BIOCHEMICAL STUDIES ON THE ANTI-LITHOGENIC EFFECT OF DIETARY FENUGREEK SEEDS (*Trigonella foenum-graecum*)

A thesis submitted to the
UNIVERSITY OF MYSORE
For the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY

by

RAGHUNATHA REDDY R.L

Department of Biochemistry and Nutrition
Central Food Technological Research Institute,
Mysore - 570 020, India
August-2010

Raghunathareddy.R.L
Research scholar
Dept. of B & N,
CFTRI, Mysore-20

DECLARATION

I hereby declare that the thesis entitled "**Biochemical studies on the anti-lithogenic effect of dietary fenugreek seeds (*Trigonella foenum-graecum*)**" submitted to the University of Mysore, Mysore, for the award of degree of **Doctor of Philosophy** is the result of work carried out by me under the guidance of **Dr. K. Srinivasan**, Scientist - G, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore – 570 020, during the period 2005 – 2010.

I further declare that the results have not been previously submitted for any other degree or fellowship.

Place: MYSORE

Date: August, 2010

RAGHUNATHA REDDY R.L

Certificate

This is to certify that the thesis entitled "**Biochemical studies on the anti-lithogenic effect of dietary fenugreek seeds (*Trigonella foenum-graecum*),**" submitted by **Mr. Raghunatha reddy.R.L,** to the University of Mysore, Mysore, for the degree of **Doctor of Philosophy** is the result of work carried out by him under my supervision in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore - 570 020 during 2005 - 2010.

Signature of the candidate

Date:

Signature of the Guide

Date:

Signature of the Chairperson/HOD with seal

To Family

Friends (at GKVK) and

Farmers of this Country…..

Acknowledgements

I would like to express my deep sense of gratitude to my Guide **Dr. K.Srinivasan**, Scientist-G, Department of Biochemistry and Nutrition for his technical support, valuable guidance, constructive criticism, also for allowing me to take up the task independently and with more freedom rendered to me during my research work.

I am very much thankful to **Dr. V.Prakash,** Director, CFTRI for his support to carry out my research work in the institute.

I wish to express my heartfelt thankfulness to **Dr. Kalpana platel**, Scientist, Dept. of B & N for her persistent reinforcement, encouragement through out my stay at CFTRI.

My sincere thanks to **Dr. P.V. Salimath,** Head, Dept. of B & N, **Dr. S.G. Bhat**, former head, **Dr. K. Sambaiah** for their constant support, encouragement, guidance and timely help during my work.

I would like to thank **Dr. Appu Rao,** Head, PCT, who was the gateway for my entry into CFTRI, **Dr. B.R. Lokesh**, HOD, Dept. of LSTF, **Dr. Varadaraj.M.C** Head HRD and **Dr. Uma V.Manjappra**, Scientist, PCT for their valuable suggestions and guidance during my entire research period.

I am really grateful to My GKVK friends (at *222*) viz., **RGR**, **LNR**, **Raghu**, **Kitty**, **Venkat**, **Shankar**, **NR**, **Naidu**, **PG, Venki**, **Nanjareddy**, **Santhu**, **Vijay** who are the driving force for me at all levels of my life especially at decisive time.

I wish to express my sincere thanks to the dept. staff viz., **Dr. Shylaja Dharmesh**, **Dr.Prasada Rao**, **Dr.Baskaran**, **Dr.Akiilender Naidu**, **Dr.Muralikrishna**, **Dr.Muralidhara**, **Dr.Venkatesh**, **Dr.Jaganath Rao**, **Dr. Nandini**, **S.Vishwanatha** and all the former and present staff of the dept. who have helped me directly and indirectly during research period. I would also like to extend my sincere thanks to all the members of animal house.

It's my pleasure to express my sincere thanks to my seniors in the lab and in department; **Vidyashankar**, **Manjunath**, **Hemalatha and Suresh**, other lab seniors include; **Raju**, **Lakshminarayan**, **Chandrashekar**, **Thyags**, **Siddaraju**, **Smitha**, **Anitha**, **Shoba**, **Ani**, **Promod**, **Srinath and Manu** for their timely help during most critical times of research.

My earnest appreciation to all my B&N friends, **Satish, Chandru**, **Ravi**, **Madhu**, **Srikanta**, **Harishbabu**, **Reevanappa**, **Kavitha**, **Jamuna Bhaskar**, **Sangeetha**, **Vasu**, **Daru**, **Malli**, **Ramesh**, **Ashok**, **Kiran**, **Mamatha**, **Rajni**, **Fatima**, **Giri, Kiran**, **Srikanth**, **Manju**, **Lalith**, **and Denny** who were extremely cordial, helping and for their encouragement. Without their help it would have been difficult to execute to work**.**

My earnest thanks to all my labmates, **Chetana Amulya**, **Suresha**, **Shubhra Pande**, **Smitha gautam**, **Bhumika**, **Usha Prakash**, **Supriya**, **Bhavyasree** & **Revathi** from whom I learnt many things needed not only for the research but also for life.

My sincere thanks to all my friends in other departments; **Sreedhar, Lokesh, Santosh, Manju, Shibin, Sharma, Danny, Rangarao, Kisan, Harsha, Mahendra, Mallikarjun, Sriranga, Ramakrishna, Farhan Zameer, Vasanth, Reena, Divya** & **Suga** who have helped me during hard times.

My special thanks to my close friends **Mamatha.A.M, Shubha, Shamala, Kanchan singh, Sowjanya** & **Suma,** for their moral support, affection and encouragement through out my PhD programme.

I would like to express thanks our mess team with whom I had a nice time in mess all these days. Members of mess team include, **Kumaresan**, **Desai**, **Gangadhar**, **Vidya**, **Venkat**, **Raju**, **Surya**, **Banergee** & **Harsha**.

I would like to express my sincere thanks to **Vittal & Shivu** (Health center)**, Girish,** food engineering**, Anbalagan, Vatcharavelu, Shivaswamy,** other members of CIFS, chemical stores for their timely support. I also would like to thank all the staff of *FOSTIS, Google, Pubmed, Science directs* for the digital libraries and making my search uncomplicated and time saving.

My heartfelt gratitude to all members of my family **"***SHREENILAYA***",** especially *AswathaReddy.K* for baring with me all these days and also I would like to memorize my childhood family teacher ***Mr. Narayanaswamy.K.N,*** with out his guidance I would not have travelled this long and I am what today is because of his direction.

I sincerely acknowledge the **CSIR, New Delhi** for the award of **Junior/Senior Research Fellowship**.

(RAGHUNATHA REDDY.R.L)

Biochemical Studies on the Antilithogenic Effects of Dietary Fenugreek Seeds (*Trigonella foenum-Graecum*)

Thesis Abstract

Cholesterol gallstone (CGS) disease ─ one of the major contemporary health problems, is a highly prevalent gastroenteronological disorder resulting from alteration in hepatic and biliary cholesterol homeostasis. The prevalence of this disease is very high in Europe and USA (10-15% of population), lesser in Asia (3-15%) and very low (< 5%) in Africa. The pathophysiology of CGS involves alteration in the delicate equilibrium between the three lipid components of bile, viz., cholesterol, bile acids and phospholipids. Increase in the concentration of cholesterol or decrease in the concentration of phospholipids and bile acids would push the CSI towards crystallization.

Fenugreek seed has been documented to have hypocholesterolemic property, which is mainly attributable to the intrinsic dietary fiber constituent. The other constituents of fenugreek having the hypocholesterolemic property include saponins, diosgenin, trigonelline and 4-hydroxyisoleucine. With the aim of extending our knowledge on the possible anti-lithogenic influence of dietary fenugreek seeds mediated through hypocholesterolemic effect, the present research programme envisaged. Animal studies were carried out to study the effect of dietary fenugreek seeds on the induction of CGS, regression of pre-established CGS, effect on biliary proteins and also its combination with another known hypocholesterolemic spice onion on the incidence and severity of CGS.

The antilithogenic potential of fenugreek seeds was evaluated at different dietary doses both in their raw and heat processed forms during HCD induced CGS formation in mice. CGS was induced were induced by feeding lithogenic diet (0.5% cholesterol and 0.25% bile salts) for 10 weeks. Fenugreek seed powder was included at 5, 10 and 15% of this lithogenic diet. Dietary fenugreek significantly lowered the incidence of CGS; the incidence was 63, 40 and 10% in 5, 10 and 15% fenugreek group respectively, as compared to 100% in lithogenic control. Serum cholesterol level was decreased by 26–31%, hepatic cholesterol was lowered by 47–64%. Biliary cholesterol was reduced to 8.73–11.2 mM with dietary fenugreek from 33.6 mM (HCD) and CSI was reduced to 0.77–0.99 with fenugreek addition as compared to 2.57 in HCD group.

CGS was induced by feeding a HCD for a period of 10 weeks. After the CGS induction, groups of these animals were maintained for further 10 weeks on high cholesterol/ basal control diet/ 6% fenugreek powder / 12% fenugreek powder diets. Dietary fenugreek significantly lowered incidence of CGS, the extent of regression being 61 and 64% in the lower and higher dose groups when compared to 10% regression in basal control group. Serum cholesterol reduced by 35%, hepatic cholesterol decrease by 53-63%, also decreased C: P ratio (0.40 - 0.44 as compared to 0.79 in the basal control group). Biliary C: BA ratio lowered by 67 and 73%. The CSI was 0.90 and 0.42 as compared to 1.86 in the basal control diet group). Activities of liver functioning enzymes in serum increased with HCD feeding and this effect was countered by fenugreek feeding. Fenugreek addition reduced hepatic lipid peroxides, increased antioxidant molecules and activities of hepatic antioxidant enzymes ─ glutathione reductase, glutathione-S-transferase and glutathione peroxidase compared to HCD.

Incorporation of fenugreek into HCD decreased the cholesterol content (70.5%), total protein (58.3%), glycoprotein (27.5%), lipid peroxides (13.6%) and CSI (from 1.98 to 0.75), increased the bile flow rate (19.5%), prolonged the cholesterol NT, reduced the vesicular form of cholesterol (65%), increased smaller vesicular form (94%), increased phospholipid (33%) and total bile acid (49%) in HCD + fenugreek group as compared to HCD group. Electrophoretic separation of LMW proteins showed the presence of high concentration of 28 kDa protein which might be responsible for the prolongation of cholesterol NT in the fenugreek fed groups.

The results of the effect of combination of fenugreek seeds and onion showed that fenugreek and onion individually exerted antilithogenic effect, the effect was higher in the case of fenugreek seeds but the combination was not more than that of fenugreek seeds alone.

The present study has evidenced the antilithogenic potency of fenugreek seeds is attributable to its hypocholesterolemic effect. Fenugreek not only reduced incidence, but also regressed the existing CGS, thus preventing possible recurrence. The anti-lithogenicity of the fenugreek was considered to be due not merely to their ability to lower CSI, but also to their influence on biliary proteins. Among the studied spices fenugreek showed better antilithogenic effect.

SYNOPSIS

Biochemical Studies on the Antilithogenic Effects of Dietary Fenugreek Seeds (*Trigonella Foenum-Graecum*)

Cholesterol gallstone (CGS) disease ─ one of the major contemporary health problems, is a highly prevalent gastroenteronological disorder resulting from alteration in hepatic and biliary cholesterol homeostasis. The prevalence of this disease is very high in Europe and USA (10-15% of population), lesser in Asia (3-15%) and very low (< 5%) in Africa. The pathophysiology of CGS involves alteration in the delicate equilibrium between the three lipid components of bile, viz., cholesterol, bile acids and phospholipids. Increase in the concentration of cholesterol or decrease in the concentration of phospholipids and bile acids would push the CSI towards crystallization.

Among spices, turmeric, red pepper, fenugreek, garlic and onion are well documented for their hypolipidemic potential in various experimental animal models. The beneficial hypocholesterolemic property of the spices – turmeric and red pepper are attributable to their active principles ─ curcumin and capsaicin respectively. Curcumin and capsaicin, and spices fenugreek and onion have also been shown to be cholagoguic agents. It is believed that formation of CGS in the gall bladder is preceded by a supersaturation of bile with cholesterol. Hence, lowering of cholesterol concentration in the bile could prevent its supersaturation. A cholesterol lowering agent such as spices may therefore be able to reduce the incidence of CGS.

Earlier studies in our laboratory on experimental induction of CGS in mice by feeding a HCD have revealed that the incidence of gallstones is 25-65% lower when the animals were maintained on 0.5% curcumin / 0.015% capsaicin / 0.6% garlic powder / 2.0% onion powder containing diet. Biliary cholesterol concentration was significantly reduced and the CSI was considerably reduced in spice supplemented groups. Animal studies have also revealed significant regression of preformed CGS by these spices. The anti-lithogenicity of these spices was considered to be due not merely to their ability to lower CSI, but also to their influence on biliary proteins.

