

Original article

In vitro bile acid sequestering properties of *Morus indica* L. leaves

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Abstract

Binding of bile acids and increasing their fecal excretion have been hypothesized as a possible mechanism to lower cholesterol. Also, bile acid binding potential has been related to lowering the risk of heart disease and that of cancer. In the present study, leaves of three varieties of *Morus indica*, viz., M5, V1 and S36 were studied for the bile acid binding capacity (BABC) at different concentrations (200, 400 and 600 mg) and compared with cholestyramine, diosgenin, xanthan gum, guar gum and wheat bran. The BABC was determined by hydration and dialysis diffusion method. The total fiber content was in the order of V1 > M5 > S36. The saponins content ranged from 103 to 136 mg/g extract. The bile acid retardation index (BARI) of the samples was dose dependent and heat treatment improved the BARI significantly ($p \geq 0.05$). Among varieties, BARI of M5 at 400 mg (89.24%) was significantly higher ($p \geq 0.05$) than V1 (65.23%) and S36 (61.24%). In comparison with standards, BARI of M5 was higher than guar gum and wheat bran and comparable with cholestyramine and xanthan gum. The BARI of all the three *Morus* varieties was significantly higher ($p \geq 0.05$) than that of wheat bran. The results indicated *Morus* possess better BARI than standards and can be promoted as natural source of saponin and fiber expressing the mechanisms for the lipid lowering properties.

Key words: Bile acid, dietary fiber, saponins, cholic acid

1. Introduction

Dietary factors profoundly influence lipoprotein levels and metabolism, which, in turn, alter an individual's susceptibility to atherosclerosis. Several major dietary factors have been identified including fat, CH, fiber, phytosterols, protein, alcohol consumption, and energy balance (Hegsted *et al.*, 1965). Plasma cholesterol levels are determined by inputs from both diet and *de novo* biosynthesis, utilization of cholesterol, especially in the liver and steroidogenic tissues and excretion of either cholesterol or bile acids (Izzat *et al.*, 2000). Dietary fiber is an additional factor influencing CH (Rideout *et al.*, 2008). Fiber exhibits CH-lowering action by at least three mechanisms, one of which is bile acid sequestration within the small intestine. Hypercholesterolemia induces oxidative stress leading to generation of reactive oxygen species (ROS) which plays a key role in coronary artery disease (CAD) and atherosclerosis, that affects millions of people annually throughout the world (Izzat *et al.*, 2000; Steinberg, 1997; Tiwari, 1999). Reducing plasma cholesterol levels is the major public concern. Statins, fibrates, niacin, cholyseramine, *etc.*, are cholesterol lowering drugs and has its own mode of action in reducing the lipid levels. For example, statins act as HMG CoA reductase inhibitors by decreasing liver cholesterol synthesis (Kostner *et al.*, 1989) and cholyseramine bind bile acids in the

intestine forming complex with the bile acids. Due to the adverse effects of these drugs, need for an alternative natural drug raised.

A number of plants with potent therapeutic components such as fibers, sterols, saponins, polyphenols, flavonoids, *etc.*, have been investigated for their antihyperlipidemic, antioxidant and antiatherosclerotic properties. These compounds are reported to be beneficial with great variation in magnitude and mechanism of action and, hence have a potential therapeutic value in combating multifactorial atherosclerotic disorders (Nishant *et al.*, 2009).

Exploration of the dietary factors and chemical constituents of the plants and pharmacological screening will, thus provide the basis for developing new life saving drugs and functional foods. Phytochemicals such as phenolics, flavonoids, terpenoids, saponins, alkaloids, *etc.*, are the bioactive components present at micro level in our daily diet and have received much attention in disease treatment and due to their *in vivo* and *in vitro* lipid lowering capabilities (Morton *et al.*, 2000). *M. indica* (Mulberry) and *M. indica* L. (Mulberry tree) of the family Moraceae has been widely cultivated in countries all over the world including temperate to tropical areas. Plant is well explored for antihyperglycemic potency in streptozotocin induced diabetic rats (Andallu and Varadacharyulu, 2003; Devi and Urooj, 2008). It is rich source of phytochemicals, plants antioxidant properties in food and biological substrates, lipid lowering properties such as HMG CoA reductase inhibition using *in vitro* and *ex vivo* methods are reported (Reddy and Urooj, 2013; Reddy and Urooj, 2013a). In the present study, as a good source of dietary fiber and phytochemicals.

