5.6450** | 249 ± 7.3393** |
| 5 | HFD + MP 200 mg/kg | 220 ± 3.7237** | 241 ± 6.7131** | 254 ± 4.9564** | 252 ± 4.0702** |
| 6 | HFD + MP 400 mg/kg | 224 ± 5.8309** | 238 ± 15.4229** | 247 ± 5.8906** | 245 ± 5.0497** |

Table 2: Effect of mycoprotein on body weight in high fat diet induced hyperlipidemic rats. [Values are mean ± SEM of 6 animals.
*P* Values: @ <0.001 compared to NC group.* <0.05, ** <0.01 compared to HFD control group.

### Effect of Mycoprotein on AST and ALP

As shown in (Figure 6b) the levels of ALP, SGOT and SGPT in high fat diet treated group were significantly higher ($p<0.01$) when compared to normal and test treated groups. Under normal conditions, the enzymes aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) are predominantly contained within liver cells. However, if the liver is injured or damaged, the liver cells spill these enzymes into the blood. This elevates the enzyme blood levels, signaling liver disease. It was observed that the mycoprotein-treated groups (100, 200, 400 mg/kg) and standard simvastatin (10 mg/kg) significantly decreased the levels of ALP, SGOT and SGPT.

### Effect of Mycoprotein on LDL/HDL Ratio and Atherogenic Index

It was found that the high fat diet (HFD) treated group elevated the level of LDL/HDL ratio and atherogenic index compared to normal and mycoprotein-treated groups (Figure 6c). However, the standard and biomass treated groups demonstrated significant decrease in level of LDL/HDL ratio and atherogenic index. The results indicate that the biomass treatment may help to decrease the cardiac risk indicator Atherogenic Index due to decrease in cholesterol level with increase in HDL level.

### Histopathological Studies

In HFD induced hyperlipidemic model, a significant fatty infiltration, granular degeneration and increased mononuclear cell infiltration within lobules was observed in the liver sections of HFD control rats as compared to normal rats.

Microscopic examination of liver of normal control group did not reveal any lesion of pathological significance. Microscopically, liver of treated rats revealed various lesion of pathological significance. Multifocal mild per vascular lymphocytic infiltration, multifocal moderate fatty change of hepatocytes in liver of Group 2 animals fed with HFD. Animals of Group 4 (Test 1, receiving biomass 100 mg/kg, po) and Group 5 (Test 2, receiving biomass 200 mg/kg, po) respectively showed focal moderate and mild cytoplasmic vacuolation of hepatocytes and hepatocellular swelling in the liver. In comparison, no abnormality of pathological significance was detected in Group 3 (Test 3, receiving biomass 400 mg/kg, po) and Group 6 (Standard treated) respectively.

Patho-morphological observation in present report suggests that treatment of High fat diet produce inflammatory and degenerative lesions in liver. Treatment of mycoprotein at 400 mg/kg and standard simvastatin reverse the effect of HFD. Whereas, partial mitigation is noted in rats treated with mycoprotein at 100 and 200 mg/kg body weight (Figure 7).

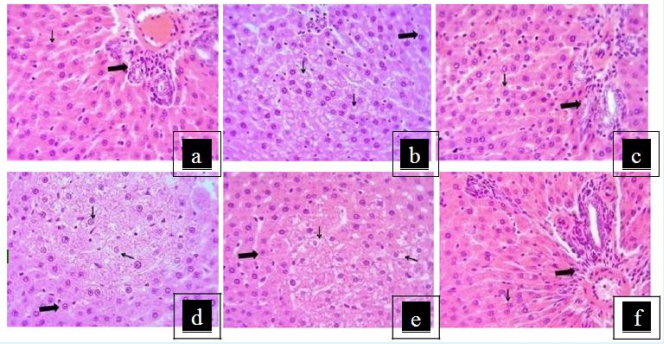


Figure 7: Transverse section of hepatic tissue of rats (a) Normal control group showing normal architecture; (b) high fat diet (HFD) group; (c) HFD + Simvastatin (10 mg/kg) group;(d) HFD + mycoprotein (100 mg/kg) group; (e) HFD+ mycoprotein (200 mg/kg) group; (f) HFD+ mycoprotein (400 mg/kg) group.

