

Research Article

Inhibition of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (*Ex Vivo*) by *Morus indica* (Mulberry)

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Phytochemicals are the bioactive components that contribute to the prevention of cardiovascular and other degenerative diseases. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase would be an effective means of lowering plasma cholesterol in humans. The present study explores the HMG CoA reductase inhibitory effect of extracts from leaves of *Morus indica* varieties, M5, V1, and S36, compared with the statin, using an *ex vivo* method. The assay is based on the stoichiometric formation of coenzyme A during the reduction of microsomal HMG CoA to mevalonate. Dechlorophyllised extract of three varieties was studied at 300 μg . The coenzyme A released at the end of assay in control (100.31 nmoles) and statins (94.46 nm) was higher than the dechlorophyllised extracts of the samples. The coenzyme A released during the reduction of HMG CoA to mevalonate in dechlorophyllised extracts of the samples was as follows: S36 < M5 < V1. The results indicated that the samples were highly effective in inhibiting the enzyme compared to statins (standard drug). The results indicate the role of *Morus* varieties extracts in modulating the cholesterol metabolism by inhibiting the activity of HMG CoA reductase. These results provide scope for designing *in vivo* animal studies to confirm their effect.

1. Introduction

Cholesterol as a constituent of all eukaryotic plasma membranes is essential for the growth and viability of higher organisms. For healthy state cholesterol homeostasis is very important and is accomplished by a regulatory complex network [1]. The hypercholesterolemia and elevated LDL-C (LDL-cholesterol) concentration are the major risk factors for the development of atherosclerosis and coronary heart disease [2]. In this consequence, reducing plasma cholesterol levels is one of the major aims of public health organizations [3]. Hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) is the rate-limiting step in the biosynthesis of cholesterol in humans; inhibition of this enzyme would be an effective means of lowering plasma cholesterol. Drugs like statins, fibrates, niacin, and so forth are used to lower cholesterol. Due to the prevalence of these drugs adverse effects, search was made for an alternative natural drug [4].

Medicinal plants are good sources of therapeutic components and their use for medical purposes has a long history. Over past years, extracts of medicinal plants have

gained increasing importance as a source of herbal drugs [5]. Exploration of the chemical constituents of the plants and pharmacological screening will thus provide the basis for developing new life saving drugs and functional foods. HMG CoA reductase inhibitors have been identified from many herbs, medicinal plants, microorganisms, and so forth. Phytochemicals such as phenolics, flavonoids, terpenoids, saponins, and alkaloids are the bioactive components present at microlevel in our daily diet and have received much attention in disease treatment due to their *in vivo* and *in vitro* antioxidant capabilities [6].

Many studies have reported the hypocholesterolemic property of medicinal plants at biological level [7–9]. Some studies have also reported the *ex vivo* inhibition of HMG CoA reductase activity by herbs specially medicinal plants, namely, *Quercus infectoria*, *Rosa damascene*, *Myrtus communis*, *Andrographis paniculata*, *Anthocephalus indicus*, and *Ocimum sanctum* [10]. Our team has explored the antioxidant properties and potency of the phytochemicals and polyphenols of different medicinal plants from western Ghats on *ex vivo* inhibition of the HMG CoA reductase activity

[11–15] and hypocholesterolemic effect of *Moringa oleifera* leaf powder in animal models [7].

Morus indica (Mulberry tree) of the family Moraceae has been widely cultivated in countries all over the world including temperate to tropical areas. It is nontoxic natural therapeutic agent and different parts of the plant shown to possess hypoglycemic, hypotensive, cholesterol and lipid peroxides reduction, antiphlogistic, diuretic properties and expectorant effects. It occupies an important position in the holistic system of Indian medicine “Ayurveda” which has its roots in antiquity and has been practiced for centuries. The leaves of mulberry are nutritious, palatable, nontoxic, and also enriched with different active principles [16–18].

Reports indicate *Morus* as a good source of phytochemicals and various solvent extracts of its leaves showed varying degrees of antioxidant activity in both *in vitro* and *ex vivo* models. Also, the extracts have exhibited stability to high temperature and different pH [19, 20]. Few reports are available on the antihyperglycemic and antioxidant effect of *Morus* leaves in streptozotocin-induced diabetic rats. The *Morus* effectively reduced the increased oxidative stress in diabetic rats and the leaves exerted a rapid protective effect against lipid peroxidation by scavenging O_2^- (superoxide) and elevating the activities of antioxidant enzymes by virtue of antioxidant flavonoids (quercetins) [21, 22]. In another study, *Morus* therapy to human subjects with diabetes has resulted in a significant decrease in the concentration of serum total cholesterol, triglycerides, plasma free fatty acids, LDL-cholesterol, VLDL-cholesterol, plasma peroxides, and urinary peroxides, while increasing HDL-cholesterol, and also in diabetic rats reduced the retinopathy [16, 17]. The available literature indicates the antidiabetic and antioxidant properties of *Morus*. However, studies on the hypocholesterolemic effect are lacking and studies are needed to prove its efficacy at biological level especially in cholesterol metabolism in animals. As an initiative, preclinical studies have been undertaken to study the ability of *Morus* varieties to inhibit HMG CoA reductase, a key enzyme in cholesterol metabolism. The *Morus* extracts were compared with the commercial hypocholesterolemic drug, Atorvastatin.