2. Materials and Methods

All chemicals used were of analytical grade. Cholic acid and dialysis tubes were purchased from Hi Media, Bangalore, India.

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2.1 Collection and preparation of samples

M. indica leaves (MI-S36) were collected from Centre for Sericulture Research and Technical Institute (CSRTI), Mysore district of Karnataka, India and subsequently identified by Dr. G. R. Shivamurthy, Department of Studies in Botany, University of Mysore, Mysore, India. The samples were thoroughly washed under running water, dried overnight (50°C), powdered, passed through 60 mesh sieve (BS) and stored in airtight container at 4°C till further use.

Dietary fiber was estimated by enzymatic AOAC (1995) method and saponins were estimated by Majob *et al.* (2003) method.

2.2 Bile acid binding by hydration and diffusion method

M. indica leaf sample was taken at different concentrations, 200, 400 and 600 mg and standards - cholesteramine (25 mg), xanthan gum (50 mg), guar gum (50 mg), wheat bran (200 mg), diosgenin (saponin-25 mg) were weighed. To the samples, 10 ml cholic acid solution (15 mM) in phosphate buffer (0.01 M, pH 7) with 1% sodium azide was added. All the contents were transferred into dialysis bags and hydrated for 14 h. After hydration, dialysis bags were transferred into the beaker with 100 ml of phosphate buffer at 37°C and aliquots of 1 ml were collected from dialysate at 0, 30, 60,

120 and 180 min. Aliquots were dried at 50°C and 2% vanillin (200 µl) in alcohol was added and dried again. To the dried contents, 5 ml of 79% ortho phosphoric acid was added and incubated for 30 min at 50°C and was read at 465 nm. The cholic acid diffused was calculated from standard graph. A control was run only with cholic acid without *M. indica* (Adiotomre *et al.*, 1990).

2.3 Heat treatment of *M. indica* samples

The dehydrated leaf powder (2000 mg) of *M. indica* was treated with hot water for 10 min. and was subjected to the above procedure of bile acid binding capacity.

Bile acid retardation index (BARI) (Adiotomre *et al.*, 1990)

$$= 100 - \left\{ \frac{\text{Total bile acid diffused from the dialysis bag with sample}}{\text{Total bile acid diffused from dialysis bag without sample}} \times 100 \right\}$$

3. Results and Discussion

Dietary fiber and saponin content of the *M. indica* varieties is presented in Figure. The percentage of dietary fiber (g/100 g) was in the order of V1 > M5 > S36 and saponins (mg/100 g) were in the order of M5 > S36 > V1.

Table 1: Bile acid binding capacity of *M. indica* by hydration method

CONC.	30 min	60 min	120 min	180 min
CHOLIC ACID – CA (CONTROL) - µM				
15 mmol CA	45 ±0.57	65± 10	95±9.29	131± 7.76
MI-M5 (raw)				
200 mg +15 mmol	7.9± 0.23	475± 0.5	62± 2.0	98± 3.6
400 mg+15 mmol	8.3± 0.79	10.7± 1.55	16.06± 1.69	16.96±11.44
600 mg+15 mmol	14.76± 0.61	24.76± 6.4	46.7± 2.64	44.4±4.78
MI-V1 (raw)				
200 mg+15 mmol	7.45± 0.10	48.75± 0.5	60.65± 1.25	91.35± 1.01
400 mg+15 mmol	10.8± 0.17	22.95± 0.34	32.85± 2.74	41.7± 5.57
600 mg+15 mmol	11.85± 0.89	18.75± 1.58	32.8± 0.50	45.35± 3.92
MI-S36 (raw)				
200 mg+15 mmol	8.98± 1.02	54.85± 8.80	62.7± 0.52	105.5± 4.72
400 mg+15 mmol	13.4± 2.64	19.2± 4.13	43.45± 4.07	43.65± 5.50
600 mg+15 mmol	15.2± 0.35	25.7± 4.14	27.6± 4.79	46.4± 4.88
MI-M5 (Heat treated) *				
200 mg +15 mmol	5.5±1.61	11.87±3.52	13.15±0.85	20.67±1.84
MI-V1 (Heat treated)*				
200 mg+15 mmol	13.17±1.32	17.22±2.94	21.3±4.58	25.93±2.81
MI-S36 (Heat treated)*				
200 mg+15 mmol	10.7±2.47	16.8±4.19	21.02±2.45	29.6±0.75