Fenugreek seed is another spice documented to have hypocholesterolemic property, which is mainly attributable to the intrinsic dietary fibre constituent. Fenugreek seed is another spice documented to have hypocholesterolemic property, which is mainly attributable to the intrinsic dietary fiber constituent. The other constituents of fenugreek having the hypocholesterolemic property include saponins, diosgenin, trigonelline and 4-hydroxyisoleucine. With the aim of extending our knowledge on the possible anti-lithogenic influence of dietary fenugreek seeds mediated through hypocholesterolemic effect, the present research programme envisaged:

1. Evaluation of the beneficial effect of dietary fenugreek seeds on Cholesterol gallstone (CGS) with respect to:

   a) Influence of dietary fenugreek seeds on the incidence and severity of CGS
   b) Influence of dietary fenugreek seeds on regression of pre-established CGS

2. Studies on the mechanism of anti-lithogenic effect of fenugreek seeds involving the cholesterol nucleating factors such as biliary proteins making use of *in-vitro* models of cholesterol saturated bile.

3. Examination of the synergistic antilithogenic influence of a combination of fenugreek seeds and another known hypocholesterolemic spice onion.

The thesis is presented in **five chapters** as follows:

Chapter-I: General Introduction

This encompasses the following: Epidemiology of CGS, Pathogenesis of CGS, Bile (Composition, importance, functions, and biliary disorders), Cholesterol homeostasis, CGS, Role of diet in the prevention of CGS and Scope of present investigation.

Chapter-II: Reduction of atherogenic diet induced cholesterol gallstone formation in mice by dietary fenugreek (*Trigonella foenum-graecum*) seeds

With the aim of extending the knowledge on possible anti-lithogenic influence of dietary fenugreek seeds mediated through its hypocholesterolemic effect. In this context, the antilithogenic potential of fenugreek seeds was evaluated at different dietary doses both in their raw and heat processed forms in laboratory mice. CGS was induced in groups of mice by maintaining on a HCD (0.5% cholesterol and 0.25% bile salts) for 10 weeks.

Fenugreek seed powder was included at 5, 10 and 15% of this lithogenic diet. Dietary fenugreek significantly lowered the incidence of CGS; the incidence was 63, 40 and 10% in 5, 10 and 15% fenugreek group respectively, as compared to 100% in lithogenic control. The antilithogenic influence of fenugreek is attributable to its hypocholesterolemic effect. Serum cholesterol level was decreased by 26–31% by dietary fenugreek, while hepatic cholesterol was lowered by 47–64% in these HCD animals. Biliary cholesterol was 8.73–11.2 mM as a result of dietary fenugreek, as compared to 33.6 mM in high cholesterol feeding without fenugreek. CSI in bile was reduced to 0.77–0.99 in fenugreek treatments as compared to 2.57 in HCD group. Thus, fenugreek seeds offer the health beneficial antilithogenic potential by virtue of its beneficial influences on cholesterol metabolism.

Chapter-III: Regression of preestablished cholesterol gallstones in experimental mice by dietary fenugreek seed (*Trigonella foenum-graecum*)

The regression of pre-existing gallstones is a very important aspect, and in the absence of much information on the dietary strategies to achieve regression of preexisting CGS, there is a need to explore hypocholesterolemic dietary components for this potential. An exhaustive animal study was carried out to evaluate the beneficial influence of dietary fenugreek seeds in terms of regression of pre-established CGS. CGS was induced by feeding a HCD for a period of 10 weeks. After the CGS induction, groups of these animals were maintained for further 10 weeks on high cholesterol/ basal control diet/ 6% fenugreek powder / 12% fenugreek powder diets. Incidence of CGS and its severity were evaluated at the end of this feeding regimen. The incidence of CGS was significantly lowered as a result of dietary fenugreek seeds, the extent of regression being 61 and 64% in the lower and higher dose groups when compared to 10% regression in basal control group. The antilithogenic influence of dietary fenugreek was accompanied by significant reductions in serum cholesterol concentration which was more than 35%. Hepatic cholesterol concentration was also profoundly lowered by dietary fenugreek, the decrease being 53-63% compared to basal control diet. Biliary cholesterol concentration was significantly lower as a result of dietary fenugreek during post-CGS induction period resulting in decreased C: P ratio (0.44 and 0.40 as compared to 0.79 in the basal control group). Biliary C: BA ratio was lowered upon feeding fenugreek (by 67 and 73%) much more than in the

basal control group. The CSI in the bile was also beneficially lowered by fenugreek treatment during post-CGS induction period (which was 0.90 and 0.42 as compared to 1.86 in the basal control diet group). The present study has evidenced the potency of hypolipidemic fenugreek seeds in regressing the pre-established CGS and this beneficial antilithogenic influence is attributable to its primary influence on cholesterol levels. This finding is significant in the context of evolving a dietary strategy to address CGS, which could help in the prevention of incidence, regression of existing CGS and preventing possible recurrence.

This chapter also presents data on hepatoprotective and antioxidant effect of fenugreek seeds in mice under lithogenic condition. Activities of serum aspartate aminotrasferase, alanine aminotransferase, lactate dehydrogenase and alkaline phosphatase increased with prolonged feeding of HCD. Activities of these enzymes were lower in animals fed basal control/ fenugreek containing diets after initial exposure to HCD, and were prominent in fenugreek groups. Hepatic lipid peroxides decreased and antioxidant molecules increased in fenugreek fed groups. Activities of hepatic antioxidant enzymes – glutathione reductase, glutathione-S-transferase and glutathione peroxidase were higher in fenugreek treatment. These results suggested hepatoprotective and antioxidant potential of fenugreek seeds under conditions of lithogenicity.

Chapter-IV: Effect of dietary fenugreek seeds on biliary proteins which influence nucleation of cholesterol crystals in bile

Dietary fenugreek seed was evidenced to possess antilithogenic potential in mice both in terms of preventing the experimental induction of CGS and also regressing the pre-established CGS. The antilithogenic influence of fenugreek seeds was attributable to their cholesterol-lowering effect in blood and liver, and the ability to lower cholesterol saturation index by altering the biliary lipid composition, and in otherwords biliary cholesterol homeostasis. Formation of CGS in gallbladder is controlled by procrystallizing and anticrystallizing factors present in bile. Apart from beneficial modulation of biliary CSI, fenugreek may also influence cholesterol nucleating and antinucleating proteins that contribute to their antilithogenic potential. In view of this, the influence of dietary

fenugreek on biliary proteins and glycoproteins in particular, was evaluated in an animal study, the results of which are presented in this chapter. In order to understand the mechanism of cholesterol crystal nucleation and the probable effect of proteins present in biles of rats fed fenugreek, they were tested with supersaturated model biles for cholesterol nucleation. An animal experiment was carried out to evaluate the effect of dietary fenugreek on the compositional changes in the bile, particularly effect on glycoproteins, low molecular weight (LMW) and high molecular weight (HMW) proteins, cholesterol nucleation time, and cholesterol crystal growth. Groups of Wistar rats were fed for 10 weeks with diets: (1) Basal control, (2) Basal control + Fenugreek (12%), (3) High cholesterol diet (HCD), and (4) HCD + Fenugreek (12%). Incorporation of fenugreek into HCD decreased the cholesterol content (70.5%), total protein (58.3%), glycoprotein (27.5%), lipid peroxides (13.6%) and CSI (from 1.98 to 0.75), increased the bile flow rate (19.5%), prolonged the cholesterol nucleation time, reduced the vesicular form of cholesterol (65%) accompanied with an increase in the smaller vesicular form (94%), improvement in contents of phospholipid (33%) and total bile acid (49%) in HCD + fenugreek group as compared to HCD group. Electrophoretic separation of LMW proteins showed the presence of high concentration of 28 kDa protein which might be responsible for the prolongation of cholesterol nucleation time in the fenugreek fed groups. These findings indicate that the beneficial anti-lithogenic effect of fenugreek which is primarily by reducing the cholesterol content in the bile is also affected through a modulation of the nucleating and anti-nucleating proteins which in turn affect the cholesterol crystallization.

Chapter-V: Study on the antilithogenic influence of a combination of fenugreek seeds and onion

This chapter presents results of an animal study where the possible additive / synergistic antilithogenic effect of a combination of fenugreek seeds and onion was examined during experimental induction of CGS in mice. While fenugreek and onion individually exerted antilithogenic effect, the effect was higher in the case of fenugreek seeds. The antilithogenic effect of the combination was not more than that of fenugreek seeds alone.

CONTENTS **PAGE NO.**

List of tables

Chapter - V

List of figures

Chapter -IV

Chapter -V

List of Abbreviations

%	: Percent
µg	: Microgram
µg/ml	: Microgram/milliliter
µl	: Microlitre
3α HSD	: 3α-hydroxysteroid dehyderogenase
ABC	: ATP binding cassette
ADP	: Adenosine diphosphate
AIN	: American institute of nutrition
ALT	: Alanine amino transferase
Apo-AI	: Apolipoprotein AI
Apo-AII	: Apolipoprotein AII
AST	: Aspartate amino transferase
BSA	: Bovine serum albumin
BSEP	: Bile salt export pump
C/BA	: Cholesterol: bile acids ratio
C/PL	: Cholesterol: phospholipid ratio
CBB	: Commassie brilliant blue
CCK	: Cholecystokinin
CDNB	: Chlorodinitrobenzene
CGS	: Cholesterol gallstone
CHD	: Coronary heart disease
cm	: Centimeter
CoA	: Coenzyme-A
Con-A	: Concanavalin-A
CSI	: Cholesterol saturation index
CVD	: Cardio vascular disease
CYP27A1	: Sterol 27-hydroxylase
CYP7A1	: Cholesterol 7α-hydroxylase
dL	: Decilitre
DNPH	: 2, 4-dinitrophenylhydrazine
DPPC	: Dipalmitoyl phosphatidyl choline
DTNB	: Dithionitrobenzoic acid
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra-acetic acid
EGF	: Epidermal growth factor
Fig	: Figure
FXR	: Farnesoid X receptor
g	: Gram
g/dL	: Gram by deciliter
g/Kg	: Gram by kilogram
GSH	: Glutathione reductase
GSSG	: Glutathione oxidase
H	: Hour
H_20_2	: hydrogen peroxide

HCD	: High cholesterol diet
HDL	: High density lipoprotein
HMG-CoA	: 3-hydroxy-methyl-glutaryl coenzyme-A
HMW	: High molecular weight
HPLC	: High Performance liquid chromatography
I_c	: Crystal index
Ig A	: Immunoglobulin-A
Ig M	: Immunoglobulin-M
I_g	: Growth index
IR	: Insulin resistance
I_t	: Time index
IU	: International unit
Kb	: Kilobase
KD	: Kilo daltons
Kg	: Kilogram
L	: Litre
LDH	: Lactate dehydrogenase
LDL	: Low density lipoprotein
LDLR	: Low density lipoprotein receptor
LG	: Lithogenic
LMW	: Low molecular weight
LXR	: Liver X receptors
M	: Micelles
M	: Molar
MDA	: Malondialdehyde
mg	: Milligram
Min	: Minute
mL	: Millilitre
ml/min	: Millilitre by minute
mm	: millimeter
mM	: milli Molar
MW	: Molecular weight
N	: Normal
NAD	: Nicotinamide adenine dinucleotide
NADP	: α- Nicotinamide adenine dinucleotide phosphate
NADPH	: Reduced α- Nicotinamide adenine dinucleotide phosphate
NaN_3	: Sodium azide
ND	: Not detected
ng	: Nanogram
nm	: Nanometer
NT	: Nucleation time
OD	: Optical density
PAGE	: Polyacrylamide gel electrophoresis
PE-10	: Polyethylene tubing-10
PPARγ	: Peroxisome proliferator-activated receptor gamma
PUFA	: Polyunsaturated fatty acids

ROS	: Reactive oxygen species
rpm	: Revolutions per minute
RT-PCR	: Reverse Transcriptase Polymerase Chain Reaction
SD	: Standard deviation
SDS	: Sodium dodecyl sulphate
SEM	: Scanning Electron Microscopy/ Standard error mean
SOD	: Superoxide dismutase
SREBP	: Sterol regulatory element binding protein
STDC	: Sodium tauro deoxycholate
S-V	: Small vesicles
TAE	: Tris-acetate-EDTA
Taq	: Thermus aquaticus
TBA	: Thiobarbituric acid
TBARS	: Thiobarbituric acid reactive substances
TBE	: Tris-Borate-EDTA
TBS	: Tris-buffer saline
TCA	: Trichloro acetic acid
TCDA	: Taurochenodeoxy cholic acid
TEMED	: NNNN-tetramethyl ethylenediamine
TMP	: Tetra methoxypropane
TNF	: Tumor necrosis factor
Tris	: Tris (hydroxymethyl) amino methane
U	: Unit enzyme
UV	: Ultraviolet
V/V	: Volume by volume
W/V	: Weight by volume
W/W	: Weight by weight
Wt.	: Weight
α	: Alpha
β	: Beta

1.0 CGS disease

Cholesterol gallstone (CGS) disease is one of the most common gastrointestinal diseases. The etiology is considered as failure of cholesterol homeostasis and is the consequence of final stage of supersaturation of cholesterol in bile. By definition, CGS were abnormal mass of solid mixture of cholesterol crystals, mucin, calcium bilirubinate and proteins, which contains at least 70% of cholesterol by weight. There are three different types of gallstones, depending on their major constituents; CGSs, pigment gallstones and mixed gallstones. About 85-90% of gallstones are CGS, which is common in western countries, while the other two constitutes 10-15%. Both pigment and mixed gallstones are more frequent in Asia and Africa, where more chances of infection caused by prevailing unhygienic conditions (Portincasa *et al.,* 2006). Different classes of CGS and their characteristics are given in Table-1. The size of gallstones varies depending on the species and the complex involved. Size varies from as small as a sand particle to as large as a golf ball, where texturally they resemble mulberry fruit. Majority of the subjects experience no symptoms and only in 1-2% of these stones are symptomatic. Some of the problems are: obstruction of bile duct caused by lodging the stone, hindrance to free flow of bile, severe abdominal pain, inflammation and infection of biliary tact, which is some times fatal.

