



# Bioactive Pigments of *Monascus purpureus* Attributed to Antioxidant, HMG-CoA Reductase Inhibition and Anti-atherogenic Functions

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### Specialty section:

This article was submitted to  
Sustainable Food Processing,  
a section of the journal  
Frontiers in Sustainable Food Systems

**Received:** 01 August 2020

**Accepted:** 13 January 2021

**Published:** 15 February 2021

### Citation:

Mohankumari HP, Naidu KA,  
Narasimhamurthy K and  
Vijayalakshmi G (2021) Bioactive  
Pigments of *Monascus purpureus*  
Attributed to Antioxidant, HMG-CoA  
Reductase Inhibition and  
Anti-atherogenic Functions.  
*Front. Sustain. Food Syst.* 5:590427.  
doi: 10.3389/fsufs.2021.590427

*Monascus purpureus* is known to produce pigment molecules. The pigments were extracted from *M. purpureus* fermented rice. *In-vitro* antioxidant effects of pigments were observed and presumed to alleviate oxidative stress related atherosclerosis effect in rats fed with high fat diet (HFD) for 14 weeks. The formation of lipid peroxide due to the oxidation of serum lipid was higher in rats fed with HFD. While, the feeding of fermented rice (groups III-V) significantly lowered the formation of lipid peroxide (27.1–51.7%) in serum of rats, indicated antioxidative effect of pigments. In addition, feeding of fermented rice lowered serum cholesterol and triacylglycerol by 44.82 and 45.30%, respectively. Whereas, LDL-cholesterol levels were decreased by 70.12% and HDL-cholesterol increased by 34.58%. The atherogenic indices (LDL/HDL and TC/HDL) were reduced by 77.80 and 61.05%, respectively, in rats fed with fermented rice. These data confirmed the anti-atherosclerotic effect of pigments. Further liver enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was significantly inhibited up to 54%. The identification of statins, sterols and fatty acids in fermented rice revealed the HMG-CoA reductase inhibitory activity. This was confirmed by synthesis of lower levels of cholesterol and triacylglycerol in liver of rats fed with fermented rice. Accordingly antioxidant, inhibition of HMG-CoA reductase, anti-atherogenic functions of *M. purpureus* fermented rice is attributed to the collective effect of bioactive metabolites.

**Keywords:** antioxidant, HMG-CoA reductase, *Monascus*, pigment, anti-atherogenic

## INTRODUCTION

Red mold rice (RMR) is a traditional food additive of the Chinese for thousands of years. In many Asian countries, *Monascus* fermented rice has been consumed as food and folk medicine. *Monascus* spp. are well-known for various bioactive metabolites, viz., pigments (red pigments: monascorubramine and rubropunctamine, orange pigments: monascorubrin and rubropunctatin, yellow pigments: ankaflavin and monascin), isoflavones, polyketides, lipids, dimeric acid an antioxidant (Aniya et al., 2000),  $\gamma$ -Aminobutyric Acid (GABA), an antihypertensive metabolite (Feng et al., 2012), metabolites with antibacterial, antitumor, and immunosuppressive properties (Martinkova et al., 1999) and, structural analogs of lovastatin (pravastatin, compactin, monacolin J). Monacolins, in particular monacolin K (MK), inhibitor of 3-hydroxy-3-methylglutaryl coenzyme

A (HMG-CoA) reductase is marketed as lovastatin drug (Hachem et al., 2020). Apart from food color, *Monascus* pigments extended its use in biological activities, like anti-inflammatory, anticancer and antihyperlipidemic activities (Lin et al., 2011).

Monacolin K, a new hypocholesterolemic agent was first isolated from *M. ruber* by Endo (1979a), that competes with HMG-CoA reductase due to its structure similarity with HMG-CoA (3-Hydroxy 3-Methylglutaryl Coenzyme A). Thus, inhibits the cholesterol synthesis by prohibiting the formation of mevalonic acid an essential intermediate product for the cholesterol biosynthesis.

Dihydromonacolin-MV and dehydromonacolin MV2 isolated from *M. purpureus* and its hyper pigment mutant have shown biological activities (Dhale et al., 2007a,b). Dihydromonacolin-L, a potent inhibitor of cholesterol biosynthesis was isolated from the *M. ruber* (Endo et al., 1985). Our earlier toxicological studies of RMR did not show any adverse effect in rats (Mohan-Kumari et al., 2009).

In recent years, a high fat and cholesterol diet has been implicated as one of the risk factors for cardiovascular diseases (CVD) related problems in developed and developing countries. Atherosclerosis is the accumulation of cholesterol-rich plaques in arteries, a key contributor to CVD, prevents sufficient blood flow to the heart. Atherosclerosis greatly progress due to the elevation of blood lipids from circulating lipoproteins and inflammation (Hansson, 2005). The atherogenic index (AI) is expressed for cholesterol level in the blood can be a risk factor for heart disease and atherosclerosis. Atherogenic risks also include oxidative stress, inflammation, obesity, diabetes, endothelial dysfunction, shear stress, homocysteine, infection, and genetic factors. Therefore, lowering the cholesterol levels in patients with cardiovascular diseases has become an important concern. Even though the diet and exercise is successful in certain patients for the management of hyperlipidemia, pharmacological intervention is required to effectively lower cholesterol. Cholesterol lowering agents that inhibit HMG-CoA reductase (EC 1.1.1.34) are prominent among the drugs of choice for treating hyperlipidemia. The available hypolipidemic drugs lowers circulating cholesterol levels either by prevention of cholesterol absorption, or increasing hepatic uptake of low density lipoproteins (LDL) or inhibit cholesterol synthesis. The HMG-CoA reductase inhibitors significantly reduce cholesterol biosynthesis in animal and human subjects owing to the structural similarity of HMG-CoA (substrate of the enzyme) and statins. Lovastatin produced by *Aspergillus terreus*, *M. purpureus*, and other fungi (Endo, 1979b) is a well-known inhibitor of HMG-CoA reductase and widely used as a hypocholesterolemic drug in humans (Alberts et al., 1980).

During the *M. purpureus* fermentation process multitude of secondary metabolites, pigments, monacolins, phytosterols, isoflavonoids, fatty acids, and others are produced. This study is aimed to investigate these metabolites effect which attributes antioxidant effect of RMR on total cholesterol (TC), triacylglycerol (TG), lipoprotein cholesterol (low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), serum lipid, hepatic lipid, and HMG-CoA reductase enzyme in hypercholesterolemic albino rats.

## MATERIALS AND METHODS

### Chemicals

Cholesterol and bile acid mixtures were purchased from Sigma chemical Co. (St. Louis, MO, USA). Mineral mix, vitamin mix and cellulose were obtained from Sisco Research Laboratories, Mumbai, India. Casein was purchased from Nimesh Corporation, Mumbai. The rice used for cultivating *M. purpureus* was purchased locally. Kits used were as follows: Total cholesterol (TC) assay kit (Product No. 11403002, Kerala, India), triacylglycerol (TG) assay kit (Product No. 11410102, Kerala, India), were purchased from Agappe Diagnostics Ltd. Kerala, India. All the solvents used were of analytical grade.

### Microorganism and Red Mold Rice Preparation

*M. purpureus* MTCC 410 obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India was maintained on potato dextrose agar (PDA, Hi Media, India) slants at 4–8°C. It was sub cultured every 30 days.

Seed culture was prepared by inoculating a loopful of *M. purpureus* MTCC 410 to 100 ml basal medium (pH 6.0) containing (dextrose (100 g), peptone (10 g), KNO<sub>3</sub>(2 g), NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (2 g), MgSO<sub>4</sub>·7 H<sub>2</sub>O (0.5 g), CaCl<sub>2</sub> (0.1 g) in 1L distilled water (Su et al., 2003). After incubation at 30°C for 48 h at 110 rpm, 5% seed medium (volume/weight) was used for solid state fermentation.