*200 mg of the heat treated samples were taken, values are mean of triplicates, bile acid retardation index, expressed as %, (n = 3); (p ≤ 0.05).

Table 2: Comparison of bile acid binding capacity of *M.indica* with the standards

CONC.	30 min	60 min	120 min	180 min	BARI*
MI-M5	8.3± 0.79	10.7± 1.55	16.06± 1.69	16.96±11.44	89.24 ^{cd}
MI-V1	10.8± 0.17	22.95± 0.34	32.85± 2.74	41.7± 5.57	65.23 ^b
MI-S36	13.4± .2.64	19.2± 4.13	43.45± 4.07	43.65± 5.50	61.25 ^b
Chol.	5.32±0.62	10.23±1.95	12.09±1.57	13.21±1.72	88.30 ^{cd}
Dios.	4.4±1.73	1.66±1.34	2.13±0.40	3.53±0.46	97.04 ^d
Xg	1.6± 0.13	19.2± 1.4	12.9± 0.06	24.1± 0.17	79.83 ^c
GG	13.91±0.28	25.85± 0.21	39.45± 0.54	57.10± 1.13	52.21 ^b
WB	23.36 ±0.77	32.03 ± 0.54	45.70 ± 0.80	77.3 ± 2.12	39.03 ^a

(MI-M5, MI-V1, MI-S36-400 mg), Dios-diosgenin (25 mg), Xg-xanthan gum (50 mg), GG-guar gum (50 mg), WB-wheat bran (200 mg), chol. cholesteramine (25 mg). Values are mean of triplicates (n=3). Values carrying different superscripts a, b, c..... differ significantly.

3.1 Bile acid binding (BAB) capacity

This method is one of the oldest and crude method and only one bile acid, *i.e.*, cholic acid was used as substrate. Different concentrations of free unbound bile acid, *i.e.* cholic acid diffused from dialysis bags at different time intervals, *i.e.*, from 0 to 180 min. is given in Table 1. From the Table 1, it can be observed that the BAB capacity is dose dependent in all the varieties of Morus and at 400 and 600 mg, the BAB was comparable in case of V1 and S36. In M5 variety at 400 mg, the BAB was maximum compared to other samples.

Heat treatment of the Morus leaf powder has resulted in a significant increase in bile acid binding than untreated samples (Table 1).

3.2 Comparison with standards

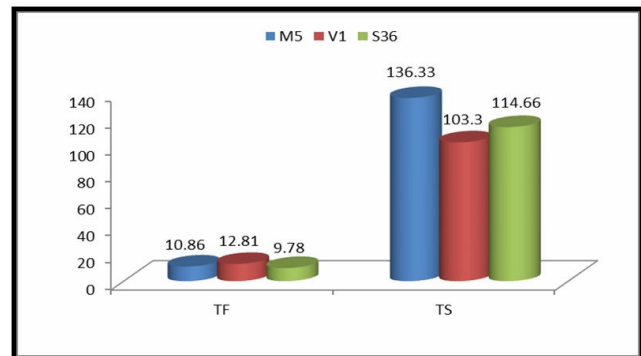
BAB capacity of the Morus leaf samples compared with the standards - cholesteramine, xanthan gum, guar gum, wheat bran and diosgenin is given in Table 2. The BAB of the Morus was comparable with the standards. Primarily, BAB capacity of M1 variety of Morus was comparable with cholesteramine and xanthan gum. The BAB capacity of guar gum and wheat bran were less than all Morus samples. However, diosgenin pure saponin has showed maximum BAB capacity.