1.1 Epidemiology

Data on the epidemiology of the disease shows that this disease is common in developed countries, representing major health burden. The prevalence is as high as 10-15% in western population including US, where more than 1.0 million new cases are being diagnosed annually in US alone (Van Erpecum, 2004). It is the leading cause of the gastrointestinal related problems. Data on the prevalence of CGS for each country is not yet established, but data available in relation to the occurrence of CGS is given in Table-2**.** The highest prevalence is in the Pima Indians from Arizona, US and the prevalence rate in Hispanics were higher than those reported in Europe. The estimated growth of gallstones was found to be 2 mm per year, thus it takes few years to attain symptomatic CGS (Cuevas *et al.,* 2004).

Table 1. Different classes of gallstones and their characteristics

Type of gallstone	CGS	Pigment stones	
		Brown	**Black**
Prevalence (%)	85-90	10-15	≤5
Main composition	50-90% Cholesterol	≈ 50% bilirubin	≥50% bilirubin
Color	Yellow to gray	Brown	Dark brown-black
Etiology	Cholesterol supersaturation	Increased deconjugation of bilirubin	Increased biliary bilirubin load
Location	Gallbladder	Bile duct	Gallbladder
Prevalence	US and Europe	Asia and Africa	Asia and Africa

(**Source**: Grunhage & Lammert 2006; Marshall & Einarsson, 2007)

Table 2. Prevalence of gallstones

City / Country	Prevalence (%)		
	Male	**Female**	**Average**
North America			
US Hispanics	5.4	19.1	13.3
Starr County, Texas	8.0	20.2	17.9
American Indians-13 tribes	29.5	64.1	-
Mexican Americans	8.9	26.7	-
Non-Hispanic whites	8.6	16.6	-
Non-Hispanic blacks	5.3	13.9	-
South America			
Santiago, Chile	14.5	37.4	28.5
Pampas de San Juan, Peru	16.1	10.7	14.3
Europe			
Sirmione, Italy	6.7	14.4	10.9
Copenhagen, Denmark	5.6	11.0	8.8
Bergen, Norway	20.3	23.3	21.9
Schwedt, Germany	13.1	24.5	19.7
Timisoara, Romania	6.1	12.8	10.9
Stockholm, Sweden	11.0	18.0	15.0
Bristol, England	6.8	8.0	7.5
Poland	8.2	18.0	-
Vidauban, France	12.5	17.8	15.7
Guadalajara, Spain	7.8	11.5	9.7
Asia			
Okinawa, Japan	2.4	4.0	3.2
Srinagar, Kashmir, India	3.1	9.6	6.1
Chandigarh, India	6.2*	21.6*	15.6*
Taipei, Taiwan	10.7	11.5	10.7
Jiaotong, China	2.3	4.7	3.6
Chiang Mai, Thailand	2.5	3.7	3.1
Africa			
Khartoum, Sudan	5.6	5.1	5.2

(**Source**: Acalovschi, 2001; Shaffer, 2006)

1.2 Pathogenesis

CGSs are the result of abnormalities in the cholesterol metabolism, caused by various factors. There are three major abnormalities which are responsible for the formation of CGS (Portincasa *et al.,* 2006). They are:

1. Supersaturation of bile with cholesterol, with no change or reduction in the concentration of other lipids i.e. phospholipids and bile salts (Fig.1).

2. Enhanced cholesterol crystal nucleation; when pronucleating factors overrule antinucleating factors.

3. Gallbladder hypomotility.

Of the three abnormalities, the primary and essential one is the supersaturation of bile with cholesterol. In the absence of sufficient phospholipids and bile salts to keep cholesterol in soluble form favors crystallization. The percentage of cholesterol supersaturation in bile is determined by the molar ratio of the three major lipid constituents of bile, *i.e.*, cholesterol, phospholipids and bile acids. It is caused by excessive cholesterol biosynthesis, excessive absorption of the dietary cholesterol and also by reduced acyl-CoA cholesterol acyltransferase (ACAT) activity inhibiting cholesterol esterification leading to excretion of more free cholesterol into the bile. Feeding of cholesterol-rich diet leads to accumulation of high levels of cholesterol in the body including bile. This activates the feedback mechanism, *i.e.*, reduced hepatic cholesterol synthesis by decreasing the activity of HMG-CoA reductase and also by activation of the cholesterol 7α-hydroxylase (CYP7A1), which enhances the conversion of cholesterol to bile acids. Uninterrupted feeding of high levels of cholesterol some times fail to activate this mechanism, thus affecting cholesterol homeostasis, which leads to the accumulation of more cholesterol not only in blood and liver but also in bile as well. Thus, lipid composition in general and cholesterol content of bile in particular is very important in the pathogenesis of CGS.

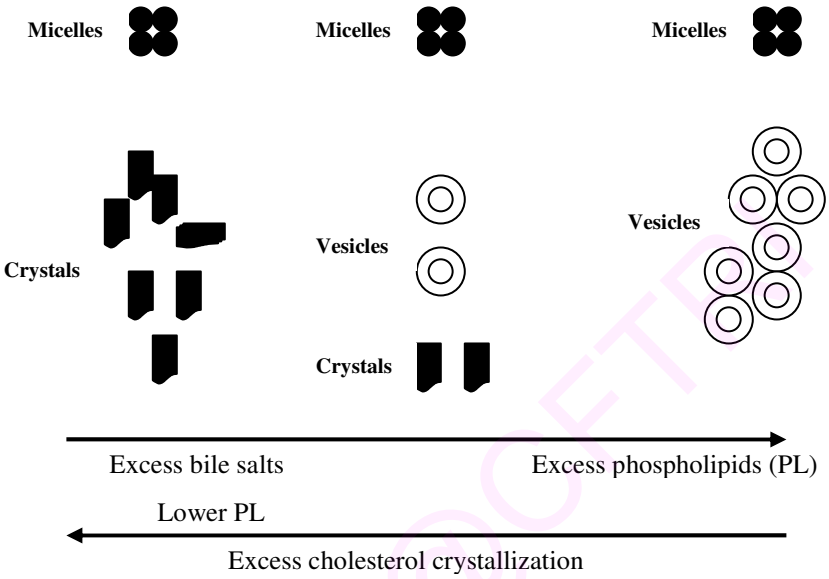


Fig.1. Relationship between bile salts, phospholipids and cholesterol crystallization in the bile

(**Source**: Van Erpecum, 2004).

1.3 Stages of CGS formation

The process of formation of CGS is more complex and it has been broadly classified into three stages,

A. Supersaturation: Supersaturation is the point where the cholesterol solubility is in a weak position and above this point cholesterol starts to accumulate rather than solublizing in bile. Supersaturation of bile with cholesterol is the primary and leading aspect of CGS formation. There are many mechanisms responsible for this and all these mechanisms or processes mainly affect the cholesterol homeostasis. It is caused by the uncontrolled and excessive secretion of cholesterol into bile and also by reduced secretion of phospholipids and bile salts. Thus, once the cholesterol carrying capacity of bile is reduced, the cholesterol will tend to accumulate in the form of crystals.

B. Accelerated crystallization: It is experimentally confirmed that all CGS patients have supersaturated bile but all supersaturated bile does not have CGS, *i.e*. supersaturated bile is a prerequisite for CGS but does not guarantee stone formation. Only 10% of the 50% population having supersaturated bile forms gallstones. Thus one has a doubt, why, how and what made others not to form stones even with supersaturated bile. An inhibitor type of proteins may help to explain this cause (Holzbach, 1995). Many factors which influence CGS formation influence the events of crystallization. Any change in these events, namely, vesicle maturation, nucleation, crystal growth, aggregation, fusion or agglomerisation of the formed crystals will have a direct bearing on cholesterol crystallization.

C. Stone formation from the crystals: The third and the final stage in the process of CGS formation is the amalgamation of formed crystals. Earlier in 1960's and 1970's sludge was used to refer necrotic collagen that obstructed bile ducts, but now sludge is used in this way is a construction of echogenic gallbladder sludge, material detectable in the gallbladder on ultrasonography. Sludge must be distinguished from sediment, as it refers to the solid material detectable by microscopy. Sludge is usually composed of bilirubinate, microcrystals and mucus with cholesterol crystals some times. Factors like fasting allow bile to stay for longer period, giving sufficient time to settle and favour acceleration of

cholesterol crystallization mechanism to take place. Sludge is a precursor for CGS and bilirubinate stones. Sludge is uncommon ultra sound finding; on the other hand pathogenic sediment always precedes stone formation.

The bile secreted is concentrated, stored and slightly acidified in gallbladder during intra digestive interval. Alterations in the relative or absolute proportions of lipid components of bile can lead to phase separation of cholesterol. With the advancement in recent years, the key to stone formation are very clear: supersaturation of bile with cholesterol, crystallization and finally stone formation with the contribution of many nucleating factors. Schematic diagram representing the pathogenesis of CGS formation is given in Fig.2.

1.4 Symptoms of CGS

CGS disease is asymptomatic and is formed through many years and once the stone reaches considerable size, symptoms become apparent. The average risk of developing symptoms for a symptomatic gallstone patient is as low as 2-5% per year, thus making it very difficult to diagnose with the symptoms, but some symptoms associated with this disease are persistent and intense pain in the upper abdominal region, associated with nausea, vomiting, fever and jaundice. Often, these occur after a heavy fatty meal and most of the times happen at night. Other associated symptoms include abdominal bloating, intolerance to fatty foods and indigestion.

1.5 Diagnosis of CGS

CGS disease is asymptomatic in majority of the cases and it takes several years to develop symptoms. The correct diagnostics of symptomatic subjects is very much essential and plays a vital role in the treatment of the disease. Because of its fundamental characteristics of slow growth, it is very difficult to know the initiation of the disease. The diagnosis of the disease involves:

a) *Laboratory tests:* This includes estimation of activity of enzymes such as alkaline phosphatase and bilirubin which are usually elevated in disease conditions.

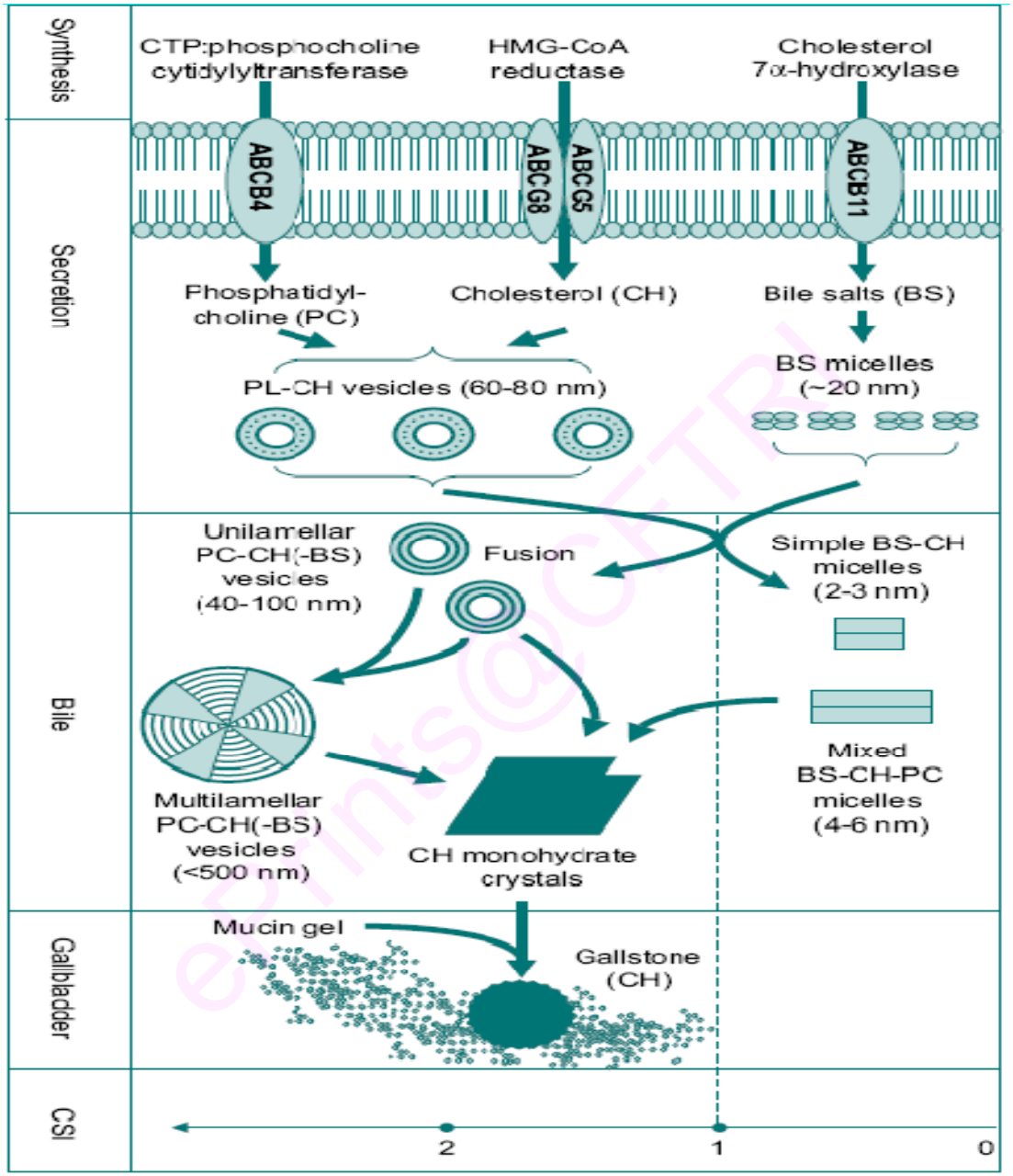


Fig.2. Schematic diagram representing the pathogenesis of cholesterol gallstone formation

(**Source**: Grunhage & Lammert, 2006)

b) *Imaging techniques:* This method of diagnosis helps to diagnose the disease correctly. Of the various imaging techniques available ultrasonography is most accurate and widely accepted standard method of diagnosis. This also helps to know the functioning of gallbladder especially with respect to its motility which is usually impaired in CGS patients.