The traditional RMR was prepared by inoculating 500 g of rice (sterilized/cooked) with 5% (v/w) seed culture of *M. purpureus* (Su et al., 2003) under solid state fermentation and inoculated rice was incubated at 30°C [Adolf Khuner Therm-Lab, Birmeldern (Basel), Switzerland] for 14 days in a slanting position with intermittent shaking. After fermentation, RMR was dried at 45–50°C for 24 h and then powdered. The powdered RMR was used for the experiments.

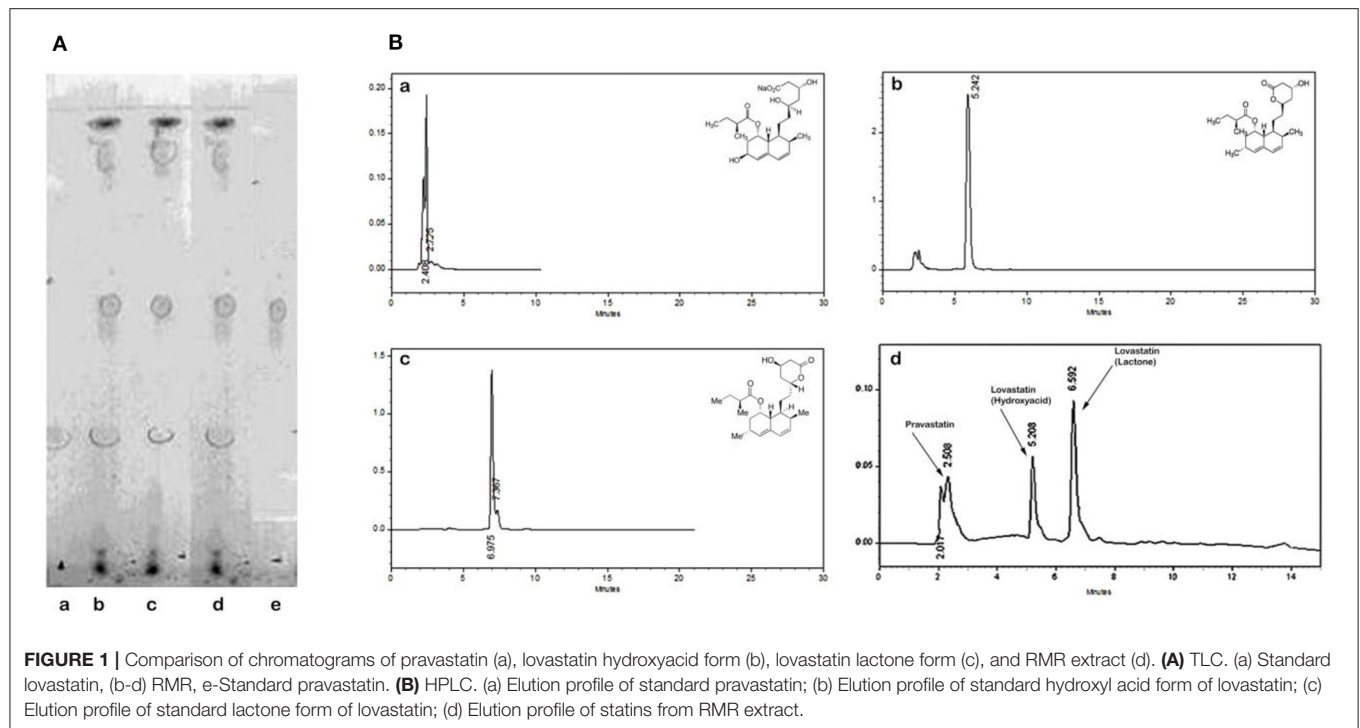
### *M. purpureus* Bioactive Molecules Pigments

The pigments were extracted with 95% ethanol at 30°C for 60 min on rotary shaker (150 rpm). After filtration, the pigments were quantified by measuring the optical density (OD) with appropriate dilution. The OD was measured at 375, 475, and 500 nm for yellow, orange, and red pigments, respectively. Pigment yield was calculated as OD Units using

**TABLE 1** | Polyketide pigment of RMR methanol extract.

Strain	OD Units/g dry cell mass			
	Red (500 nm)	Orange (475 nm)	Yellow (375 nm)	Total pigments
MTCC 410	202.8 ± 12.56	183.4 ± 9.86	120.3 ± 2.23	506.5

Values are mean ± standard deviation (SD, n = 3).



following formula.

$$\text{OD Units} = \frac{\text{OD} \times \text{Total vol of extraction} \times \text{Dilution}}{\text{Red mould rice (g)}}$$

### Statins

RMR powder added to 75% ethanol (1:10, w/v) was kept at 30°C for 1 h on rotary shaker [Adolf KhunerTherm-Lab, Birmeldern (Basel), Switzerland] at 180 rpm. After centrifuging for 10 min at 10,000 rpm, samples were filtered through a 0.45 μm millex-LH (Millipore Corp., Bedford, MA 01730) filter before injection. The identification of statins were carried out by HPLC equipped with linear isocratic system, using Shimadzu Liquid Chromatograph LC-10A (Shimadzu, Japan) fitted with water's ODS (5 μm, 25 cm × 4.6 mm i.d.) column with a UV detector (237 nm) eluted with acetonitrile:water (72:28, v/v) at a elution of 1.0 ml/min maintained column temperature at 30°C with the injection volume of 20 μl. Statins were identified by comparing with the retention time of standards.

### Fatty Acids

Total lipid extraction from dry biomass and analysis of fatty acids was carried out according to the method (Somashsekar et al., 2003). The fatty acids were identified with the retention time of authentic fatty acid standards from Sigma. Fatty acids were identified by converting the fatty acids to fatty acid methyl esters (FAMES) as described earlier (Kate, 1964) with slight modification. FAMES were separated by Shimadzu GC-15A equipped with a DEGS packed column with isothermal temperature with flame ionization detector. The injector temperature was set at 220°C, detector temperature was

maintained at 230°C and column temperature at 180°C. N<sub>2</sub> was used as carrier gas at a flow rate of 40 ml min<sup>-1</sup>.