## Discussion

India is one of the developing countries in the world which might face a major problem of malnutrition due to protein deficiency. This can lead to several health problems ranging from indigestion, fatigue to physiological changes in skin, hair texture and decline in immunity. As all hormones and enzymes are protein-based, the implications of this deficiency can be serious and life threatening. Absolute deficiency of protein may lead to an excessive intake of carbohydrates, thereby leading to degeneration accompanied with obesity, heart illnesses and diabetes. These finding make it necessary to investigate suitable alternative protein sources. Proteins extracted from yeast, fungi and bacteria are currently available in market as nutritional supplements. During the 1960s and 1970s, rapid developments in production and applications of microbial protein occurred. Extensive research was conducted on a wide range of microorganisms as possible alternate protein sources, motivated by large increase in the price of conventional animal feed [24]. Mycoprotein derived from *Fusarium venenatum* and marketed under the trade name Quorn is a widely used protein source. This protein product, designated as a source of first class protein has high fibre content, contains all nine essential amino acids, with zero cholesterol and is an excellent meat alternative [25,26]. This protein derived from fungi has been reported to possess satiety and satiation properties and exhibit blood lipid lowering effect. Turnbull WH, et al. in a study conducted in healthy individuals, has reported that the mycoprotein exerts beneficial effects on blood lipids [9]. The studies also hypothese that the mycopropein may contain bioactive that may decrease cholesterol synthesis via inhibition of the HMG Co-A reductase enzyme which catalyzes the rate limiting step in cholesterol synthesis [24,3]. However the literature reports did not contain any research being conducted to study its potential for therapeutic application in treatment of hyperlipidemic. Based on these findings and in continuation to our previous studies that suggest that the mycoprotein can reduce elevated blood lipid levels in the acute Triton 100x induced hyperlipidemic model in rats, it was decided to study the effect of the biomass using the chronic high fat diet induced hyperlipidemic model in rats.

Chronic hyperlipidemia leads to atherosclerosis which is implicated in the pathophysiology of coronary artery disease and myocardial ischemia. High fat diet induced hyperlipidemia in rats is an established and documented animal model to evaluate effects of anti-hyperlipidemic drugs. Chronic hyperlipidemia finally alters morphological and hemodynamic functions of vital organs including the heart and liver [27]. The liver plays a major role in regulating the cholesterol and plasma LDL metabolism through the secretion of cholesterol and bile acids. Consumption of high fat diet increases cholesterol input in liver and hepatic synthesis of VLDL and LDL-cholesterol, resulting in increased accumulation of cholesterol in liver [28,29].

In the present study, treatment with mycoprotein and standard simvastatin reduced increased body weight of hyperlipidemic rats. HFD caused a significant rise of serum TC, TG, LDL-TC, VLDL-TC and concomitant decrease in serum HDL-TC. Treatment of HFD fed rats with mycoprotein (100, 200 and400 mg/kg) and Simvastatin (10 mg/kg), significantly decreased the elevated TC, TG, LDL-TC, VLDL-TC levels and restored the decreased HDL-TC in serum. The treatment with mycoprotein at 400 mg/kg dose was most effective and brought the elevated lipid level back to normal values. The atherogenic index is an important and sensitive indicator to determine the cardiac risk as stated in the NCEP guidelines [30]. Treatment with mycoprotein significantly reduced the artherogenic index, thereby demonstrating its promising anti-hyperlipidemic activity.

Jeon, et al. [31] has reported that the intake of high fat diet leads to abnormal increase in lipid and lipoprotein levels. This may promote increased lipid peroxidation leading to oxidative damage due to the formation or generation of excessive free radicals in the liver. The formation of these highly reactive free radicals may cause oxidative stress there by accelerating the progress of atherogenesis. In the present study, LC-MS separation of the methanolic extract of biomass led to the preliminary identification of two compounds, S-(3-acetamidophenyl) ethanethioate and pregna-5,16-diene-20-one oxime, 3beta-hydroxy) [31].

A study of the literature also indicates that pregnenolone is a naturally occurring steroid, and is a precursor to other hormones, including cortisone, estrogen, testosterone, and progesterone [32,33]. These pregnenolone derivatives have been synthesized, and evaluated for diverse biological activities, such as anti-inflammatory, hypotensive, hypocholesterolemic, and diuretic activities [34,35]. S-(3-acetamidophenyl) ethanethioate identified by spectral matching using NIST database contains two fragments, the acetamidophenyl and ethanethioate moieties that show promising biological activities. A study carried out by Desai, et al. [36] indicates the efficacy of (E)-S-1Hbenzo[d]imidazol-2-yl 2-benzylidene) hydrazinyl) ethanethioate derivatives against different strains of bacteria, fungi and

mycobacteria [36]. A newer series of chalcone derivatives S-4-(isonicotinamido)-5-(phenoxymethyl) - 4H-1,2,4 triazole-3-yl-3(-4-chlorophenyl) propanethioate were found to be promising antitubercular [37]. S-hexyl (heptyl) ethanethioate derivatives were designed and synthesized as anti tumor agents against MCF-7 cell lines [38]. These studies substantiate the potential of the ethanethioate moiety. Meng-yuan Li, et al. in a recent study has demonstrated the efficacy of 6-((2-((3-acetamidophenyl) amino) pyrimidin-4-yl) oxy)-N-phenyl-1-naphthamide, targeting the VEGF/VEGF receptor (VEGFR) pathway for cancer treatment. Molecules containing the 3-acetamidophenyl fragment are also being investigated for the treatment of inflammation, diabetes and related disorders [39,40].