1.6 Treatment of CGS

The next most important aspect after correct diagnosis is the proper treatment. Treatment varies with complexity and the stage of disease. There are two methods of CGS treatment.

i) Surgical removal of CGS: The first treatment ever made is the surgical removal of affected gallbladder and this technique has remained as the standard therapy for symptomatic gallstones for a long period (Portincasa *et al.,* 2006). Removal of gallbladder guarantees that the patient will no longer suffer and no chance of recurrence of gallstones. But removal of gallbladder is associated with various other problems like poor fat digestion, feeling fullness, uneasiness after heavy fat meal, *etc*. So, gallbladder removal should be the last option and it is removed only when there is a threat to life.

ii) Non-surgical removal of CGS: This method involves the administration of intravenous fluids, pain killers and antibiotics. In the case of gallstone associated pain but not infection, laparoscopy and lithotripsy are performed. Drug therapy is preferred for patients who are not willing to undergo surgery. CGS can be dissolved by the oral administration of bile acids like ursodeoxycholic acid. The main problem associated with this treatment is the recurrence once the treatment is stopped and around 25-30% of recurrence has been reported after five years of the treatment termination (Bellows *et al.,* 2005; Portincasa *et al.,* 2006).

1.7 Factors which influence CGS formation

CGS disease is a very complex disease, the etiology of which is considered as multifactorial. Factors which affect the process of CGS formation are broadly classified into two groups, *viz*.,

1) Genetic / unmodifiable / independent factors
2) Environmental / modifiable / dependent factors / man made factors.

Some of the factors which influence CGS are:

1.7.1 Age: CGS incidence increases with age, especially above 40years. There is a 4-10 times higher incidence of CGS in the older subjects than young. This is due to the decreased production of bile acids caused by reduction in the activity of CYP7A1, which favour biliary cholesterol saturation (Acalovschi, 2001).

1.7.2. Ethnicity/race: Prevalence of gallstone disease is very high in some ethnic groups: 73% of female Pima Indians aged 25 years and older have CGS. In South America, a high prevalence of gallstones (35.2%) is present in Chilean Mapuche Indians, who migrated from Asia (Portincasa *et al.,* 2006).

1.7.3. Gender and Estrogen: Women are more prone to CGS compared to men. Sex hormones, use of oral contraceptives, pregnancy favour gallstone formation through hormonal influence on the bile composition. Estrogen induces increased hepatic free cholesterol pool by up-regulating the LDL receptor (LDLR). Decrease in the gallbladder motility during pregnancy favour nucleation and growth of stones. Post-menopausal women on estrogen replacement therapy have a higher risk (Shaffer, 2006; Acalovschi, 2001).

1.7.4. Diabetes/Triglycerides/Insulin/Obesity: All these factors are interlinked and people who have high triglycerides are prone to obesity and those who are obese are very much prone to diabetes. People who have type 2 diabetes or insulin resistance (IR) have the tendency to synthesize more cholesterol which increase the CSI of bile. Thus all these

are major risk factors affecting CGS and this is more prevalent in women and increases with age. IR is characterized by hyperinsulinemia with progressive tendency to hyperglycemia, hypertriglyceridemia and type 2 diabetes mellitus. Epidemiological studies indicate that IR predisposes to subsequent CGS, a link that supports the hypothesis that insulin plays a significant role in the regulation of cholesterol metabolism and enterohepatic circulation of biliary lipids (Biddinger *et al.,* 2008). Dubrac *et al.,* (2001) have reported that insulin increases CSI of bile mainly by decreasing the concentration of biliary anti-nucleating proteins like, apo-A1, which also modulates major enzymes of cholesterol and biliary metabolism thus enhancing CGS incidence. Insulin is known to stimulate hepatic lipid synthesis by selectively up-regulating the SREBP-1 expression. PPARγ gene is also a potential candidate involved in the pathogenesis of CGS since this nuclear transcriptional factor regulates the expression of multiple genes involved in lipid metabolism and is associated with hyperlipidemia, obesity, IR or type 2 diabetes (Cuevas *et al.,* 2004).

1.7.5. Rapid weight loss: During this period body fat is mobilized, metabolized rapidly and this leads to higher secretion of extra cholesterol into bile by the liver which favours CGS. In this way up to 50% of population who undergo rapid weight loss will have CGS. Some of the CGS formed by this process will dissolve once the weight is regained in long run but not in all cases. Weight loss pattern is also associated with CGS and >1.5 kg weight loss per week has higher rate of CGS incidence (Bellows *et al.,* 2005; Shaffer, 2006). The increased risk associated with rapid weight loss may be due to an increase in the ratio of cholesterol to bile salts in the gallbladder and bile stasis resulting from decreased contraction of gallbladder (Gaby, 2009).

1.7.6. Fasting/parental nutrition: Both these conditions decrease the motility of gallbladder, which gives time to settle cholesterol, favouring CGS formation.

1.7.7. Crohn's disease: Patients who underwent total colectemy have cholesterol saturated bile. Impaired bile acid enterohepatic circulation and metabolism are affected, so that there will not be sufficient bile acids to keep cholesterol in soluble form causing crystal formation and favoring CGS formation.

1.7.8. Clinical factors: Some clinical factors make patients more vulnerable to gallstones. Thirty percent of individuals with cirrhosis have CGS. Resertion/surgical removal of ileum leads to limited absorption of bile acids leading to more chances of CGS formation (Portincasa *et al.,* 2006).

1.7.9. Cholesterol lowering drugs: Some of the cholesterol lowering drugs like, cholestyramine, clofibrate, ceftriaxone, octreotide and oestrogens act mainly by preventing the reabsorption of bile acids. It is also reported that many of these drugs remove cholesterol from blood and at the same time they increase the cholesterol content of the bile.

1.7.10. Smoking: It is reported that smoking has a protective effect and is due to the decreased prostaglandin synthesis and mucus production, which are causative factors of CGS (Halldestam *et al.,* 2009). However, Acalovschi (2001) has reported that smoking is associated with low plasma HDL cholesterol, a risk factor for CGS.

1.7.11. Cholecystokinin (CCK): Thompson *et al.,* (1982) reported that reduced CCK release and more sensitivity of gallbladder to CCK in patients with gallstones, humoral motility abnormality is also evident; but it was not possible to determine whether CGS is the result of this abnormality. Van Erpecum *et al.,* (2006) reported that increased gallbladder wall thickness with the increased duration of feeding a lithogenic diet. In contrast, mice resistant to CGS displayed no or mild increase in gallbladder thickness. It is also suggested that gallbladder undergoes various changes that ultimately result in reduced motility, increased edema and altered function of gallbladder and affect gallbladder motility (Grunhage & Lammert, 2006).

1.7.12. Role of infection, inflammation and immune system: Maurer *et al.,* (2009) have reported that genetically identical mouse models fed with high cholesterol/lithogenic diet show differences in their susceptibility to form CGS. They suggested that differences in their colonization status with gastrointestinal microbes and also said that some enterohepatic *Helicobacter Spp.* (*H. pylori*) contribute to lithogenicity. There are some contradicting reports with respect to the *H. pylori* infection and also not very clear that

those *H. pylori* is the cause or the consequence of the CGS. Since growth of this organism is inhibited by bile salts both *in vitro* and *in vivo* and chemotactic assay reported that bile salts actually repel the organism. Never the less many identified *H. pylori* DNA in bile and gallstones and also mentioned that gallbladder bile of patients with chronic cholecystitis with consistent obstruction has *H. pylori* DNA but not in symptomatic patients. It was also mentioned that *H. pylori* influence may be partially due to their ability to promote inflammation and also precipitate calcium salts. It was reported that response of the adaptive immune system is essential, since Rag-deficient mice, which lack mature T and B cells, rarely (< 10%) develop CGS and this is one of the contributing factors for lithogenicity. Recently it is reported that mice having only T cells has a greater frequency of CGS development while only B cells do not recapitulate the prevalence of CGS. Critical contributions of the gallbladder epithelium and immune system are known to modulate CGS. Most of the immunological factors describe prolithogenic alterations in liver, gallbladder and bile; without these inflammations it would not be sufficient for the formation of CGS. Biliary epithelial cells composed of all the necessary cellular components which are required to participate in the immune system (innate) which appear to predispose to biliary infection.

1.7.13. Immunoglobulins: *In vitro* studies with model bile have shown that Ig's, particularly IgM and IgG, promote the nucleation of cholesterol. The source of Ig's are important, i.e. IgM and IgA from commercial source are incapable, while the commercial IgG has pronucleating activity. The anatomical source Ig's causes crystal nucleation more specifically, Ig's of bile produce more influence than the Ig's of blood. The order of influence of Ig's on nucleation is: IgA < IgG < IgM (Maurer *et al.,* 2009). Van Erpecum *et al.,* (2006) have however reported that Ig's have no role to play in CGS formation.

1.7.14. Mucin glycoprotein: One of the most important and thoroughly studied factors affecting the pathogenesis of CGS is the biliary mucin glycoprotein. The major mucin genes which are expressed in biliary epithelia include MUC_1, MUC_2, MUC_3, MUC_5AC, MUC_5B, and MUC_6. Mucin glycoproteins have proven to affect CGS formation both in humans as well as in animal models and its upsurge precedes crystallization. Studies have

shown that mucin accumulation and their gene expression increased following exogenous addition of lipopolysaccharides and TNF-α in cell culture system. The exposure of lipopolysaccharides and TNF-c expressed MUC_2 and MUC_5AC genes via protein kinase C stimulation, which signals increased activation of the two genes (Maurer *et al.,* 2009). Finzi *et al.,* (2006) reported that TNF-α regulate the expression of MUC_5AC via epidermal growth factor (EGF) mediated pathway. The EGF binds to EGF-receptor and this interaction favour MUC_5AC expression. Thus these findings indicate the production of glycoproteins is influenced by inflammatory mediators (Maurer *et al.,* 2009).

1.7.15. Chronic inflammatory condition: Chronic hepatic C virus (HCV) infection is associated with gallstone formation and chronic hepatic damage might be responsible for this effect, leading to deranged liver function and cirrhosis. Some studies indicated that HCV infection is independent of cirrhosis but increased the CGS prevalence. HCV influences on CGS depend on its replication in the biliary epithelia causing inflammation and altered gallbladder motility (Maurer *et al.,* 2009).

1.7.16. Intestinal transit: Xu *et al.,* (1996) reported that cholesterol feeding increases CSI, deoxycholic acid fraction, with unchanged total bile acid pool, reduces smooth muscle contractility, prolonged small intestinal transit time, with reduced gallbladder contractility and sluggish recycling of bile salt favour CGS formation. Prolonged transit has been equated with higher production of deoxycholic acid. Fan *et al.,* (2007) studied the influence of intestinal transit time on CGS formation and observed that there was a possible relationship between dysfunction of intestinal transit time, gall bladder motility and formation of CGS. There was also a close relationship between intestinal migrating motor complex (MMC) and gallbladder motility (Portincasa *et al.,* 2006). Gall bladder dysfunction and increased concentration of bile favour crystal and stone formation. HCD feeding facilitates CGS formation by significant hindering of intestinal transit. Fig.3 shows the role of liver, gallbladder and intestine on the formation of CGS.