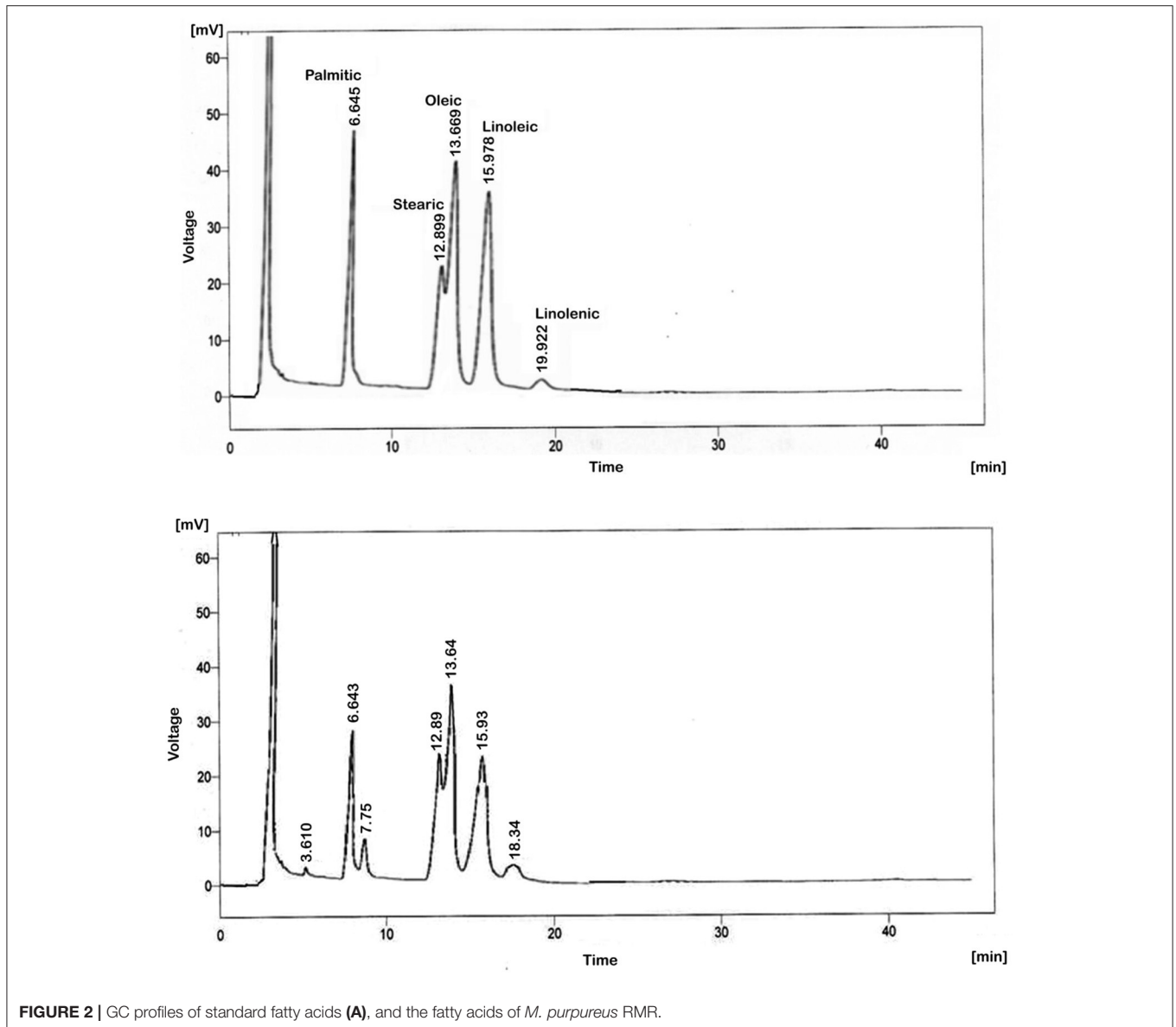
### Phytosterol

About 10–20 mg of extracted lipid was subjected to saponification by methanolic KOH (6%, w/v) and refluxed for 2 h at 80°C in boiling water bath, cooled and diluted with 1 vol. of water. Total sterol was repeatedly extracted 3 times with hexane. The total extract was pooled together and evaporated to dryness. The final residue was dissolved in 1 ml chloroform and estimated by Liberman-Burchard method (Syed Mubbasher et al., 2003). Briefly to 200 μl sample, 800 μl chloroform and 2 ml of the Liberman-Burchard reagent (5 ml of H<sub>2</sub>SO<sub>4</sub> dissolved in 10 ml acetic anhydride) was added in a graduated test tube. The final volume was made up to 7 ml with chloroform and incubated in dark for 15 min. The absorbance of resultant solution was determined spectrophotometrically at 640 nm (Shimadzu 160 UV A). Sitosterol was used as standard for comparison.

### Antioxidant Activity

Antioxidant activity of *M. purpureus* extract was determined by estimating DPPH radical scavenging activity, inhibition of ascorbate autoxidation, lipid peroxidation, and reducing activity. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls. Butylated hydroxyl anisole was used standard as for comparison.

The DPPH scavenging activity (Blois, 1958) of was measured accordingly (Moon and Terao, 1998). The inhibition of ascorbate autoxidation was determined according to Mishra and Kovachich (1984). Lipid peroxidation inhibitory activity was measured according to Kulkarni et al. (2004). The reducing power



was determined according to Oyaizu (1986). Percentage of antioxidant activity was determined using the standard formula.

## IN VIVO HYPOLIPIDEMIC ACTION OF RMR

### Experimental Animals

Sixty four male albino rats (CFT-Wistar strain) weighing 150–180 g were used as experimental animals. The rats were bred in animal house facility at Central Food Technological Research Institute, Mysore, India. The experimental protocol was approved by the Institute animal ethical committee. Animals were grouped by randomized design on a body weight basis and were kept (12 h light/dark cycles, RH 60% and at  $25 \pm 2^\circ\text{C}$ ) in stainless steel cages for 14 week. Four animals in control group were sacrificed immediately to analyze cholesterol and

triacylglycerol levels in serum and liver and established a base line lipid profile data.

### Treatment Groups and Dosages

American Institute of Nutrition (AIN)-76 diet formulation was followed for the preparation of experimental diets with slight modification. Animals were divided into six groups of 10 animals each. The control group rats (I group) were fed a normal diet of AIN-76 formulation. Group II were fed with high fat diet (HFD, cholesterol). Group III to V rats were fed with HFD supplemented with 8, 12, and 16% of RMR powder, while Group VI rats were fed with HFD supplemented with lovastatin (0.08 g/kg). Appropriate proportion of cholesterol, bile salts, RMR and standard lovastatin were supplemented to the control diet at the expense of corn starch. Cholesterol (1.0%) and bile acids (0.15%) were added to the diet to induce hyperlipidemia in rats. Diets

**TABLE 2** | Antioxidant activity of RMR methanol extract.

	Phenolics mg/100 g	DPPH scavenging (IC <sub>50</sub> )	Inhibition of ascorbate autoxidation (%)	Reducing (equivalent cysteine, μM)	Lipid peroxidation (IC <sub>50</sub> )
Methanol extract	141.32 ± 0.01	100.78 ± 2.66	21.10 ± 0.95	1.35 ± 0.05	47.02 ± 1.91
BHA	–	18.96 ± 1.28	9.76 ± 1.45	0.65 ± 0.11	38.02 ± 1.53

Values are mean ± standard deviation (SD, n = 3).

**TABLE 3** | Food intake of male rats fed dietary *M. purpureus* RMR for 14 weeks.

Concentration of RMR mixed with diet (w/w)	Average food Intake (g/rat/day)							
	0th week	2nd week	4th week	6th week	8th week	10th week	12th week	14th week
Control	12.67 ± 1.43	13.50 ± 1.42	14.89 ± 2.20	13.93 ± 1.44	9.62 ± 0.84	14.31 ± 2.35	15.42 ± 0.69	14.37 ± 0.97
HCD	13.20 ± 1.30	13.74 ± 1.37	14.82 ± 1.73	14.41 ± 1.76	8.78 ± 1.22	14.28 ± 0.84	15.79 ± 0.79	13.98 ± 1.06
HCD + 8% RMR	13.11 ± 1.71	13.68 ± 1.63	14.75 ± 1.43	14.08 ± 1.94	9.87 ± 0.70	15.06 ± 0.54	15.86 ± 1.11	13.82 ± 0.33
HCD + 12% RMR	11.58 ± 2.31	13.41 ± 1.46	14.24 ± 1.65	14.24 ± 1.84	9.81 ± 0.30	14.95 ± 2.32	16.13 ± 0.55	14.08 ± 1.41
HCD + 16% RMR	13.36 ± 1.10	13.25 ± 1.62	14.25 ± 1.45	14.36 ± 1.94	9.86 ± 0.61	15.13 ± 0.81	15.90 ± 0.79	14.50 ± 0.42
HCD + 80 mg Lovastatin	12.61 ± 1.28	13.38 ± 1.36	14.70 ± 2.15	14.32 ± 2.07	9.41 ± 0.60	15.19 ± 1.39	16.34 ± 0.71	15.84 ± 0.58

Values are mean ± SEM of six animals. No significant difference between control and *M. purpureus* red mold rice fed groups ( $p < 0.05$ ).

were prepared every week and stored at 4°C. Throughout the study (14 weeks), the animals had free access to food and water *ad libitum*. The animals were observed thoroughly for the onset of any signs of toxicity. Daily food intake and gain in weekly body weight gained was monitored. At the end of the experimental period (7th and 14th week), overnight fasted rats were sacrificed. Blood samples were collected by cardiac puncture and serum separated by centrifugation at 6,000 g. Organs like liver, heart, brain, kidney, adipose, lungs, spleen and testis were excised, blotted, weighed and stored at –20°C till they were processed. A portion of the liver was stored in 10% neutral formalin and processed for histological examination.