2. Materials and Methods

2.1. Chemicals. All the chemicals were of analytical grade. HMG CoA, dithiothreitol, triethanolamine, and NADPH were obtained from Sigma Aldrich Chemicals, Bangalore.

2.2. Plant Material. Three varieties of *Morus indica* (MI), namely, M1, V1, and S36 leaves, collected from Central Sericultural Research and Training Institute (CSRTI), Mysore, and identified by Dr. Shivamurthy, Department of Studies in Botany, University of Mysore, and voucher specimen was retained in the laboratory for future. The collected leaves were washed and dried in a hot air oven at 55°C. Dry leaves were ground separately and passed through a 60 μ m mesh sieve and kept in air-tight containers at 4°C until further use.

2.3. Preparation of Dechlorophyllised Extract. 15 g sample of each sample was extracted separately with 100 mL 80%

methanol (methanol 80 mL and water 20 mL). To avoid the interference of chlorophyll, it was separated as per the method of Rich A and Rich C [23]. Briefly, hexane was added to the 80% methanol extract and shaken for 30 min and the chlorophyll-rich hexane top layer was separated. The remaining extract was further evaporated (Rotary evaporator) and oven-dried (50°C) and stored in air-tight container at 0°C until used.

2.4. Preparation of Microsomes. A healthy male adult rat was obtained from the Central Animal House, Department of Zoology, University of Mysore, after availing clearance from the University Animal Ethics Committee of University of Mysore (number MGZ/2620/2011-12; dtd: 31.01.2012). The rat was fasted for 24 hrs and sacrificed after 9:00 pm to obtain the active HMG CoA reductase enzyme. The liver was immediately removed from the rat and placed in cold triethanolamine HCl buffer (0–4°C) at pH 7.4. The liver was thoroughly chilled and homogenized. The homogenate was centrifuged at 60,000 g for 60 min; the supernatant was separated and microsomal pellet was then rinsed with buffer and frozen at –20°C. The resuspended microsomes to be used for the assay were diluted with buffer to give a protein concentration of 5–10 mg/mL. The procedure described here is partially modified from that reported by Shapiro and Rodwell, 1971 [24].

2.5. 3-Hydroxy-3-methylglutaryl CoA Reductase Assay. For the assay of 3-hydroxy-3-methylglutaryl CoA reductase, the incubation mixtures contain 0.5–1.0 mg of microsomal protein, 150 nmoles of HMG CoA, and 2 μ moles of NADPH. These components are added to 0.8 mL of 0.1 M triethanolamine-0.02 M EDTA buffer at pH 7.4 without dithiothreitol. The dithiothreitol (0.2 μ mole) was added along with the microsomal preparation. The final incubation volume was 1 mL. Contents in tubes were as follows: (a) all components, (b) all components except NADPH, (c) all components except NADPH with 300 μ g of *Morus* leaves extracts separately, and (d) all components except NADPH with statins (Atorvastatin-10 μ g) [25]. After a series of steps, the coenzyme A released was calculated using the following formula:

$$\text{nmoles / min} = \frac{[A \text{ reaction} - A \text{ control}] \times 1.43}{0.0136 \times \text{time}} \quad (1)$$

The value of 1.43 is the dilution factor, and A is the absorbance at 412 nm. The difference in absorbance between the complete reaction and that of all components except NADPH represents the activity due to HMG CoA reductase.

2.6. Statistical Analysis. Each experiment was conducted in triplicates and data are expressed as mean \pm SD. The data was subjected to Student *t*-test ($P \leq 0.05$).