3.3 Bile acid retardation index (BARI)

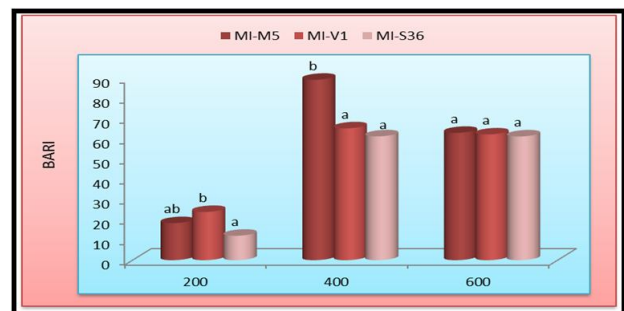
The effect of fiber and saponins of Morus and other standards on the bile acid binding is indicated by BARI in Figures 2, 3 and 4 where percentage of cholic acid passing into the dialysate is compared with the cholic acid control solution. Figure 2 shows the comparison of BARI between the *M. indica* varieties where M5 variety at 400 mg is significantly ($p \geq 0.05$) higher than the other two variety samples. Heat treatment of Morus leaf powder has significantly ($p \geq 0.05$) increased the BARI in all the samples. In case of standards, the BARI of Morus M5, no significant ($p \geq 0.05$) difference was observed between the BARI of standards cholesteramine, diosgenin and xanthan gum.

Serum cholesterol is in part controlled through the enterohepatic circulation of the bile acids. Bile acids are conserved through absorption in the ileum. Serum cholesterol may decrease when there is an increase in the loss of bile acids from the ileum to the cecum and feces (Adiotomre *et al.*, 1990). Dietary fiber and saponins may

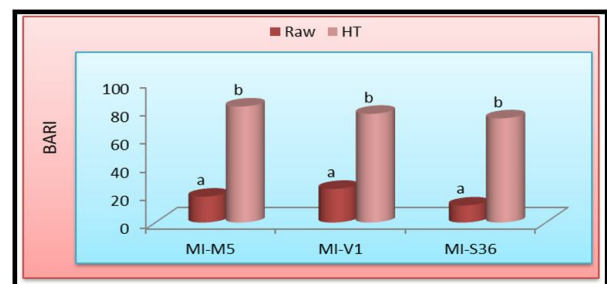
reduce the absorption of bile acids from ileum and this was modeled here in our system mimicking the GI conditions and by a dialysis method.



TF-total fiber (g/100 g); TS-Total saponins (mg/100 g)

Figure 1: Total dietary fiber and saponin content of the MI dehydrated samples.

(n = 3); ($p \leq 0.05$). Values carrying different superscripts a, b, c..... differ significantly

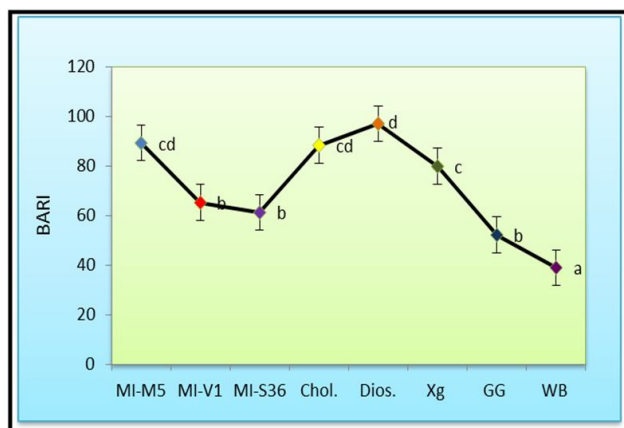
Figure 2: Bile acid retardation index (BARI) of the *M. indica* leaves at different concentrations (mg).