## Serum Cholesterol and Triacylglycerol Analysis

Serum cholesterol and triacylglycerol were measured using commercial enzymatic kits, respectively. HDL cholesterol was estimated after precipitation of LDL with heparin-MnCl<sub>2</sub> reagent (Warnick and Albers, 1978).

## Liver Cholesterol and Triacylglycerol Analysis

Triacylglycerol in liver was estimated by the method of Fletcher (1968). Total lipids from liver after extraction was estimated by the method of Folch et al. (1957). Total cholesterol content of liver was estimated by the method of Searcy and Bergquist (1960).

## 3-Hydroxy-3-Methylglutaryl-CoA Reductase Activity

Preparation of microsomes and HMG CoA reductase activity was assayed according to the method of Hulcher and Oleson (1973). The reaction mixture was containing 0.5–1.0 mg of microsomal

protein, 150 nmoles of HMG-CoA, 2 μmoles of NADPH and 0.8 ml of 0.1 M triethanolamine in 0.02 M EDTA buffer (pH 7.4) without dithiothreitol. The final volume was made up to 1 ml and kept for incubation at 37°C for 30 min (Hulcher and Oleson, 1973).

## Statistical Analysis

The statistical analyses of experimental data were subjected to one-way analysis of variance (ANOVA) using Duncan's test. Statistical significance was considered when  $P < 0.05$ . Statistical analysis were performed by software SPSS, release 9.0 (SPSS, Inc.).

## RESULTS

### Identification of Bioactive Molecules

Major bioactive molecules were identified using various techniques like HPLC, GCMS and UV visible spectroscopy.

### Pigments

Production of more red pigment (202.8 OD units) was observed compared to orange (183.4 OD units) and yellow pigments (120.3 OD units). These results indicated the production of three major colored pigment like yellow, orange and red (Table 1).

### Statins

#### Qualitative and Quantitative Analysis by TLC and HPLC

Statins (lovastatin and pravastatin) from *M. purpureus* RMR, extracted with ethanol, were identified after TLC and HPLC analysis. For TLC, pravastatin and lovastatin standards obtained from sigma were used. After performing TLC, R<sub>f</sub> values were determined in relation to the standards. Standards showed an R<sub>f</sub>

**TABLE 4** | Body weight gain of male rats fed with *M. purpureus* RMR for 14 weeks.

Concentration of RMR mixed with diet (w/w)	Average body weight gained (g)							
	0th week	2nd week	4th week	6th week	8th week	10th week	12th week	14th week
Control	177.40 ± 2.12 <sup>f</sup>	219.9 ± 10.05 <sup>c</sup>	267.4 ± 21.56 <sup>c</sup>	297.3 ± 26.70 <sup>c</sup>	315.4 ± 22.7 <sup>a</sup>	318.2 ± 35.38 <sup>a</sup>	332.8 ± 44.05 <sup>a</sup>	339.0 ± 63.79 <sup>a</sup>
HCD	169.00 ± 1.05 <sup>d</sup>	218.8 ± 8.84 <sup>c</sup>	266.2 ± 16.86 <sup>c</sup>	298.1 ± 20.21 <sup>c</sup>	313.8 ± 20.36 <sup>a</sup>	324.8 ± 15.55 <sup>a</sup>	342.6 ± 17.04 <sup>a</sup>	355.6 ± 20.07 <sup>a</sup>
HCD+8% RMR	164.80 ± 1.40 <sup>c</sup>	204.3 ± 15.71 <sup>b</sup>	247.4 ± 18.65 <sup>b</sup>	279.4 ± 19.48 <sup>bc</sup>	316.8 ± 26.45 <sup>a</sup>	326.4 ± 27.35 <sup>a</sup>	343.6 ± 23.68 <sup>a</sup>	349.2 ± 21.92 <sup>a</sup>
HCD+12% RMR	161.00 ± 1.05 <sup>b</sup>	184.7 ± 8.55 <sup>a</sup>	223.6 ± 17.63 <sup>a</sup>	245.8 ± 20.48 <sup>a</sup>	316.8 ± 26.45 <sup>a</sup>	299.8 ± 27.55 <sup>a</sup>	315.4 ± 35.42 <sup>a</sup>	331.8 ± 19.59 <sup>a</sup>
HCD+16% RMR	155.70 ± 1.42 <sup>a</sup>	185.1 ± 10.07 <sup>a</sup>	226.8 ± 14.97 <sup>a</sup>	244.9 ± 14.56 <sup>a</sup>	286.4 ± 12.97 <sup>a</sup>	298.2 ± 11.43 <sup>a</sup>	321.8 ± 12.76 <sup>a</sup>	328.4 ± 11.59 <sup>a</sup>
HCD+80 mg Lovastatin	172.50 ± 0.71 <sup>e</sup>	200.8 ± 7.24 <sup>b</sup>	246.8 ± 13.55 <sup>b</sup>	267.4 ± 21.36 <sup>b</sup>	326.8 ± 40.34 <sup>a</sup>	321.2 ± 17.09 <sup>a</sup>	338.2 ± 17.71 <sup>a</sup>	330.8 ± 8.04 <sup>a</sup>

Values are mean ± SD of six animals. Mean values within each column with different superscripts are significantly different Duncan's test at  $p < 0.05$ .

**TABLE 5** | Effect of feeding *M. purpureus* RMR for 7 weeks on organ weight of male rats.

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100 g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.08 ± 0.16 <sup>a</sup>	0.41 ± 0.10 <sup>ab</sup>	0.73 ± 0.07 <sup>ab</sup>	0.27 ± 0.03 <sup>a</sup>	0.90 ± 0.14 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>	0.62 ± 0.06 <sup>a</sup>	0.26 ± 0.03 <sup>b</sup>
HCD	4.30 ± 0.23 <sup>b</sup>	0.40 ± 0.06 <sup>a</sup>	0.71 ± 0.02 <sup>a</sup>	0.28 ± 0.01 <sup>ab</sup>	0.84 ± 0.04 <sup>a</sup>	0.02 ± 0.01 <sup>a</sup>	0.60 ± 0.15 <sup>b</sup>	0.21 ± 0.04 <sup>a</sup>
HCD + 8% RMR	3.76 ± 0.25 <sup>a</sup>	0.43 ± 0.04 <sup>ab</sup>	0.73 ± 0.05 <sup>bc</sup>	0.31 ± 0.02 <sup>bc</sup>	0.83 ± 0.15 <sup>a</sup>	0.02 ± 0.01 <sup>a</sup>	0.59 ± 0.59 <sup>c</sup>	0.26 ± 0.05 <sup>b</sup>
HCD + 12% RMR	3.55 ± 0.39 <sup>a</sup>	0.47 ± 0.07 <sup>ab</sup>	0.75 ± 0.06 <sup>ab</sup>	0.32 ± 0.04 <sup>c</sup>	1.18 ± 0.20 <sup>b</sup>	0.03 ± 0.01 <sup>a</sup>	0.60 ± 0.12 <sup>c</sup>	0.23 ± 0.05 <sup>ab</sup>
HCD + 16% RMR	3.42 ± 0.30 <sup>ab</sup>	0.49 ± 0.08 <sup>b</sup>	0.74 ± 0.06 <sup>bc</sup>	0.34 ± 0.02 <sup>c</sup>	1.14 ± 0.05 <sup>b</sup>	0.03 ± 0.00 <sup>a</sup>	0.68 ± 0.68 <sup>c</sup>	0.24 ± 0.05 <sup>ab</sup>
HCD + 80 mg Lovastatin	3.49 ± 0.25 <sup>a</sup>	0.47 ± 0.10 <sup>b</sup>	0.73 ± 0.06 <sup>c</sup>	0.30 ± 0.02 <sup>c</sup>	0.98 ± 0.20 <sup>b</sup>	0.02 ± 0.00 <sup>a</sup>	0.63 ± 0.10 <sup>c</sup>	0.20 ± 0.02 <sup>ab</sup>