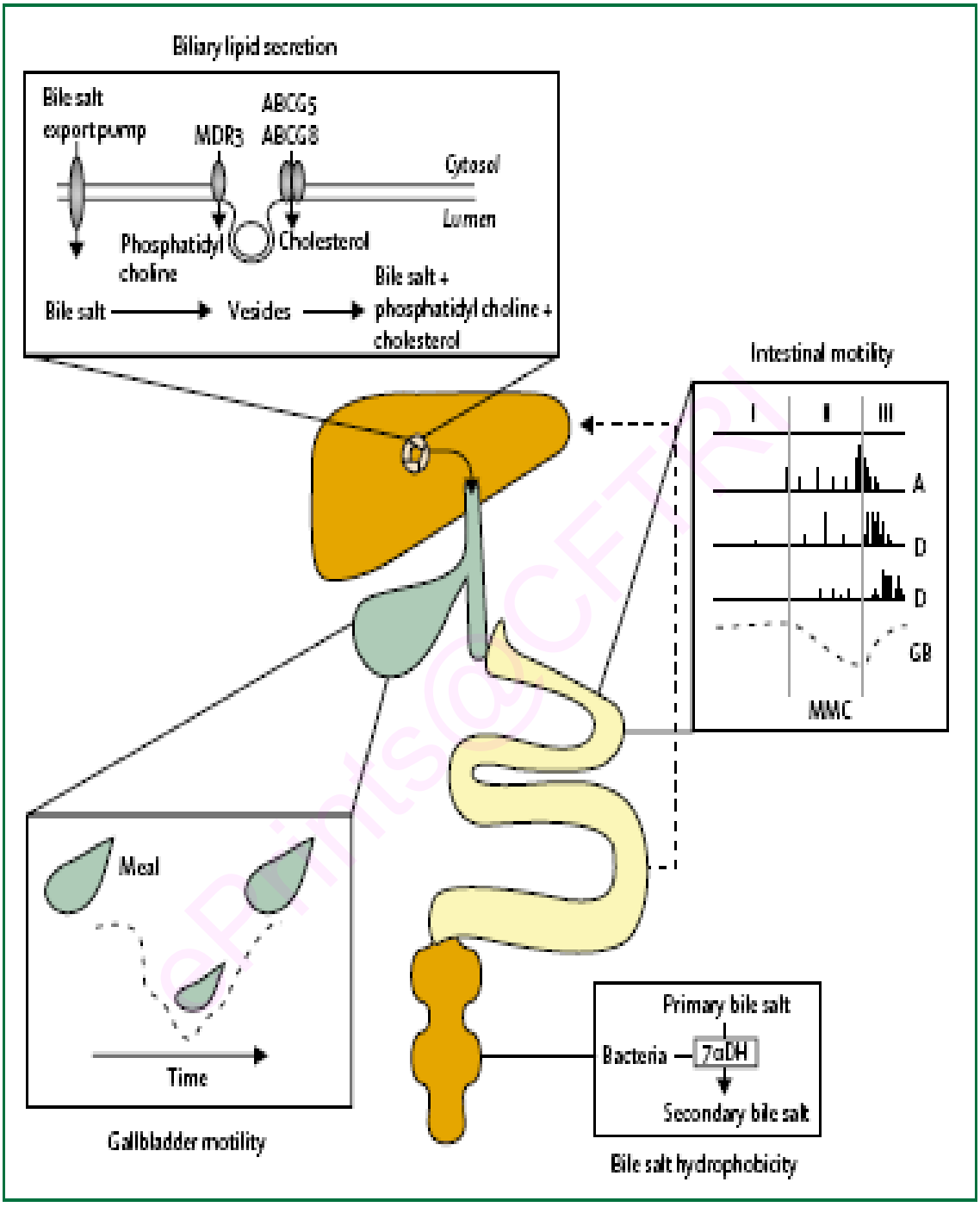


Fig.3. Role of liver, gallbladder, intestine and enterohepatic circulation in CGS formation (**Source**: Portincasa *et al.,* 2006).

1.7.17. Pro-nucleating and antinucleating factors: The critical balance between these two factors will determine the nature of bile, *i.e*. the higher concentration of pronucleating agents than the antinucleating agents will render bile more susceptible to form crystals and higher the concentration of anti-nucleation factors than the pronucleating factors prolong the process of cholesterol crystal formation and nucleation of formed crystals. In the last decade, much work has been done in this field with regard to various factors affecting crystallization, *viz*., mucin, glycoprotein, bilirubin, C: P ratio, bile salt: lecithin ratio, CSI, calcium, calcium binding proteins, fibronectin, biliary proteins especially low molecular proteins, Con-A binding proteins, *Helix pomatia* binding proteins, phospholipase-C/A_2, immunoglobulins IgA, IgG, IgM, α-acid glycoprotein, apoliproteins-A1/AII, and *H. pylori* (van Erpecum, 2004).

1.7.18. Genetic control of the disease

CGS is a very complex disease with the interaction of genetics and environmental factors. Thus, for the same reason it is possible to put forth diet that modulate these regulatory genes will influence CGS formation. Several studies indicated the role of few genes ($ABCG_5/_8$, FXR, LDL-R, CYP7A1, Apo-B, ApoE, and cholecystokinin (CCK) as common genetic determinants for CGS in humans (Portincasa *et al.,* 2006). In the last few years the list of regulatory genes involved in lipid metabolism is growing and this has opened new horizons in the investigation of the relationship between diet, lipid homeostasis and disease. Since regulatory genes regulate lipid metabolism and involved in biliary lipid secretion they play a significant role in CGS formation. The genes related to the regulation of lipid metabolism plays a crucial role in the process of CGS formation. One of the most important group of regulatory proteins that corresponds to this is sterol regulatory element binding protein (SREBP's). This group is formed by three different isoforms (SREBP-1a, SREBP-1c and SREBP2), which directly activate the expression of more than 30 genes controlling synthesis and transportation of cholesterol. The other nuclear receptor group of regulatory factors also plays a vital role in the metabolism of lipids. Members of this group include peroxisome proliferator-activated receptor (PPARγ), liver X receptors (LXR) and farnesoid X receptor (FXR). Biliary lipid secretion is a

regulated network of ATP binding cassette (ABC) transporters on the hepatocyte canalicular membrane. The ABC transporter ($ABCB_{11}$), known as the bile salt export pump (BSEP), which serves as the major canalicular bile salt export pump. The human multi drug resistant 3p-glycoprotein, also know as $ABCB_4$ (MDR_3–p-glycoprotein) functions as a flippase, translocate phosphatidylcholine molecules from inner to outer canalicular membrane. Finally, ABC transporters $ABCG_5$ and $ABCG_8$ pump cholesterol into bile. Two nuclear receptors, bile acid receptors or FXR and the oxysterol receptor or LXRs play an important role in transcriptional regulation of the genes encoding these proteins. The expression of $ABCB_4$ and $ABCB_{11}$ are under FXR control, while $ABCG_5$ and $ABCG_8$ are controlled by LXRs. PPARγ is another key lipid sensor, which regulates the expression of several genes related to fatty acid oxidation and peroxisome proliferation. PPARγ also modulates positively the expression of several genes related to biliary lipid secretion including the hepatic canalicular phospholipid and cholesterol transporters $ABCB_4$ and $ABCG_5/G_8$ respectively. PPARγ also regulate the genes associated with increase in phospholipid and bile salt content of bile. PPARγ gene is potentially involved in CGS pathogenesis since this nuclear transcription factors regulate the expression of multiple genes involved in lipid metabolism and it is also associated with hyperlipidemia, obesity, IR or type 2 diabetes, all these factors affect CGS (Cuevas *et al.,* 2004).

The rate limiting enzyme of hepatic cholesterol synthesis and bile salt synthesis are HMG-CoA reductase and CYP7A1, respectively (Table-3). These enzymes are regulated by the SREBP and nuclear receptor (NR) signaling pathways. Bile salts serve as natural ligands of the nuclear receptor FXR, which represents the bona fide hepatic bile salt sensor of the liver. FXR represses CYP7A1 expression, but stimulates the expression of the ABC transporters for bile salts ($ABCB_{11}$) and phospholipids ($ABCB_4$), whereas the cholesterol transporter $ABCG_{5/8}$ is induced by the nuclear receptor for oxysterols (LXR). FXR null mice have a reduced expression of $ABCB_{4/11}$ with decreased concentrations of phospholipid and biliary bile salts favor the process of CGS formation. LXR induced expression of $ABCG_{5/8}$ which controls the secretion of biliary cholesterol thus increase cholesterol secretion increases the cholesterol saturation.

Table 3. Major genes involved in lipid metabolism and their functions.

Gene	Symbol	Potential mechanism involved
1.ATP binding cassettre transporter B_4	$ABCB_4$	reduced biliary phospholipid secretion
2.ATP binding cassettre transporter B_{11}	$ABCB_{11}$	reduced biliary bile salt secretion
3.Apolipoprotein B	APO-B	reduced hepatic VLDL synthesis
4.Apolipoprotein E	APO-E	increases intestinal cholesterol absorption
5.Cholecystoknin-A receptor	CCKAR	gallbladder hypomotility
6.Cytochrome P450 7A1	CYP7A1	reduced bile acid synthesis

(**Source**: Grunhage & Lammert, 2006)

that many of the genes involved in hepatic metabolism affect CGS *in vivo*.

Bile acids are the end products of cholesterol metabolism; the entire process involves 17 different enzymes, many of which are preferentially expressed in the liver. Primary bile acids are the immediate products of these pathways, structures of these primary bile acids vary widely between the species. In case of humans and rats cholic acid and β-muricholic acid are the predominant primary bile acids. The expression of selected enzymes in this pathway is highly regulated by nuclear hormone receptor and other transcriptional factors.

Farnesoid X receptor: Bile acid synthesis suppression is mediated by FXR, which binds bile acids and activates the transcription of genes involved in bile acid and lipid metabolism. Bile acid pool of an organism varies in their ability to activate FXR, in their susceptibilities to metabolism by gut flora and in their enterohepatic circulation. Mice deficit in FXR over-express CYP7A1 and CYP27A1 mRNA; resulting in high synthesis of bile acids (Russell, 2003).

Liver X receptor: LXR activates the expression of CYP7A1 gene but not CYP27A1 gene. It also plays a crucial role in integrating the pathways of cholesterol supply and catabolism by regulating the expression of the SREBP-1c gene**.** SREBP-1c is a transcription factor that activates many genes involved in cholesterol and lipid biosynthesis. It also stimulates the transcription of genes encoding cholesterol efflux proteins (ABCA1, ABCG5 and

ABCG8 transporters); numerous other genes involved in lipid metabolism are targets of LXR (Russell, 2003).

1.8 Dietary factors which influence the formation of CGS

Diet having high fiber has proved to reduce the risk of CGS formation. The incidence of CGS is very high in western countries while it is low to very low in Asians and Africans in general and Indians in particular. Some authors attribute this to the consumption of regular spice-rich diet. Studies in experimental animals in recent years have indicated that consumption of spices namely fenugreek, garlic, onion, red pepper and turmeric caused not only reduction in the incidence but also regression of pre established CGS as well

(Hussain & Chandrasekhara, 1993; 1994; Vidyashankar *et al.,* 2009). Consumption of diet containing spices not only increased bile flow but also enhanced bile acids and phospholipid content, while there was a decrease in the biliary cholesterol concentration and biliary total lipids. Spices mainly act by stimulating the activity of CYP7A1 (Srinivasan & Sambaiah, 1991). The secretion and composition of bile was influenced by various factors, which in turn influence the formation of CGS.

Diet is very crucial from the point of health, nutrition of an individual. Proper diet in many cases acts not only acts as preventive but can also as curative for many diseases and at many times. Like in other diseases too, diet has an important role to play in the process of formation of CGS. It not only affects directly, but can affect indirectly by influencing the many factors which control cholesterol crystal nucleation. Thus the diet plays an important role not only in the management CGS, but also in other events related to high cholesterol content in the body like atherosclerosis, CHD, fatty liver and related diseases. Some of the dietary components which influence the CGS are:

1.8.1. Dietary fiber: Dietary fiber is that portion of food which is derived from the cellular walls of plants and these are not digested in humans. Dietary fiber helps in bringing serum lipid levels to near normal. It was reported that fiber slows down enzymatic activity and small intestinal absorption. Bile salts binding capacity and reabsorption are altered and it was observed that fiber-rich foods bind bile salts in the intestine, prevents their absorption and increases fecal bulk and stool frequency. Similarly, micelle and chylomicron formation together with absorption of dietary cholesterol was reduced. Dietary fiber intake is inversely associated with CGS formation. There were several mechanisms by which fiber protects the formation of CGS, the first and foremost effect was by reduction in the surface area of absorption caused by increased particle size in the intestine and thus reduces absorption of cholesterol. Apart from this reduction in intestinal transit time (Platel & Srinivasan, 2001), generation of secondary bile acids such as deoxycholate also inhibit CGS formation by reducing biliary cholesterol saturation (Cuevas *et al.,* 2004). In an observational study higher intake of fiber was associated with a lower prevalence of gallstones (Gaby, 2009). Deconjugated bile salts were bound to pectic substances by

hydrogen bonding which may be responsible for the cholesterol lowering mechanism of fiber. Dietary fiber reduces biliary CSI, but the mechanism was not clear. It was hypothesized that reduction in the circulating pool of secondary bile acid, deoxycholic acid was responsible for this effect. A number of experiments have consistently proven the cholesterol lowering effect of fiber in cholesterol fed condition, but in animals which receive no dietary cholesterol this effect was almost absent.