The decreased lipid peroxidation may be one such avenue that can reduce the chances of atherosclerosis development due to hyperlipidemia. The bioactives (s) present in mycoprotein, may have resulted in hypolipidemic effect. However, the other probable mechanism (s) such as effect on cholesterol synthesis, metabolism and its excretion, by the phytochemical (s), especially sterols need to be investigated [41-44].

In conclusion, the present study demonstrate that the administration of mycoprotein elicited promising anti-hyperlipidemic activity, that may be attributed to the bioactive (s) present, which has reversed the genesis of hyperlipidemia, manifested due to oxidative stress on cellular system. This needs further isolation of the bioactives and further investigation of their anti-hyperlipidemic activity in order to confirm the precise role of biomass in triggering hypolipidemic activity.

## Acknowledgements

The authors would like to thank Principal, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, India, for providing the necessary infrastructural facilities to carry out the work.

**Compliance with ethical standards**

**Competing Interests** The authors declare that there are no competing interests.

## References

1. Olaf M, Michael K (2005) Malnutrition and health in developing countries, Review Synthese, CMAJ 173(3): 279-286.
2. Swaminathan S, Vaz M, Kurpad AV (2012) Protein intakes in India. Br J Nutr 108(2): 50-58.
3. Gour S, Nupur M, Singh A, Bhatnagar P (2015) Single cell protein production: a review. Intl J Curr Microbiol App Sci 4(9): 251-262.
4. Trinci APJ (1990) Mycoprotein: a 20 year overnight success story. Mycological Research 9: 1-13.
5. Trinci APJ (1994a) Evolution of the Quorn myco-protein fungus, Fusarium graminearum A3/5. Microbiology 140: 2181-2188.
6. Edelman J, Fewell A, Solomons G (1983) Myco-protein - a new food. Nutr Abstr Rev Clin Nutr 53: 471-480.
7. Miller SA, Dwyer JT (2001) Evaluating the safety and nutritional value of mycoprotein. Food Technology Chicago 55(7): 42-47.
8. Turnbull WH, Leeds AR, Edwards DG (1990) Effect of mycoprotein on blood lipids. Am J Clin Nutr 52(4): 646-650.
9. Turnbull WH, Leeds AR, Edwards DG (1992) Mycoprotein reduces blood lipids in free- living subjects. Am J Clin Nutr 55(2): 415-419.
10. Burley VJ, Paul AW, Blundell JE (1993) Influence of a high-fibre food (myco-protein) on appetite:effects on satiation(within meals) and satiety (following meals). Eur J Clin Nutr 47(6): 409-418.
11. Turnbull WH, Bessey D, Walton J, Leeds AR (1991) The effect of myco-protein on hunger satiety and subsequent food consumption. Obesity in Europe 91 London: Libbey 67-70.
12. Thomas AB, Shetane TD, Singha RG, Nanda RK, Poddar SS, et al. (2017) Employing Central Composite Design for Evaluation of Biomass Product-ion by Fusarium venenatum: In Vivo Antioxidant and Antihyperlipidemic Properties, Appl Biochem Biotechnol 183: 91-109.
13. Hosseini SM, Khosravi-Darani K, Mohammadifar MA, Nikoopour H (2009) Production of mycoprotein by *Fusarium venenatum* growth on modified vogel medium. Asian J Chem 21(5): 4017-4022.
14. Khandelwal KR (2009) Practical Pharmacognosy Techniques and Experiments. Nirali publication 149-156.

15. Suriyavathana M, Pakutharivu T (2011) Phytochemical Analysis and Antioxidant profile of Hippo-08 an Oral Ayurvedic Formulation. International Symposium on Functional Foods and Health, Periyar University. Salem-11, appendix-1 and appendix-4.

16. Elleuch L, Shaaban M, Smaoui S, Mellouli L, Karray-Rebai I, et al. (2014) Bioactive secondary metabolites from a new terrestrial Streptomyces sp. TN262. Applied Biochemistry and Biotechnology 174: 1535-1547.

17. (2011) Indian National Science Academy New Delhi-110002, Man, Animal and Science September 15.

18. (2001) GRAS notification for mycoprotein.

19. (2001) OECD guidelines for testing of chemicals, 423 Adopted: 17th December.

20. Lee JS, Lee MK, Ha TY, Bok SH, Park HM, et al. (2006) Supplementation of whole persimmon leaf improves lipid profiles and suppresses body weight gain in rats fed high-fat diet, Food Chem Toxicol 44(11): 1875-1883.

21. Yugarani T, Tab B, Teh M, Das NP (1992) Effects of polyphenolic natural products on the lipid profiles of rat fed high fat diet. Lipids 27(3): 181-186.