(n = 3); ($p \leq 0.05$). Values carrying different superscripts a, b, c..... differ significantly

Figure 3: Effect of heat treatment on bile acid retardation index (BARI) of *M. indica* leaves.

Bile acid binding capacity of a sample can be attributed to its total fiber content (TDF) and phytochemical composition especially saponin content. In the present study, M5 with high saponin content exhibited significantly higher ($p \geq 0.05$) BARI than V1 and S36. Here, V1 with high fiber content did not exhibit better BARI than MI-M5 which was rich in saponins, compared to V1 and S36. Such diverse results indicate that both TDF and saponins of the sample could be responsible for showing high bile acid by M5. Compared to non-nutritive fibers, bran, cholesteramine, diosgenin (saponin

standard), the BARI of diosgenin was higher than the other samples, followed by MI-M5 and cholestyramine whose BARI was comparable and significantly higher than Xg, MI-V1, MI-S36, GG and wheat bran. Our results are similar to the observations reported by researcher (Kritchevsky, 1974), where bile acid binding capacity of non-nutrient natural fibers was lesser than that of cholestyramine.



(n = 3); ($p \leq 0.05$). Values carrying different superscripts a, b, c.... differ significantly

Figure 4: Comparison of BARI of *M. indica* with the standards.

Many studies have reported the *in vitro* and *in vivo* bile acid binding capacity of different fibers and saponins rich foods such as soybean, lucerne and chickpea (Oakenful, 1986; Oakenfull and Sidhu, 1990). The authors suggest that large mixed micelles formed by the interaction of saponins with the bile acids, account for their increased excretion, resulting accelerated metabolism of cholesterol in the liver by causing low serum cholesterol levels.

However, studies on the *in vitro* bile acid binding capacity of saponins are scarce especially in medicinal plants. Morehouse *et al.* (1999) given the mechanism of action of saponins, which was luminal and did not involve stoichiometric complexation with the cholesterol. Heat treatment results in an increase in particle size due to water absorption and, thereby increase in the surface area for the bile acid binding. Presently, heat treatment significantly increased in all the samples and MI-M5 with high BARI. Similarly, heat treatment of wheat grain also increased bile acid binding significantly compared to uncooked wheat (Kahlon, 2009). In another study, steam cooking of different vegetables, *viz.*, collard greens, mustard greens, kale, cabbage, *etc.*, resulted in an increase in the bile acid binding and also found to be more healthful. The bile acid binding in different vegetables was related to their phytochemicals, hydrophobicity or active bonding sites. As a good source of phytochemicals, bile acid binding capacity of *Morus* can be correlated to their content. These results also indicate the stability of *Morus* to heat treatment, as food is consumed after cooking (Kahlon, 2008). Another possibility suggests bacterial fermentation of soluble fibre of *Morus* in the colon form gases and short chain fatty acids (acetate, butyrate and propionate). The complete absorption of these short chain fatty acids might promote the synthesis of cholesterol by the liver. Finally, brans may accelerate the removal of cholesterol from peripheral circulation by acting as bile-acid binding agent (Francis *et al.*, 2002).

In the present experiment, the method followed may help in developing a new model in understanding the mechanism more clearly especially the stability of *Morus* to different chemicals and extreme pH conditions.

4. Conclusion

Development of additional cholesterol-lowering agents with mechanisms of action distinct from statins and cholestyramine will probably be necessary to achieve cholesterol target levels in many individuals. Concurrently, it is essential to arrive at the dietary dosages of the samples with high fiber and saponin showing potent hypocholesterolemic property. In present study, *Morus* is proved with potent bile acid binding property, further exploring the *M. indica* as functional food by supplementing in disease specific food formulations basically lipid lowering is needed.

Conflict of interest

We declare that we have no conflict of interest.

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