1.8.2. **Proteins:** High plasma triglyceride and low plasma HDL-C concentrations were positively associated with CGS (Mendez-Sanchez *et al.,* 2007). Substitution of dietary carbohydrate by protein significantly increases HDL-C, decreases triglyceride concentration and improves insulin sensitivity. This biological action has forwarded the protective effect of proteins against CGS in humans, also supported by animal models.

1.8.3. **High calorie/energy/carbohydrates intake:** High calorie and carbohydrates intake was directly related to the incidence of CGS. High calorie (>2500 kcal/day) positively associated with CGS formation, but significant only in men. Energy intake contributes to the development of obesity. High intake of refined sugar mainly affects cholesterol synthesis in liver. Increase in cholesterol synthesis is secondary only to an increase in insulin secretion and also may be due to the lipid metabolism induced modifications in bile composition. It was proved that sugar equivalent to 40 g per day doubles the risk of symptomatic gallstones (Cuevas *et al.,* 2004).

1.8.4. Saponins: Saponins are a heterogeneous group of glycosides which are widely distributed in plants and are bitter in taste. Of the various properties they possess, the most striking property was the reduction of plasma cholesterol which has a direct impact on CGS and coronary heart disease. Saponins in the gastrointestinal tract directly interact with dietary cholesterol forming less soluble to insoluble complex which reduces cholesterol absorption. Some saponins affect cholesterol metabolism indirectly by interacting with bile acids and thus increase fecal excretion of bile acids. Fecal loss of bile acids would be recompensated by newly synthesizing bile salts from cholesterol.

1.8.5. Vitamins and minerals: Vitamin C is a cofactor for the CYP7A1 and facilitates the conversion of cholesterol to bile acids thereby decreasing the lithogenicity of bile. It was reported that vitamin C deficiency reduces the activity of CYP7A1 in liver. Supplementation of vitamin C (2 g/day for 2 weeks) showed changes in bile composition and also prolongs the crystal nucleation time. It was also mentioned that alcohol consumption, ascorbic acid supplementation were independently associated with 50% reduction in the prevalence of gallstones and a 62% reduction in cholecystectomy. Serum ascorbic acid level was inversely related to the prevalence of clinical and asymptomatic gallbladder disease among women.

Some studies have found an inverse association between the dietary calcium and gallbladder disease by binding secondary bile acids mainly the deoxycholate (Cuevas *et al*., 2004). Recent findings have showed that the deficiency of iron favors the CGS formation. Iron-deficient diet had a higher incidence of cholesterol crystals in their bile than animals fed a control diet. The activity of hepatic CYP7A1 was lower by 64 percent in iron-deficient dogs than in controls. These findings suggest that iron deficiency have a role to play in the pathogenesis of CGS formation in humans (Acalovschi, 2001; Gaby, 2009).

1.8.6. Coffee: Effect of coffee on CGS have contradicting results, but many reported that coffee consumption stimulates CCK release, enhances gall bladder motility, inhibits gall bladder fluid absorption, increases the intestinal motility and decreases cholesterol crystallization in bile. Coffee diterpenes may down regulate the hepatic LDL-receptor and reduces HMG-CoA reductase activity. The protective effect of caffeine appears to be due to stimulation of bile flow and increased enterohepatic circulation of bile acids (Acalovschi, 2001; Cuevas *et al.,* 2004; Gaby, 2009).

1.8.7. Plant sterols: The effect of plant sterols on CGS formation was been well studied and it was reported that β-sitosterol inhibits CGS formation in lithogenic diet fed animals mainly by reducing intestinal cholesterol absorption. It was reported that some of the plant sterols come in the way of cholesterol absorption. Phytosterols are structurally similar to cholesterol but have slight modification in the aliphatic side chain. The principal molecular

forms are sitosterol, campesterol and stigmasterol and they are thought to act primarily in the intestine. As cholesterol analogs, they compete with cholesterol in absorptive micelles resulting in reduced solubility and absorption of cholesterol. Plant sterols have high affinity than cholesterol for micelles and it was reported that 30-40% decrease in cholesterol absorption and also reduction of 10-15% in LDL-cholesterol was observed with high phytosterol doses.

Plant sterols displace cholesterol from bile salt micelles. Plant sterols are not competitive inhibitors of cholesterol absorption but observed that solubility reduction of cholesterol was an important event in the inhibition of cholesterol absorption (Ikeda *et al.,* 1988; Cuevas *et al*., 2004). Nuclear receptors LXR and FXR regulate the absorption of dietary sterols by modulating the transcriptional regulation of important genes involved in cholesterol metabolism. One of these genes encodes a molecule (ATP binding cassette transporter) that transports dietary cholesterol from enterocytes back to the intestine lumen, thereby limiting the amount of cholesterol absorbed thus producing hypocholesterolemic effect in diet induced hyper-cholesterolemic condition (Chen, 2001).

1.8.8. Fatty acids and fatty acid composition of lecithin: There was a high risk associated with the consumption of large amounts of lipids, mainly saturated fatty acids. Epidemiological studies suggest that monounsaturated fatty acids (MUFA) and fish oil have a protective role (Cuevas *et al.,* 2004). The degree of saturation was directly related to the nucleation and crystal growth rate and while unsaturated fatty acids binds less tightly to cholesterol compared to saturated fatty acids and higher C: P ratio (>1.0) was associated with crystal formation in bile.

1.8.9. Nuts: It was observed that women who consume ≥5 oz of nuts/week had a significantly lower risk of cholecystectomy (Mendez-Sanchez *et al.,* 2007).

2.0 Bile: its importance, functions, composition and biliary disorders

Bile is a complex colloidal aqueous system containing organic and inorganic compounds secreted by the liver, which has a wide range of physiological functions. Bile is formed in hepatic canaliculi, small space formed between the tight junctions of

hepatocytes. Everyday 500 ml of bile is secreted and passed. Bile acids are biologically precious molecules restricted to the enterohepatic circulation, so as to reutilize many times. Bile is stored in the gallbladder after the concentration it was secreted into the duodenum in response to the food passage.

Initially, hepatocytes secrete bile into canaliculi, from which it flows into bile ducts. This hepatic bile contains large quantities of bile acids, cholesterol and other organic molecules. As bile flows through the bile ducts it is modified by addition of water, bicarbonate-rich secretion from ductal epithelial cells.

2.1 Functions

Bile is stored in the gall bladder and pumped into the duodenum in response to food in the intestine. Bile has a significant role in the physiology of body: and some of the important functions of bile are:

i) Emulsification: Bile salts are strong detergents which emulsify the fat in the intestine and helps in absorption of fat and fat soluble vitamins. Emulsification is not digestion perse, but it is of importance because it increases the surface area of fat, making it easily available for digestion and absorption.

ii) Solubilization and transport of lipids in an aqueous environment: Bile salts solubilize many lipids by forming micelles - aggregates of lipids that remain suspended in water. Bile acids are also critical for transport and absorption of vitamins which are fat-soluble.

iii) Excretion: Bile is an important vehicle for excretion. Drugs that are mainly excreted in the bile are usually have high molecular weight (>300) and have a strong polar group. Some of the substances excreted through bile include cholesterol, bile acids, biliary pigments, several drugs, toxins and other harmful substances. Drugs and chemicals excreted into bile enter the intestine and are subsequently either reabsorbed or eliminated in the feces. This process of excretion into bile and re-absorption in the intestine is known as enterohepatic circulation.

2.2 Mechanism of bile formation

Bile is an aqueous solution of lipids with bile salts (67% of solutes by weight), phospholipids (22%) and cholesterol (4%) representing main lipid species. Hepatocytes express specific ATP-dependent transport proteins-known as ABC transporters for each of these three biliary lipids at canallicular membrane domain. The $ABCB_{11}$ transporter is the bile salt export pump, $ABCB_4$ is the transporter for the major biliary phosphatidyl choline (lecithin) and $ABCG_5$/ $ABCG_8$ form obligate heterodimer for biliary cholesterol secretion (Grunhage & Lammert, 2006).

In many species which have gallbladder (man and most domestic animals except horses and rats) further modification of bile occurs. Gallbladder stores and concentrates bile during intra digestive intervals. Typically bile is concentrated (≈ five-fold) in the gallbladder by absorption of water and small electrolytes. In humans bile flow varies from 1.5 to 15.4 µl $min^{-1}kg^{-1}$ depending on bile secretion rate. The driving force for the movement of water into the bile canaliculus was provided by the osmotic gradient, mainly generated by active bile acid transport into the canaliculus. This mechanism, bile acid dependent bile flow accounts for 60% of the total bile flow and the other 40% is the bile acid independent bile flow generated by active transport of Na^+ and HCO_3^-. Critical composition of bile plays important role in the normal functioning.

2.3 Composition of bile

The major constituents of bile are: water, cholesterol, phospholipids and bile acids. The difference between the hepatic and gallbladder bile. Composition-wise there is not much difference between the gall bladder bile and hepatic bile. The main difference is gallbladder bile is more concentrated than the hepatic bile thus the contents are quantitatively more per liter (Table-4).

Major components of bile include phospholipids, bile acids and cholesterol.

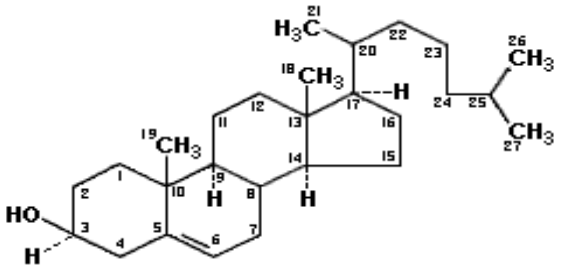
1. Phospholipids: Phospholipids are an integral part of all living cells as part of cellular membrane. The most abundant biliary phospholipid is phosphatidylcholines (lecithins), which account for 80-95% of phospholipids content in human bile. Phospholipids are

exported by liver in two secretions; bile and plasma lipoproteins. In humans, phospholipids exported per day are 10-20% of liver phosphatidylcholine pool, while 12 g/day is secreted in bile. These phospholipids play a significant role in the micellar solubilization of cholesterol, fatty acids, steroids and vitamins which are fat soluble.

*2. Bile acids***:** Bile acids are polar derivates and catabolic end products of cholesterol. During this process hydroxyl and carboxyl groups are added to sterol ring and to the side chain respectively. The spatial orientation of these polar hydroxyl and carboxyl groups are of most importance from the physiological point of bile salts. Bile salts are the major constituents of bile, which help to solublise dietary lipids. Approximately 500 mg of cholesterol was converted into bile acids each day in an adult human. The bile acids were secreted into the bile and delivered to lumen of small intestine. Bile acids were transported from intestine to the liver via portal circulation and then re-secreted into the bile. About 5% of the bile acids are lost during each cycle of the enterohepatic circulation and are replaced by new synthesis in the liver. Of the daily total cholesterol that was metabolized in the body, 90% was used for bile acid production and the remaining accounts for the steroid hormone synthesis. Excess bile acids in organism repress further synthesis and conversely when bile acids were in short, synthesis was increased, *i.e*. regulated by feedback mechanism. In the case of rats and mice accumulation of cholesterol enhances the bile acid out put. The bile salts were quite soluble in water and their aqueous solubility is ≈ 10^{-3} mol/liter, which is far exceeding cholesterol and phospholipids and this concentration, is referred to as the critical micellar concentration. The critical concentration, solubility and form of these principle lipids of bile were very important. At any given time the biliary concentration of phospholipids and bile salts are very important since their concentration dictate the form of cholesterol in bile, uncontrolled high levels of cholesterol compared to phospholipids and bile salts leads to cholesterol not being completely soluble in bile.

3. ***Cholesterol*****:** Cholesterol was first found in gallstones and derives its name from *Greek* words i.e. *Khole (bile)* and *stereos (solid).* It was a sterol that occurs in animal fats, bile, gallstones, nerve tissues, blood, brain, liver and egg yolk. It is the most widespread animal sterol. Normal body has 0.2% cholesterol by weight and most of it is in brain and nervous

system and approximately 25% of total brain lipid constitutes cholesterol. It acts as insulator and is a structural component of muscles. Cholesterol is the source for many other steroids like vitamin D_2, bile acids, adrenocorticoid hormones and sex hormones. The optimum concentration of cholesterol is vital for normal functioning of our body. Any alteration in its concentration may lead to various complications. When it was present in smaller amounts in the diet, the remaining requirement of the body is met though endogenous synthesis, while the excess was balanced by excreting out in the form of bile salts. Continuous supply of diet rich in cholesterol will activate the feedback mechanism where the activity of HMG-CoA reductase activity was reduced thus the endogenous synthesis was almost stopped and also there was a spurt in the conversion of cholesterol to bile acids. Sometimes if these mechanisms fail to activate, there will be more accumulation of cholesterol in serum, liver and bile. In 1759, Francois Poultetior dela Sale was the first to identify cholesterol in solid form from gallstones; later in 1815, Eugene Chevreul named the compound "cholesterine". The form of cholesterol plays important role in CGS; the form of cholesterol in bile directly depends on the concentration of cholesterol and was indirectly dependent on concentration of phospholipids and bile salts. Determination of cholesterol in foods and body was important because of its implication in the etiology of CGSs, atherosclerosis, coronary heart disease and fatty liver. The very high levels of cholesterol, particularly the LDL-cholesterol in blood causes atherosclerosis and cardiovascular disease. Likewise increased cholesterol levels in bile make the way for the formation of cholesterol crystals or aggregates and finally end up in CGS disease.