Values are mean ± SD of six animals. Mean values within each column with different superscripts are significantly different Duncan's test at  $p < 0.05$ .

values of 0.52 and 0.39 for pravastatin and lovastatin, respectively. Spots appearing at this R<sub>f</sub> with the ethanol extract of *M. purpureus* identified (Figure 1A) the presence of two statins. Hence for conformation, HPLC was carried out.

Further the HPLC chromatograms confirmed the presence of lovastatin (hydroxyacid and lactone form) and pravastatin in RMR extract. The retention time (*R<sub>t</sub>*) was 5.22 and 6.97 min for hydroxyacid and lactone form of lovastatin, respectively. The pravastatin showed elution time at 2.40 min when compared with standard (Figure 1B). HPLC data shows, the lactone form was the major form of lovastatin.

These results indicated the biosynthesis of lovastatin (hydroxyacid and lactone form) and pravastatin in RMR.

### Fatty Acids

Unsaturated fatty acids such as oleic, linoleic, and linolenic acids also help to reduce serum lipids (Ma et al., 2000). In order to quantitate useful fatty acids in *M. purpureus* RMR, the profile were estimated by GC. Each of the fatty acid was identified using the retention time with reference to that of the standard. Palmitic, stearic, oleic, linoleic and linolenic acids were identified as the major fatty acids in *M. purpureus* RMR (Figure 2). RMR had higher concentrations of unsaturated fatty acids (linoleic and oleic acids) than saturated fatty acids (palmitic and stearic acid). Production of linolenic acid (0.2 ± 0.66%) concentration was very less compared to other fatty acids like oleic acid (27.90 ± 0.90%) and Linoleic acid (24.60 ± 0.66%).

### Antioxidant Activity

A compound can exert its antioxidant activity by scavenging radicals, decomposing peroxides, or chelating metal ions. The antioxidant activity of crude methanol extract was carried out by *in vitro* assays for DPPH radical scavenging, inhibition of ascorbate autooxidation, reducing activity, and lipid peroxidation (Table 2). All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

The phenolic content of methanol extract of RMR was 141.32 ± 0.01 µg ml<sup>-1</sup> and expressed as mg gallic acid equivalent/100 g dry weight. The IC<sub>50</sub> value to scavenge DPPH radical for methanol extract, and BHA were found to be 100.78, 50.01 and 18.96 µg ml<sup>-1</sup>, respectively (Table 2).

Inhibition of ascorbate autooxidation and reducing activity by crude methanol extract of RMR was found to be more compared to BHA. The BHA showed lipid peroxidation inhibition activity of 38.02 ± 1.53 µg/ml (Table 2).

## IN VIVO HYPOLIPIDEMIC ACTION OF RMR

### Effect of RMR on Food Intake and Body Weight Gain in Rats

The normal, high fat and RMR supplemented diets were prepared. Cholesterol and bile acids were added to diet at 1.0 and 0.15% respectively to induce hypercholesterolemia in rats. Effects of RMR supplementation to HCD on food intake and body weight gain were presented in Tables 3, 4. No significant differences in average food intake were observed between control

**TABLE 6** | Effect of feeding *M. purpureus* RMR for 14 weeks on relative organ weight of male rats.

Concentration of RMR mixed with diet (w/w)	Relative organ weight (g/100 g body weight)							
	Liver	Lungs	Kidney	Heart	Testis	Adrenal	Brain	Spleen
Control	3.35 ± 0.36 <sup>a</sup>	0.40 ± 0.07 <sup>a</sup>	0.69 ± 0.16 <sup>a</sup>	0.35 ± 0.06 <sup>a</sup>	1.01 ± 0.18 <sup>a</sup>	0.02 ± 0.00 <sup>b</sup>	0.54 ± 0.11 <sup>a</sup>	0.25 ± 0.05 <sup>a</sup>
HCD	5.12 ± 0.45 <sup>c</sup>	0.43 ± 0.04 <sup>a</sup>	0.67 ± 0.06 <sup>a</sup>	0.32 ± 0.02 <sup>a</sup>	0.92 ± 0.05 <sup>a</sup>	0.02 ± 0.01 <sup>a</sup>	0.56 ± 0.07 <sup>a</sup>	0.22 ± 0.02 <sup>a</sup>
HCD+8% RMR	4.64 ± 0.59 <sup>b</sup>	0.38 ± 0.09 <sup>a</sup>	0.62 ± 0.04 <sup>a</sup>	0.22 ± 0.09 <sup>a</sup>	0.86 ± 0.18 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>	0.49 ± 0.10 <sup>a</sup>	0.27 ± 0.02 <sup>a</sup>
HCD+12% RMR	4.42 ± 0.09 <sup>b</sup>	0.55 ± 0.12 <sup>a</sup>	0.68 ± 0.09 <sup>a</sup>	0.33 ± 0.08 <sup>a</sup>	1.16 ± 0.04 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>	0.68 ± 0.11 <sup>a</sup>	0.26 ± 0.07 <sup>a</sup>
HCD+16% RMR	4.34 ± 0.51 <sup>b</sup>	0.61 ± 0.15 <sup>a</sup>	0.67 ± 0.10 <sup>a</sup>	0.35 ± 0.04 <sup>a</sup>	1.13 ± 0.23 <sup>a</sup>	0.03 ± 0.00 <sup>ab</sup>	0.69 ± 0.05 <sup>a</sup>	0.29 ± 0.06 <sup>a</sup>
HCD + 80 mg Lovastatin	4.37 ± 0.25 <sup>b</sup>	0.53 ± 0.07 <sup>a</sup>	0.68 ± 0.02 <sup>a</sup>	0.37 ± 0.10 <sup>a</sup>	1.11 ± 0.15 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>	0.67 ± 0.10 <sup>a</sup>	0.27 ± 0.04 <sup>a</sup>

Values are mean ± SD of six animals. Mean values within each column (liver) with different superscripts are significantly different Duncan's test at  $p < 0.05$ .