3. Results

In the present study, liver microsomes were treated with dechlorophyllised extracts (300 μ g) of three *Morus indica*

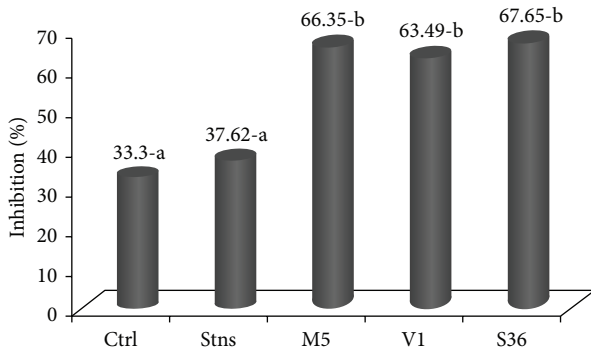


FIGURE 1: Percent inhibition of HMG CoA reductase by *Morus indica* varieties Ctrl—control; Stns—Atorvastatin (10 μ g); M5—300 μ g; V1—300 μ g; S36 - 300 μ g. ($P \leq 0.05$).

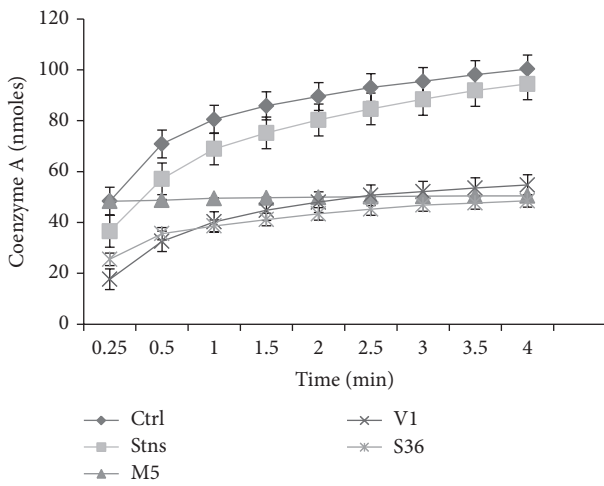


FIGURE 2: Effect of *Morus indica* on the activity of HMG CoA reductase and release of coenzyme. Ctrl—control; Stns—Atorvastatin (10 μ g); M5—300 μ g; V1—300 μ g; S36—300 μ g.

varieties (M5, V1, and S36) and their effect on the activity of 3-hydroxy-3-methylglutaryl CoA reductase was measured. The assay was run against a control and compared with the statins. The results indicate that all the three extracts were highly effective (63–67%) compared to statins (37%) in inhibiting the HMG CoA reductase in the liver microsomes (Figure 1).

The coenzyme A released during the assay was higher in control (100.31 nmoles) than in the microsomes treated with *Morus* and statins (Figures 2 and 3) and, among all, S36 was more potent in inhibiting the enzyme activity (48.52 nmoles). Figure 2 shows the time course of activity of HMG CoA reductase for a period of 4th min; it can be observed that the rate of release of coenzyme A was constantly increasing from zeroth to 4th min, both in control and statins. In M5, the coenzyme A released at zeroth min (M5—48.36 nmoles) was higher than other samples and statins. At the end of 4th min, the rate of release of coenzyme A was less in M5 (50.47 nmoles) than in the control (100.31 nmoles), statins (94.46 nmoles), and V1 (54.76 nmoles). In case of the other two varieties, that is, V1 and S36, the coenzyme A released at zero min was less (V1—17.36 and S36—25.55 nmoles) than

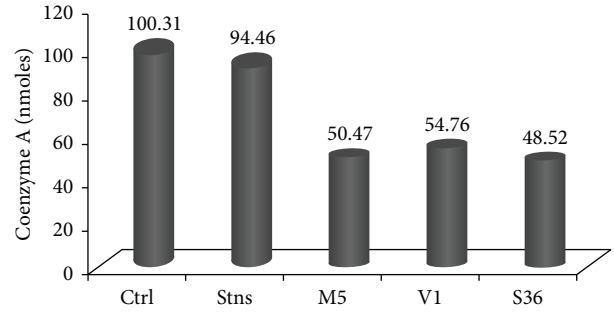


FIGURE 3: Total coenzyme A (nmoles/mg protein) released during the reduction of HMG CoA to mevalonate. Ctrl—control; Stns—Atorvastatin (10 μ g); Ctrl—control; Stns—Atorvastatin (10 μ g); M5—300 μ g; V1—300 μ g; S36—300 μ g.

the other samples and rate of release of coenzyme was high in first 2 min and decreased from 2nd to 4th min. However, the rate of release of coenzyme A in all the samples, statins, and control was high in first 2nd min and decreased from 2nd to 4th min.

Figure 3 shows the total HMG Co A reductase activity as nmoles of coenzyme A released at the end of 4th min in the order of control (100.31 nmoles) > statins (94.46 nmoles) > V1 (54.76 nmoles) > M5 (50.47 nmoles) > S36 (48.52 nmoles). The low amount of CoA released by *Morus* varieties is indicative of the inhibitory effect on HMG CoA reductase.