Structure of cholesterol ($C_{27}H_{45}OH$)

Table 4. Compositional difference between hepatic and gallbladder bile

Constituent	**Hepatic bile**	**Gallbladder bile**
Color	Golden yellow orange	Dark brown to greenish brown
Water (%)	95-97	85-90
pH	7.5	6.0
Cholesterol (g/L)	1-3.2	6.3
Phospholipids (g/L)	1.4-8.1	34
Bile acids (g/L)	3-45	32
Total phosphorus (g/L)	0.15	1.4
Total fatty acids (g/L)	2.7	24
Proteins (g/L)	2-20	4.5
Bilirubin (g/L)	1-2	3
Ca^{2+}(mM)	1.2-3.2	15
Na^{+}(mM)	141-165	220
K^{+}(mM)	2.7-6.7	14
Cl^{-}(mM)	77-117	31
HCO_3^{-}(mM)	12-55	19

(**Source:** http://en.wikipedia.org/wiki/Bile)

2.4 Absorption of cholesterol

Cholesterol is one of the most ubiquitous compounds in the body and vital structural component of every cell. Cholesterol present in intestine was derived principally from two sources, one from the diet, which contributes 300-400 mg and the other from bile which contributes to 750-1250 mg daily. Biliary cholesterol was derived from hepatic synthesis which amounts to about 9-13 mg per kg body weight per day. The amount of cholesterol absorption ranges from 30-60%. The principal pathway for the absorption of fats was by way of micellar solution. A human lipid micelle contains 1.0 mol of bile acid, 1.4 mol of fatty acid, 0.15 mol of lysolecithin and 0.06 mol of cholesterol. The major site of cholesterol synthesis was the liver, followed by intestine, but each mammalian tissue can synthesize cholesterol. A clear understanding of how dietary cholesterol may relate to LDL-cholesterol is very important. Daily dietary cholesterol intake varies; biliary cholesterol is large and is the principal component of intestinal cholesterol. Only 25% of plasma cholesterol is derived from the absorbed dietary cholesterol, while 75% of it was of endogenous origin. However, dietary cholesterol is very important because dietary cholesterol and the endogenously synthesized cholesterol are inversely correlated, suggesting that they are co-regulated. Cholesterol absorption varies between individuals and highly reproducible when measured repeatedly in individuals under standard conditions. It was reported that the dietary intake of cholesterol varies widely within or between the species and observed that there was a positive relationship between the dietary cholesterol and the plasma cholesterol and it was mentioned that, for every 100 mg of dietary cholesterol intake there was an increase of 4-5 mg of plasma cholesterol. Cholesterol absorption capacity of humans is much less compared to that of many animals (Kansal, 1995). Some individuals are hypersensitive while others are hyposensitive to dietary cholesterol and are quiet common in a variety of animal species including man (Gurr, 1983).

2.5 Transportation of cholesterol

Cholesterol and other lipids are transported in the plasma as lipid-protein complexes known as “lipoproteins” and this complex helps in easy mobility of lipids and the proteins

associated. There are four major classes of lipoproteins involved *viz*., chylomicrons, VLDL, LDL and HDL (Table-5). Lipoproteins were spherically shaped clusters containing both lipid and protein part. The protein components of these macro molecule aggregates have two important roles to play; they solubilize hydrophobic lipids and carry cell targeting signals. Cholesterol, triglycerides and other lipids from the diet were absorbed and carried away in the form of large chylomicrons. Chylomicrons deliver dietary triglycerides to muscle and adipose tissue and dietary cholesterol to liver (Smith *et al.,* 1978). Triglycerides and cholesterol in excess of liver's own use are exported into the blood in the form of VLDL. The triglycerides in VLDL are hydrolyzed by lipases on capillary surfaces. The resulting remnants, which are rich in cholesterol esters, are called IDL. These particles have two fates; half of them are taken up by liver, while the other half is converted to LDL. LDL is the major carrier of cholesterol in blood, having 22 nm (220 A°) and having molecular mass of about 3 million Daltons and plays role in the transportation of cholesterol to peripheral tissues and regulates *denovo* cholesterol synthesis. LDL is considered as bad cholesterol while, HDL is considered as good cholesterol since it functions opposite to LDL as it removes cholesterol from various tissues and transports it to liver. *i.e.*, HDL functions as cholesterol scavenger and involve in reverse cholesterol transport. In humans, most of the cholesterol present in the gallstones is of dietary origin. Constant observations have shown that hepatic biosynthesis is mediated by the scavenger receptor B-1 (SRB1) for HDL, which contribute most of the biliary cholesterol under physiological conditions, the apolipoprotein (apo) B/E receptor for LDL and the LDL-receptor related protein for chylomicron remnants, which carry exogenous cholesterol from the intestine to the liver. The inverse relation between serum HDL levels and CGS suggests that cholelithiasis is associated with an induced reverse cholesterol and hepatic catabolism of HDL (Grunhage & Lammert, 2006). LDL cholesterol, total cholesterol content and triglycerides are closely associated with CGS and it is reported that increase in their content is positively related to the incidence of CGS (Halldestam *et al*., 2009).

Table 5. Different classes of serum lipoproteins and their characteristics

Class	Density (g/mL)	Diameter (nm)	Protein	Cholesterol	Triglycerides
			%		
HDL	>1.063	5-15	33	30	4
LDL	1.019-1.063	18-28	25	50	8
IDL	1.006-1.019	25-50	18	29	31
VLDL	0.95-1.006	30-80	10	22	50
Chylomicrons	<0.95	100-1000	<2	8	84

(**Source**: http://en.wikipedia.org/wiki/Lipoprotein)

2.6 Excretion of cholesterol: Dietary cholesterol is not fully absorbed and some quantity is eliminated through feces as unabsorbed cholesterol but major quantity is eliminated from the body as bile salts. A large portion of biliary bile salts which are on the way to excretion are reabsorbed and taken up by the liver and re-secreted into the bile. Unabsorbed bile salts are excreted through feces. Thus it is a way of elimination of cholesterol from the body in the form of bile salts.

2.7 Accumulation of cholesterol: Presence of additional cholesterol in the body more than the requirement affects the body cholesterol homeostasis. The inter play of three aspects viz., cholesterol absorption, reduced endogenous synthesis and accelerated excretion plays an important role. Overall fate of cholesterol in the body is given in the Fig.4. Cholesterol rich diet makes the way for more absorption and in turn increases cholesterol to bile salt synthesis and reduces the endogenous cholesterol synthesis. The accumulation of cholesterol more than required for physiological function makes a way for some of the problems like CGS, atherosclerosis, coronary heart disease, fatty liver, *etc*. High level of cholesterol in serum is a causative factor for CVD and its complications, while supersaturation of bile with cholesterol is the foremost and primary causing factor of CGS and its related problems.

2.8 Problems associated with high cholesterol content in the body

a) **Atherosclerosis:** It is a leading cause of deaths caused by uncontrolled high level of cholesterol and specifically LDL-cholesterol, which play a significant role in plaque formation and blockage of arteries (Fig.5). The causes of this process appear to be lipid retention, oxidation, and modification, which provoke chronic inflammation at susceptible sites in the walls of all major conduit arteries. When the problem is untreated, the high level of LDL-cholesterol also causes problems and the rate of development is faster in patients with risk factors such as hypertension, smoking, diabetes mellitus, obesity, and genetic predisposition. Young population have average cholesterol content of 1.6 g/liter of blood while it is almost double (2.5 g/liter) in people who are ≥ 55 years of age.

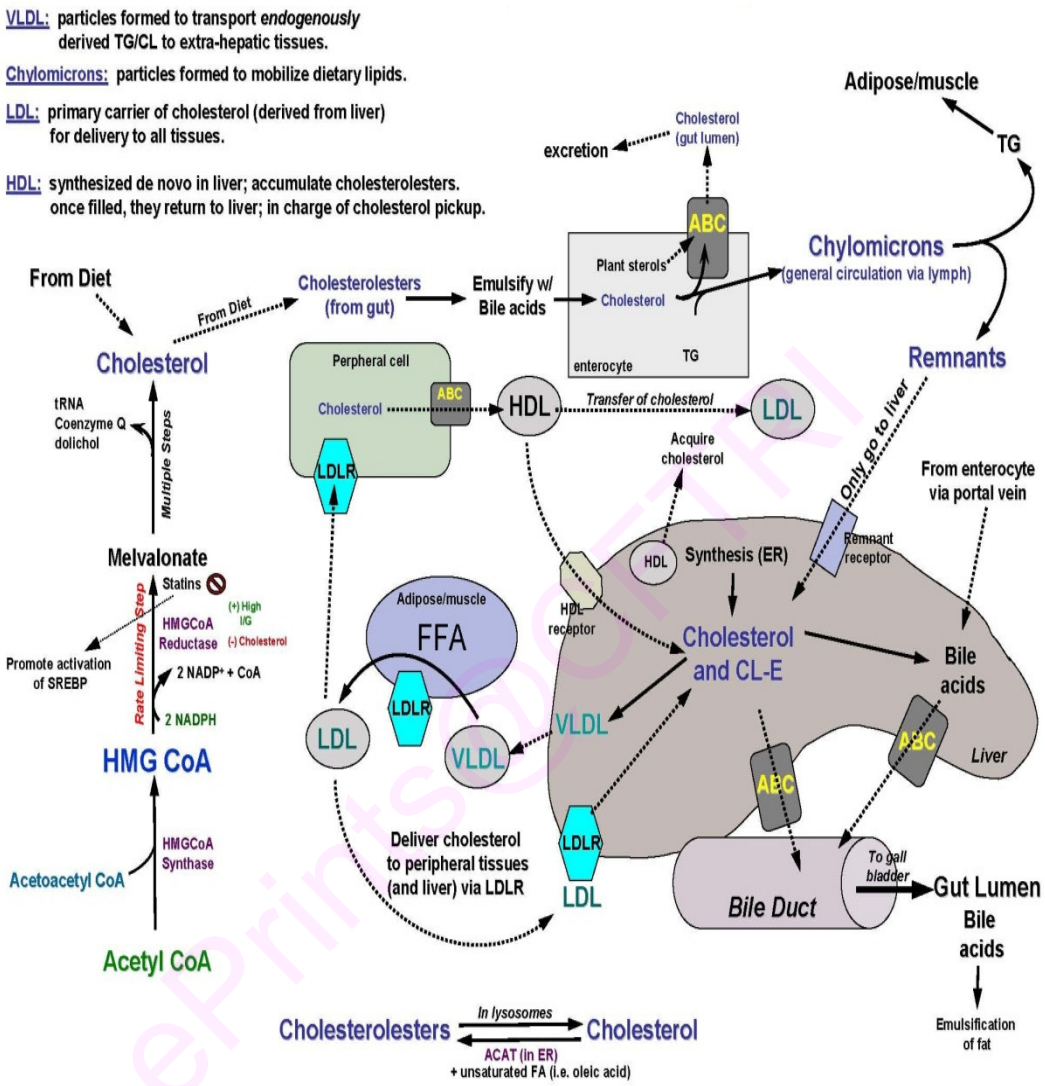


Fig.4. Schematic diagram representing the cholesterol metabolism in the body**.**

(**Source:** http://img84.imageshack.us/i/biochemcholesterolmetabxl2.jpg)

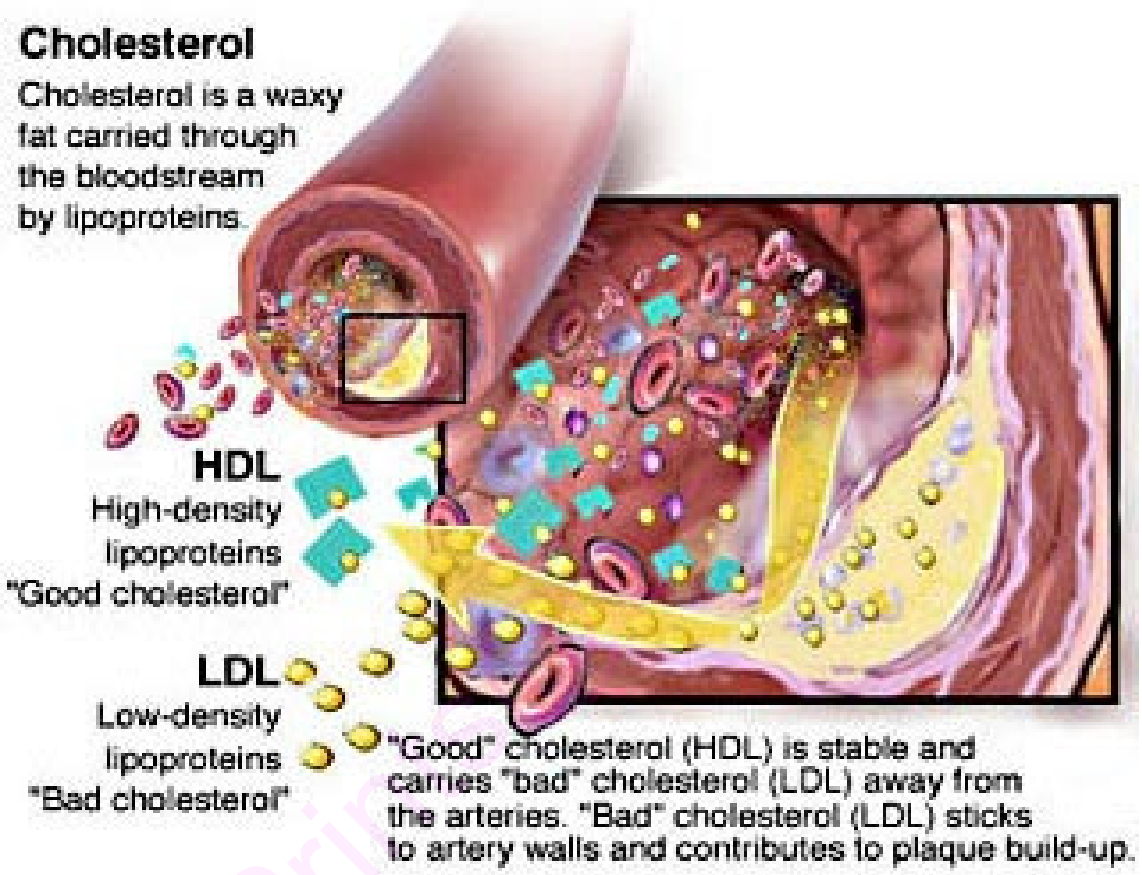


Fig.5. A schematic diagram showing the condition of atherosclerosis

(**Source:** http://aboutyourcholesterol.com/3_GoodvsBad.asp)

b) **Coronary heart diseases (CHD):** CHD is a major cause of ill health and deaths in developed countries. High total blood cholesterol, advancing age, obesity, sedentary life and diabetes mellitus are the major coronary risk factors (Hopkins & William, 1981). Many of the problems mentioned are also the causative factors for the formation of CGS.