**TABLE 7** | Effect of RMR on serum/Liver cholesterol and triglycerides levels in HCD fed male rats.

Conc. of RMR in diet (w/w)	Serum				Liver			
	7th week		14th week		7th week		14th week	
	cholesterol (mg dl <sup>-1</sup> )	Triglyceride (mg dl <sup>-1</sup> )	Cholesterol (mg dl <sup>-1</sup> )	Triglyceride (mg dl <sup>-1</sup> )	Cholesterol (mg dl <sup>-1</sup> )	Triglyceride (mg dl <sup>-1</sup> )	Cholesterol (mg dl <sup>-1</sup> )	Triglyceride (mg dl <sup>-1</sup> )
Control	66.24 ± 4.52 <sup>a</sup>	94.00 ± 25.36 <sup>ab</sup>	70.90 ± 13.80 <sup>bc</sup>	99.07 ± 11.43 <sup>a</sup>	96.29 ± 16.40 <sup>a</sup>	75.24 ± 8.57 <sup>a</sup>	62.96 ± 18.35 <sup>a</sup>	76.37 ± 5.24 <sup>a</sup>
HCD	150.02 ± 17.29 <sup>e</sup>	145.54 ± 38.43 <sup>c</sup>	165.81 ± 13.56 <sup>e</sup>	151.53 ± 13.02 <sup>d</sup>	189.93 ± 8.53 <sup>d</sup>	148.79 ± 58.92 <sup>b</sup>	152.19 ± 26.34 <sup>d</sup>	125.38 ± 14.93 <sup>b</sup>
HCD + 8% RMR	126.96 ± 6.37 <sup>d</sup>	110.25 ± 11.41 <sup>b</sup>	121.90 ± 6.48 <sup>d</sup>	113.77 ± 12.09 <sup>b</sup>	163.76 ± 14.10 <sup>c</sup>	107.05 ± 44.53 <sup>ab</sup>	114.67 ± 5.40 <sup>c</sup>	115.97 ± 18.78 <sup>b</sup>
HCD + 12% RMR	96.97 ± 9.78 <sup>c</sup>	94.29 ± 13.80 <sup>ab</sup>	105.20 ± 5.38 <sup>cd</sup>	103.07 ± 4.76 <sup>bc</sup>	133.20 ± 22.56 <sup>b</sup>	96.78 ± 42.44 <sup>a</sup>	96.72 ± 22.32 <sup>bc</sup>	95.05 ± 19.50 <sup>a</sup>
HCD + 16% RMR	82.78 ± 7.62 <sup>a</sup>	79.60 ± 11.27 <sup>a</sup>	83.91 ± 24.37 <sup>ab</sup>	86.35 ± 6.11 <sup>ab</sup>	97.89 ± 9.70 <sup>ab</sup>	86.17 ± 15.57 <sup>a</sup>	83.04 ± 22.68 <sup>ab</sup>	80.88 ± 10.99 <sup>a</sup>
HCD + 80 mg lovastatin	80.25 ± 5.42 <sup>ab</sup>	76.56 ± 5.25 <sup>a</sup>	77.85 ± 2.69 <sup>a</sup>	84.70 ± 1.90 <sup>a</sup>	92.50 ± 11.76 <sup>a</sup>	85.12 ± 12.17 <sup>a</sup>	79.75 ± 14.23 <sup>ab</sup>	79.80 ± 8.06 <sup>a</sup>

Control, normal diet (without cholesterol); HCD, High Cholesterol Diet (containing 1% cholesterol and 0.15% bile salts); HCD + 8 % RMR; HCD + 12 % RMR, HCD + 16 %; HCD + 80 mg Lovastatin Data are presented as mean ± SD (n = 6). Mean values within each column with different superscripts are significantly different ( $p < 0.05$ ).

and RMR fed groups. RMR fed groups results with lower average body weight gain compared to control group at initial periods of feeding (1–7 weeks), however, no significant change in body weight gain was observed during 8–14 weeks of feeding. There were no marked differences in mean relative organ weights of various vital organs except in liver of HCD and RMR fed groups compared to that of control groups. Changes in liver weight are ascribed to high fat diet. The average weight of the liver of HCD fed rats increased by 14.7% over that control diet and addition of RMR reduced liver weights ranges from 10.46 to 8.27 g in rats fed diets containing 8, 12 and 16 % RMR, respectively (Tables 5, 6). The results indicate that the daily food intake and gain in body weight of rats were normal and did not differ among various experimental groups.

### Effect of RMR on Serum and Liver TC and TG Levels

Change in serum and liver TC and TG levels of rats fed HCD and HCD supplemented with different concentration of RMR were given in Table 7. High cholesterol diet fed group showed 2.26 and 1.97 fold increase in serum and liver cholesterol levels. Similarly high cholesterol diet induced 1.54 and 1.97 fold increase in serum and liver triacylglycerols, respectively. RMR supplementation in high cholesterol diet resulted in a dose dependent decrease in

serum and liver cholesterol and triacylglycerol levels compared to high cholesterol fed group at 7 and 14 weeks. The cholesterol and triacylglycerol levels in serum were significantly decreased by 44.82 and 45.30%, respectively, in rats fed with 16% RMR for 7 weeks. Similarly, 48.45 and 42.08% decrease in liver cholesterol and triacylglycerols were observed in 16% RMR fed group for 7 weeks compared to high cholesterol fed group. However, at the end of 14 week, the decrease observed in serum and liver cholesterol and triacylglycerol levels in RMR fed groups were not relatively higher than 7 weeks feeding of RMR.

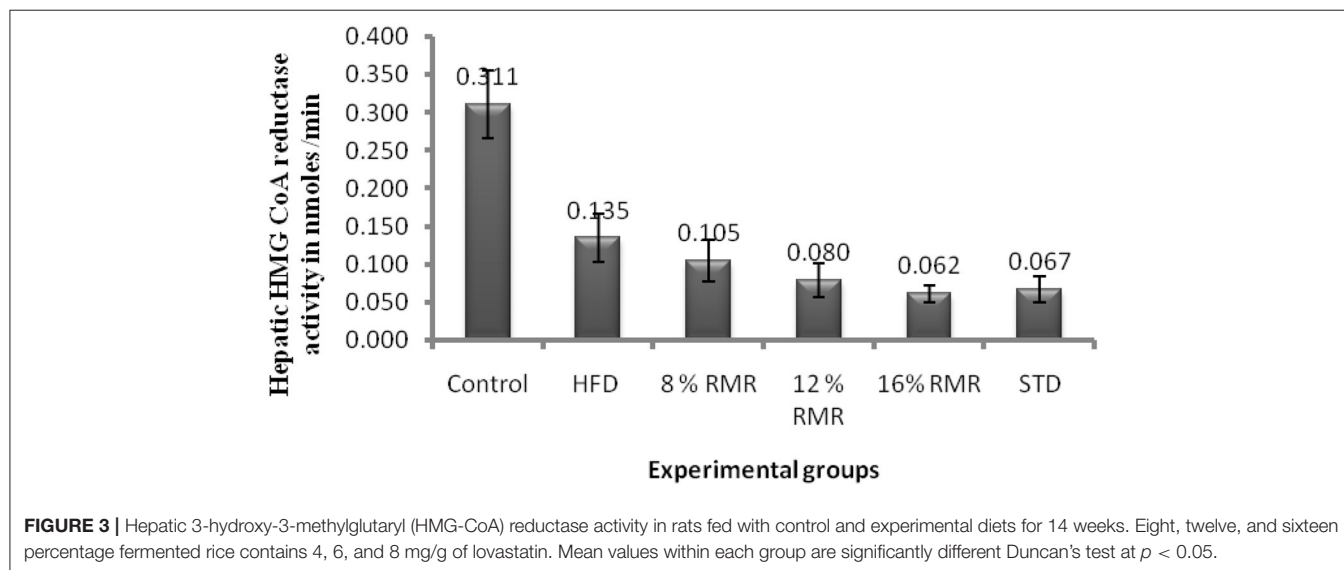
### Effect of RMR on Serum and Liver HDL and LDL-C Levels

The effects of various concentration of RMR on serum HDL and LDL-C levels are presented in Table 8. High cholesterol diet induced 3.62 fold increases in LDL-C level and 4.12 fold in LDL/HDL ratio compared to control animals. LDL-C level was increased by 5.14 fold in high cholesterol diet group. Supplementation of 16% RMR in the diet for 14 weeks significantly decreased LDL-C by 3.37 fold compared to high cholesterol control group.