22. Durekar AM, Patil RR, Naik SR (2014) Hypolipidemic and antioxidant activity of ethanolic extract of Symplocos racemosa Roxb. in hyperlipidemic rats: an evidence of participation of oxidative stress in hyperlipidemia. Indian J Exp Biol 52(1): 36-45.

23. http://webbook.nist.gov/chemistry/mw-ser.html

24. Adedayo MR, Ajiboye EA, Akintunde JK, Odaibo A (2011) Single cell proteins: as nutritional enhancer. Adv Appl Sci Res 2(5): 396-409.

25. Bhalla TC, Sharma NN, Sharma M (2007) Production of metabolites, industrial enzymes, amino acids, organic acids, antibiotics, vitamins and single cell proteins. India: National Science Digital Library.

26. Nasseri AT, Rasoul- Amini S, Morowat MH, Ghasemi Y (2011) Single cell protein: production and process. Am J Food Technol 6(2): 103-116.

27. Nachtigal P, Pospisilova N, Jamborova G, Pospechova K, Solichova D, et al. (2008) Atorvastatin has hypolipidemic and anti-inflammatory effects in apoE/LDL receptor double knockout mice, Life Sci, 82(13-14): 708-717.

28. Golan DE, Tashjian AH, Armstrong EJ, Galanter JM, Armstrong AW, et al. (2005) Principles of pharmacology, in the Pathophysiological basis of drug therapy, (Lippincott Williams and Wilkins, Philadelphia, PA) 357.

29. Brown MS, Kovanen PT, Goldstein JL (1981) Regulation of plasma cholesterol by lipoprotein receptors. Science 212(4495): 628-635.

30. (2002) NCEP guidelines: The third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final report. Circulation 106: 3143.

31. Jeon SM, Park YB, Choi MS, (2004) Anti-hypercholesterolemic property of naringin alters plasma and tissue lipids, cholesterol regulating enzymes, fecal sterol and tissue morphology in rabbits. Clin Nut 23(5): 1025-1034.

32. Maurice T, Urani A, Phan VL, Romieu P (2001) The interaction between neuroactive steroids and the sigma1 receptor function: behavioural consequences and therapeutic opportunities: Brain Res Rev 37(1-3): 116-32.

33. Vajda FJ (2002) Neuroprotection and neuro-degenerative disease. J Clin Neurosci 9(1): 4-8.

34. Edmondson RJ, Monaghan JM (2001) The epidemiology of ovarian cancer. Int J Gynecol Cancer 11(6): 423-429.

35. Tian Dong L, Jing Xia Z, Jun X, ShuJia Z, Yi Jun H, et al. (2010) Synthesis and anti-glioma activity of 25(R)-spirostan-3b,5a,6b,19-tetrol. Steroids 75(3): 224-229.

36. Desai VA, Patel DB, Patel JB (2016) Synthesis and biological evaluation of (E)-S-1Hbenzo[d]imidazol-2-yl 2-benzyli- dene) hydrazinyl)ethanethioate derivatives. IJCS 4(1): 54-60.

37. Rolf WH, Markus H, Samer H, Peter BE, Reichert W, et al. (2000) Synthesis and Evaluation of Novel Steroidal Oxime Inhibitors of P450 17 (17α-Hydroxylase/C17–20-Lyase) and 5α-Reductase Types 1 and 2. J Med Chem 43 (22): 4266-4277.

38. Wen JC, Jiang T, Bao Y, Lin XJ, Wang WQ, et al. (2014) [Synthesis and antitumor activity of S-hexyl(heptyl)

substituted ethanethioate derivatives]. Yao Xue Xue Bao 49(3): 352-358.

39. Partha Neogi, Debedranath Dey, Joseph C Fuller, Liang Chen, Ta-Kai Li (2016) Compounds for treatment of inflammation, diabetes and related disorders. WO 2004101752 A2.

40. Scharpe S, Augustyns K, Haemers A, Lambeir AM, Ingrid De M, et al. (2004) Dipeptidyl peptidase inhibitors WO2004076433 A1.

41. Mateos A, Lecumberri E, Ramos S, Goya L, Bravo L (2005) Determination of malondialdehyde (MDA) by high performance liquid chromatography in serum and liver as a biomarker for oxidative stress: Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruit. Journal of Chromatography 827: 76-82.

42. Folch J, Lees M, Sloane GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497-509.

43. Rao AV, Ramakrishnan S (1975) Indirect assessment of hydroxymethylglutaryl-CoA reductase activity in liver tissue, Clin Chem, 21(10): 1523-1525.

44. Wang X, Greilberger J, Ledinski G, Kager G, Paigen B, et al. (2004) The hypolipidemic natural product Commiphoramukul and its component guggulsterone inhibit oxidative modification of LDL, Atherosclerosis 172(2): 239-246.