4. Discussion

Cholesterol is an insoluble lipid molecule that plays a critical role in the structure and function of membrane bilayers. Membrane cholesterol contents that are either too high or too low are detrimental to cell function. When present in excess amounts in cells, cholesterol becomes toxic. Certainly, cholesterol-induced cytotoxicity represents a key initiating event leading to the development of atherosclerotic cardiovascular disease [26].

Statins are the most commonly administered class of drugs to lower plasma LDL-cholesterol. Their primary mechanism of action is to promote clearance of LDL particles from the plasma. This is accomplished because statins reduce the rate of intracellular cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, the rate-limiting step in cholesterol biosynthesis [27]. However, most commercial HMG CoA reductase inhibitors have shown adverse effects including the induction of cutaneous vasodilatation, rashes, gastrointestinal discomfort, and hyperuricemia [28].

In our laboratory, *Morus indica* has been investigated for its antioxidant, antidiabetic, and antimicrobial effects [18, 19, 21, 29]. Oral administration of *Morus* leaf powder (500 mg/kg b.w) produced a significant hypoglycaemic effect in STZ-induced diabetic rats. The concurrent effect of *Morus indica* on lipid metabolism was significant in diabetic rats; this was evidenced by the reductions in serum cholesterol, triglycerides, and lipid peroxides [30]. However, the mechanism involved in the cholesterol lowering by *Morus indica* leaf has

not been reported. Hence, an attempt was made to study the inhibitory effect of leaves from three *Morus indica* varieties (300 μg) on HMG CoA reductase activity using a simple *ex vivo* model system comprising of liver microsomes. In the rat liver microsomes, a cell-free system, *Morus indica* extracts of leaves effectively the HMG CoA reductase activity better than the statins and control. This is the first report on the *ex vivo* inhibition of HMG CoA reductase by *Morus indica*.

The enzyme inhibitory effect correlates with the medicinal properties and phytochemical components such as phenolic compounds, alkaloids, and saponins. All three *Morus* varieties are good sources of phytochemicals and dechlorophyllised extract was rich in polyphenol content [20]. Particularly, S36 with more phytochemical composition have inhibited the HMG CoA reductase with more potency than the other two varieties (M5 and V1). A similar result was reported in *Moringa oleifera* polyphenol extract on HMG CoA reductase inhibition, in a dose-dependent manner [12].

3-Hydroxy-3-methylglutaryl coenzyme A reductase, the enzyme that synthesizes mevalonate, appears to be regulated through a multivalent feedback mechanism. Full suppression of the reductase requires the presence of at least two regulators: cholesterol, which is normally derived exogenously from plasma low density lipoprotein (LDL), and a nonsterol product, which is normally synthesized endogenously from mevalonate [31]. In cultured mammalian cells such as human fibroblasts, the activity of HMG CoA reductase and hence the formation of mevalonate are controlled through a feedback mechanism mediated by cholesterol that enters cells bound to a plasma lipoprotein, low density lipoprotein (LDL). In the absence of plasma LDL, cells in culture synthesize their own cholesterol, maintaining high levels of HMG CoA reductase. This effect was demonstrated by adding LDL to the culture medium. The LDL-derived cholesterol reduced the activity of HMG CoA reductase, thereby turning off the cell's cholesterol synthesis [32, 33]. In the present study, *Morus* extracts and statins might have acted in a similar manner as LDL in inhibiting HMG CoA reductase, thus preventing formation of mevalonate.

Although several medicinal plants have been reported to possess cholesterol lowering properties, no data is reported on the cholesterol lowering mechanism [9, 34–38]. The major metabolic pathway for reducing cholesterol is via conversion to bile acids or preventing the cholesterol synthesis by inhibiting the HMG CoA reductase enzyme. To establish the cholesterol lowering mechanism of *Morus*, we are also exploring its bile acid binding capacity using *in vitro* model system. Such preclinical studies will help in proper selection of medicinal plants for promoting their therapeutic utility.

5. Conclusion

The findings of the present study provide preliminary data that suggest that *Morus indica* is capable of reducing cholesterol levels by inhibiting the HMG CoA reductase activity in hyperlipidemic condition. The study also lends support to the reported data on the hypocholesterolemic role of *Morus indica* in diabetic condition. Promoting the utilization of *Morus* leaves appears to be beneficial as it can play

an important role in the prevention and management of cardiovascular diseases.

Conflict of Interests

The authors do not have conflict of interests.

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