**TABLE 8** | Effect of RMR on serum HDL and LDL levels of HCD fed male rats.

Conc. of RMR in diet (w/w)	7th week				14th week			
	HDL-C (mg dl <sup>-1</sup> )	LDL-C (mg dl <sup>-1</sup> )	LDL-C/HDL-C	TC/HDL-C	HDL-C (mg dl <sup>-1</sup> )	LDL-C (mg dl <sup>-1</sup> )	LDL-C/HDL-C	TC/HDL-C
Control	33.30 ± 8.26 <sup>a</sup>	32.94 ± 2.77 <sup>b</sup>	0.99 ± 0.34 <sup>b</sup>	2.55	46.96 ± 3.71 <sup>ab</sup>	28.48 ± 2.10 <sup>a</sup>	0.61 ± 0.57 <sup>b</sup>	2.02
HCD	29.27 ± 8.33 <sup>ab</sup>	119.29 ± 11.07 <sup>a</sup>	4.08 ± 1.33 <sup>a</sup>	6.06	40.20 ± 2.71 <sup>a</sup>	146.61 ± 5.05 <sup>c</sup>	3.65 ± 1.86 <sup>a</sup>	5.40
HCD + 8% RMR	33.48 ± 4.07 <sup>c</sup>	92.48 ± 5.25 <sup>b</sup>	2.76 ± 1.29 <sup>b</sup>	4.42	48.51 ± 2.85 <sup>ab</sup>	92.30 ± 1.90 <sup>b</sup>	1.90 ± 0.67 <sup>b</sup>	3.37
HCD + 12% RMR	37.50 ± 3.88 <sup>bc</sup>	58.37 ± 5.18 <sup>a</sup>	1.56 ± 1.34 <sup>a</sup>	3.05	51.12 ± 1.93 <sup>ab</sup>	66.08 ± 1.51 <sup>b</sup>	1.29 ± 0.78 <sup>a</sup>	2.69
HCD + 16% RMR	42.92 ± 0.71 <sup>a</sup>	39.59 ± 1.19 <sup>b</sup>	0.92 ± 0.54 <sup>bc</sup>	2.29	54.43 ± 1.84 <sup>b</sup>	43.48 ± 1.45 <sup>ab</sup>	0.80 ± 0.79 <sup>b</sup>	2.11
HCD + 80 mg lovastatin	42.28 ± 2.35 <sup>a</sup>	38.12 ± 2.14 <sup>a</sup>	0.90 ± 0.91 <sup>c</sup>	2.26	55.28 ± 0.43 <sup>b</sup>	44.77 ± 0.89 <sup>ab</sup>	0.81 ± 2.08 <sup>b</sup>	2.11

Control, normal diet (without cholesterol); HCD, High Cholesterol Diet (containing 1% cholesterol and 0.15% bile salts); HCD + 8% RMR; HCD + 12% RMR, HCD + 16%; HCD + 80 mg Lovastatin Data are presented as mean ± SD (n = 6). Mean values within each column with different superscripts are significantly different (p < 0.05). LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol.



**FIGURE 3** | Hepatic 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase activity in rats fed with control and experimental diets for 14 weeks. Eight, twelve, and sixteen percentage fermented rice contains 4, 6, and 8 mg/g of lovastatin. Mean values within each group are significantly different Duncan's test at p < 0.05.

### 3-Hydroxy-3-methylglutaryl-CoA Reductase Activity

3-Hydroxy-3-methylglutaryl coenzyme A reductase is generally considered to catalyze the rate-limiting reaction in cholesterol biosynthesis. High cholesterol feeding decreased HMG-CoA reductase activity by 56.45% in control rats (Figure 3). RMR supplementation in high cholesterol diet resulted in a dose dependent inhibition of HMG-CoA reductase activity in liver compared to high cholesterol HCD fed group at 14 weeks. RMR feeding reduced HMG-CoA reductase activity by 22–54% compared HCD fed rats.

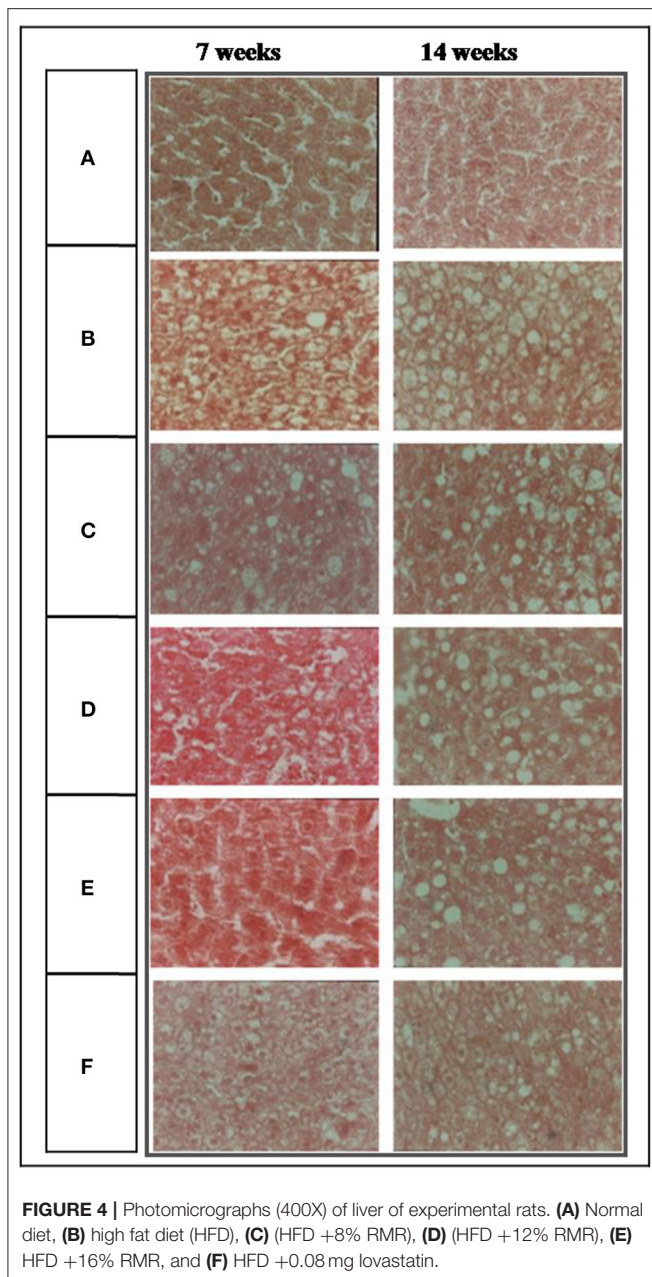
No significant damage in liver tissue of RMR groups was observed in microscopic examination compared to control group and HCD group (Figure 4). Liver sections of group III, IV, V supplemented with RMR showed relatively moderate cholesterol infiltration compared to that of group II fed with HCD. Group VI fed with lovastatin showed fatty infiltration similar to that of RMR fed groups.

### DISCUSSION

*M. purpureus* MTCC 410 RMR produces polyketides, statins (hydroxyl and lactone form of lovastatin and pravastatin), sterols and significant amount of saturated (palmitic and stearic) and unsaturated fatty acids (oleic, linoleic, and linolenic) by solid-state fermentation. Production of higher concentration of metabolites in solid-state fermentation can be attributed to better hyphal adhesion and invasion into the microstructure of the rice grain (Johns and Stuart, 1991). The hyphal mode of fungal growth is also tolerant to low water activity and high osmotic pressure conditions. Thus, the conditions apparently made the fungus competitive for efficient bioconversion of solid substrates to produce these products of interest in higher concentration.

Moreover, plant phytosterols, saponins, fungal metabolites such as mevinolin, monacolin K are being investigated for their antihyperlipidemia and antiatherosclerotic properties (Kroon et al., 1982; Harwood et al., 1993; Ikeda and Sugano, 1998). Monacolin K, the secondary metabolite produced during fermentation of rice by *Monascus* spp. has been shown to





reduce cholesterol by inhibiting HMG-CoA reductase enzyme in liver. But the safety of RMR as a dietary supplement to reduce high cholesterol was a concern due to the presence of high concentrations of a mycotoxin- citrinin in different commercial *Monascus* products.

Hypercholesterolemia is considered as the major risk factors for atherosclerosis and cardiovascular diseases. Management of hyperlipidemia with healthy less atherogenic diet, less stressful life style, and physical activity is successful for certain patients, but a majority of patients need therapeutical intervention to effectively lower hyperlipidemia. Statins that inhibit HMG CoA reductase are widely used drugs to control hypercholesterolemia (Alberts, 1990; Blankenhorn et al., 1993).

In this study, supplementation of RMR of *M. purpureus* MTCC 410 to rats at a dose of 16% for 7 weeks showed, 44.82 and 48.45% reduction in serum and liver cholesterol levels; 45.30 and 42.08% reduction in serum and liver triacylglycerol, respectively, compared to HCD fed group. LDL-C levels decreased by 66.81% while HDL-C increased by 46.63% in serum of 16% RMR fed rats for 7 weeks. RMR feeding to hypercholesterolemia rats resulted in 22–54% inhibition of HMG-CoA reductase activity in liver. Thus, RMR is very effective in reducing cholesterol and triacylglycerol and LDL-C levels in hyperlipidemia rats. The combination of dietary sterols with statin drugs was also suggested for lowering cholesterol more effectively than statin alone (Plat and Mensink, 2001). The bioactive molecules sterols and statins identified in extract were attributed to the lipid lowering activity. The RMR been developed as a health food in human dietary supplements due to its functional properties like lowering cholesterol level and blood pressure. Further, RMR reduced liver weight and fatty infiltration, suggesting inhibition of lipids/fats deposition in liver. These findings are in concurrence with reported decrease in liver weight, lipid deposition in liver and atheroma in aorta of rabbits fed with *Monascus purpureus* (Li et al., 1998). While the clinical trials with *Monascus* spp. as dietary supplement in human subjects showed an effective reduction in serum cholesterol and triacylglycerol with mild side effects (Wang et al., 1997).

Functional food product marketed in the United States as Cholestin™ by Pharmanex Inc., Simi Valley, CA (Wang et al., 1997). It is reported to contain many nutritional components including phytosterols, saponin, saponogenin, unsaturated fatty acids, isoflavones, trace elements zinc, selenium, and HMG-CoA reductase inhibitor (Wang et al., 1997). The exact mechanism by which RMR reduces serum cholesterol and triacylglycerols is not completely understood, because many *Monascus* species could synthesize small quantity ( $<500 \text{ mg Kg}^{-1}$ ) of monacolin K and only monacolin K cannot be accounted for hypolipidemic effect of *Monascus* (Chang et al., 2002; Casas-López et al., 2003). Therefore, *Monascus* species which can produce high monacolin K are being developed to apply as functional food. It is believed that, atherosclerotic index (ratio of non-HDL-cholesterol to HDL-cholesterol) is important risk factor for atherosclerosis. Our data clearly demonstrate that RMR significantly decreased the ratio, even in the lower dose group. The inhibition of HMG-CoA reductase by other forms of monacolins like, monacolin J, L, M, X, and their hydroxy acid form, as well as dehydromonacolin (MV2 and K), dihydromonacolin (MV and L), compactin,  $3\alpha$ -hydroxy-3,5-dihydromonacolin L are also accounted for the reduced cholesterol synthesis and anti-atherogenic effect of RMR (Li et al., 2004; Dhale et al., 2007a,b). Another risk factor for atherosclerosis is lower level of HDL-cholesterol. Whereas, feeding of RMR increased serum HDL-C, even in the lower dose group to increase HDL-C significantly. The HCD diet induced an increase in plasma and liver peroxides (malnaldehyde, lipid hydroperoxide and conjugated dienes) which may indicate elevated oxidative stress in the body. Monacolins, known as statins, isolated from *Monascus* spp. were found to protect body organs against oxidation of LDL and inhibit HMG-CoA reductase (Istvan and Deisenhofer, 2001). The statins play important role in atherosclerosis as

antioxidant by preventing the oxidation of LDL during oxidative stress. Cholesterol diet induces oxidative stress by conceding antioxidant molecules and antioxidant enzymes. The statins and supplementation of RMR containing statin, pigment and other secondary metabolites effectively reduced the oxidative stress by alleviating antioxidant molecules and enzymes in the liver of hypercholesterolemic rats (Mohan-Kumari et al., 2011a,b). The coronary heart disease (CHD) do not always have elevated levels of LDL but, the normal LDL levels with high levels of stress are more prone to CHD (Rosenson, 2004; Mohan-Kumari et al., 2011a). The increased oxidation of serum lipid and LDL-cholesterol in serum and its entry into artery leads to the formation of atherosclerotic lesion. The formation of atherosclerotic plaque in macrophages due to foam cells is prone to damage (Osterud and Bjorklid, 2003; Rosenson, 2004).

The feeding of RMR reduced such oxidative stress by modulating the antioxidant enzymes and molecules (Mohan-Kumari et al., 2011b). The data suggested RMR can lower or slowing down oxidative stress related atherosclerosis pathological process. However, the authentic comparison of the secondary metabolites of RMR is in their effects on severity of cholesterol-induced atherosclerosis. The cholesterol lowering and anti-atherogenic effect of RMR in this study is comparable to therapeutic drug, lovastatin. The monacolins also increase the activity of LDL-receptors and reduce total cholesterol and LDL levels in the body (Pitman et al., 1998). Thus, the significant inhibition of HMG Co-A reductase and anti-atherogenic effect of RMR observed in this study can be attributed to synergistic effect of monacolins (statins) which are suppressing cholesterol synthesis.

Commercial RMR products of *Monascus* contain high levels of citrinin, a mycotoxin affecting liver and kidney. Hence, the safety of RMR is of big concern to use it as a food supplement. Our earlier work, acute and sub-acute toxicological studies on RMR of *M. purpureus* MTCC 410 did not show any adverse effect in rats (Mohan-Kumari et al., 2009) and produced low levels of 1.427 mg kg<sup>-1</sup> of citrinin. RMR supplementation in HCD did not induce any histopathological alterations in liver but moderately reduced fatty infiltration in liver compared to HCD fed group.

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## CONCLUSIONS

In conclusion, our data suggest that *M. purpureus* RMR can be considered as a potent anti-atherogenic food supplement with no adverse effects and possess health benefits related to regulation of blood lipids by inhibiting the HMG CoA reductase. The RMR significantly decreased LDL-C level in serum. The HDL value and HDL-C to LDL-C ratio were increased in rats fed on RMR.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Ethics Committee, Central Food Technological Research Institute (CSIR), Mysore.

## AUTHOR CONTRIBUTIONS

HPMK conceptualized, executed the experiments, data analysis, and wrote the paper. KAN and GV designed the experiment and reviewed the paper. KN carried out histopathology. All the authors read and approved the final manuscript.

## FUNDING

HPMK acknowledges Council of Scientific and Industrial Research, New Delhi, India and University of Mysore for supporting research through the award of senior research fellowship. CSIR, New Delhi, University of Mysore. This work was partially supported by Ministry of Food Processing India, New Delhi (No. Q/2/2020-R&D).

## ACKNOWLEDGMENTS

We thank Director, CSIR-CFTRI, Mysore, India for encouragement and providing the facility.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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