c) **Fatty liver:** Fatty liver presents an excessive accumulation of lipids in hepatocytes

caused by enhanced *de-novo* lipogenesis, activating lipid uptake, reduction in lipid catabolism and lowering lipoprotein secretion rates. It was reported that development of fatty liver in mice was linked with the increased expression of SREBP-1 and it's down stream genes involved in lipogenesis with accompanying increased uptake of lipids.Insufficient gene expression of LDLR might contribute to the development of hyperlipidemic state which is one of the important factors in the development of fatty liver and insulin resistance. Fig.6 shows the difference in the histopathological and morphological variations between normal and fatty liver.

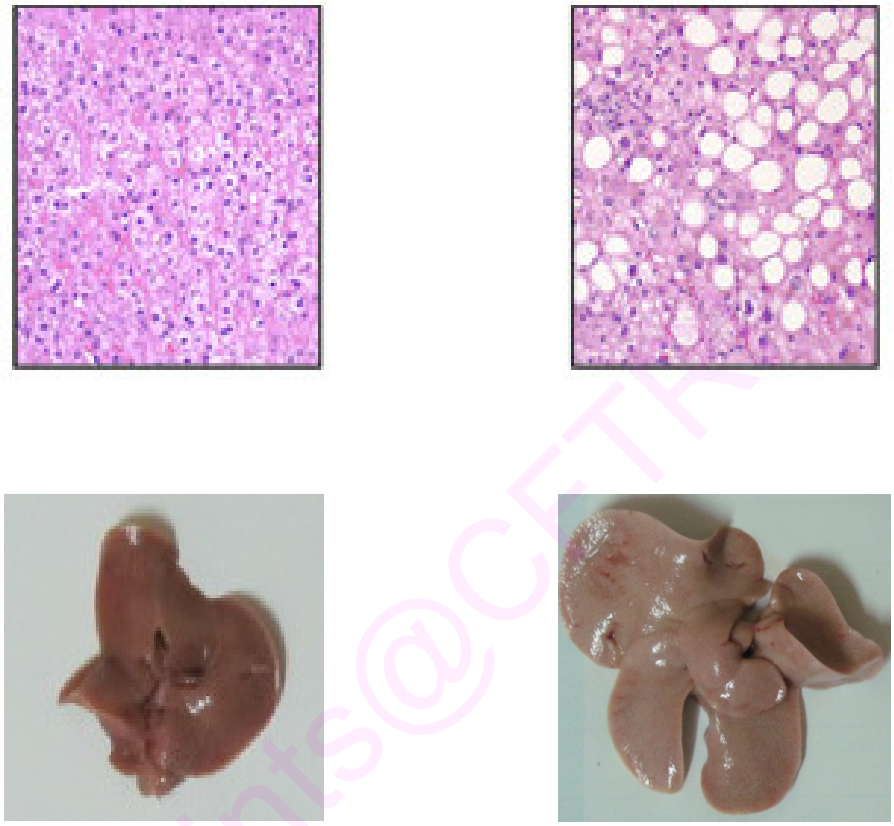
d) **CGS:** This is one of the leading diseases in western countries in recent years. The occurrence of CGS is associated with the supersaturation of bile with cholesterol.

2.9 Secretion and transportation of biliary cholesterol

Hepatic cholesterol is derived in two ways; one is synthesis within the hepatocytes and the other, taken up by liver from preformed cholesterol from the blood. Hepatic cholesterol may be directly exported into bile which is used for the synthesis of bile salts or converted into cholesterol esters, which are either exported into serum or stored as such. Most of the biliary cholesterol is preformed and only around 20-25% is newly synthesized (Strasberg, 1998).

2.10 Phase equilibrium

Phase is the form of cholesterol that exists in bile at a given point of time. There are two important phases of biliary cholesterol, i.e. monomeric (solid crystals) phase and vesicular phase, where cholesterol content is more than its solublising capacity; once the



Normal liver **Fatty liver**

Fig.6. Difference between normal liver and the fatty liver (histopathological and morphological variations).

equilibrium is reached cholesterol will remain as supersaturated vesicular phase to form cholesterol crystals. Equilibrium is a state wherein there is minimal effect of factors which influence the formation of CGS, resulting in a stable, balanced and unchanging system. The knowledge of phase equilibrium is a key in understanding the physicochemical changes that lead to the formation process of gallstone. The composition of bile varies and it will be either supersaturated or unsupersaturated with or without cholesterol crystals. This is determined by measuring the concentrations of three lipids in bile and by plotting their relative concentrations on the phase diagram. It is very difficult to plot this unless one should have a very comprehensive knowledge about this. Strasberg (1998) put forward entire information mathematically as cholesterol saturation index (CSI) and said if the CSI of bile is > 1.0 it is supersaturated and will have a cholesterol crystals in it and *vice versa*.

2.11 Pathway of cholesterol crystal formation

Secretion of more amount of cholesterol into bile with no change in phospholipids and bile acid concentration reduces its cholesterol carrying capacity and make the bile supersaturated with cholesterol. Initially the unstable bile is formed and this leads to the formation of small crystals and if this prolongs these small crystals nucleate and grow with time and attain a stable size which can be detectable under microscope. Aggregation and fusion are the two important events that bring together cholesterol-rich particles onto the vesicular surface, greatly facilitating nucleation of cholesterol crystals (Halpern *et al.,* 1986).

3.0 Spices: Nutraceuticals with multiple health beneficial effects

Spices are natural food adjuncts that have been in use for decades, mainly used to enhance the sensory quality of food. Many spices and their principles improve the quality of food not only with flavor but also aroma, color and texture as well. Spices and their principles have proven for their beneficial properties and some of the major health benefits of spices in general and fenugreek in particular are discussed here.

3.1 Stimulation of digestion: Many of the spices have proven to aid in the process of digestion mainly by influencing the secretion of saliva and gastric juice, which are

stimulated by the sense of smell. Digestive stimulant actions of spices have been studied extensively in animals (Platel & Srinivasan, 1996; 2000; 2000a; 2001a; 2004). It was established that many commonly consumed spices including fenugreek stimulate bile acid production by liver and its secretion into bile (Sambaiah & Srinivasan, 1991; Platel & Srinivasan, 2000). Spices stimulate the activity of digestive enzymes of pancreas and small intestinal mucosa (Platel & Srinivasan, 1996; 2000a; 2001a). It was reported that fenugreek had the highest stimulatory influence on the bile acid secretion in rats among the various spices examined with an increase of over 80% of the control (Srinivasan, 2006). Fenugreek increased the bile acid secretion and bile flow rate. It also increased the pancreatic lipase activity, chymotrypsin activity, and the activity of intestinal lipase.

3.2 Antioxidant property: Increased oxidative damage is the main cause for degenerative diseases like, cardiovascular disease, inflammatory diseases, cancer, neurodegenerative diseases ─ Alzheimer's disease and Parkinson's disease (Halliwell, 2001; Halliwell & Gutteridge, 2006). Many of the spices have been studied for their anti-atherosclerotic / antiatherogenic, cardioprotective, hypocholesterolemic, hypolipidemic, hypoglycemic, anti-inflammatory, anti-neurodegenerative properties and are attributed to their antioxidant properties. The beneficial antioxidant activity of curcumin, eugenol, capsaicin, piperine, gingerol, garlic, onion and fenugreek has been extensively reviewed (Srinivasan, 2010). These spices mainly act by quenching oxygen free radicals, by limiting the reactive oxygen radical production and by increasing the antioxidant enzyme activities. Oxidative damage plays an important role in diseases like CHD, inflammatory diseases, diabetes, carcinogenesis and aging. Spices have been shown to possess antioxidant activity and it is hypothesized that antioxidants play a key role in the prevention of cardiovascular diseases by preventing the oxidation of LDL.

Dietary fenugreek has been shown to counter the increase in LPO and enhances the concentration of antioxidant molecules like glutathione, ascorbic acid, thiols, β-carotene and α-tocopherol (Srinivasan, 2006; 2010). The antioxidant activity of spices is mediated by one or more of the following ways:

a. Free radical scavenging activity

b. Suppress lipid peroxidation
c. Improving the tissue antioxidant molecules levels
d. Stimulating the activities of the antioxidant enzymes and reducing LDL cholesterol oxidation

3.3 Antidiabetic potential: Many studies have shown the beneficial antidiabetic property of spices. Antidiabetic property of fenugreek, garlic and onion have been demonstrated in animal studies and clinical trials (Sharma, 1986; Sharma *et al.,* 1996) Dietary fenugreek seeds have shown a favorable effect with respect to blood glucose and glucose tolerance. The hypoglycemic property of fenugreek is attributed to the fiber constituents especially the gum (galactomannan) (Sharma & Raghuram, 1990). Fiber is believed to delay gastric emptying and directly interfere with glucose absorption. It is also presumed that dietary fiber reduces the release of gastric inhibitory peptides and insulinotropic hormones. In *Allium* spices, the antidiabetic property was attributed to the presence of disulfide compounds, di(2-propenyl) disulfide and 2-propenylpropyl disulfide, respectively, which stimulate insulin secretion. Dietary curcumin and onion have a promising ameliorating influence on renal lesions in streptozotocin induced diabetic rats (Babu & Srinivasan, 1998; 1999). Red chillies (capsaicin) have been shown to possess anti-diabetic property in terms of alleviating diabetic neuropathy. Trigonelline, an alkaloid present in fenugreek is also thought to reduce glycosuria in diabetes.

3.4 Hypolipidemic property: Fenugreek, garlic, onion, red pepper, and turmeric have been proven to possess hypocholesterolemic property. Garlic and garlic oil have been shown to reduce total cholesterol by reducing LDL-C and triglyceride levels. Dietary aged garlic extract showed better results compared to fresh garlic on lipid profile and blood pressure in moderately hypercholesterolemic subjects (Steiner *et al.,* 1996). Curcumin and capsaicin have shown to reduce LDL-cholesterol and total cholesterol and improved HDL-cholesterol in animals (Hussain & Chandrasekhara, 1992; 1994). Fenugreek seeds have been shown to possess hypolipidemic property in high-fat diet fed conditions (Singhal *et al.,* 1982). It is also observed that defatted fenugreek seed is effective in diabetic hypercholesterolemia in dogs (Valette *et al.,* 1984), rats and humans (Sharma, 1984; 1986). Atherosclerosis or plaques in blood vessels are associated with high or abnormal levels of

VLDL and LDL-cholesterol, which are important risk factors of heart related problems. It is reported that fenugreek lowers LDL-cholesterol, total cholesterol and triglycerides with not much change in HDL-cholesterol (Srinivasan, 2005). Fiber, saponin, galactomannan constituents of fenugreek seed have shown to reduce the cholesterol content. Galactomannans have been proven to reduce cholesterol concentration both in serum and liver and it is also reported to minimize the hepatic cholesterol synthesis.

3.5 Antiinflammatory effect: Several spices possess excellent anti-inflammatory activity (Srinivasan, 2005). The earliest anti-inflammatory drug/medicine to mention is the use of turmeric and its compounds. Natural anti-inflammatory compounds operate mainly by inhibiting proinflammatory stimuli with COX activity (Srinivasan, 2010). Oral administration of fenugreek extract exhibited anti-inflammatory and antipyretic effect. It is also reported the anti-ciceptive effect of this extract is the result of anti inflammatory effect (Ahmadiani *et al.,* 2001).

3.6 Anti-thrombotic property: Many spices have been documented to have anti-platelet aggregation and anti platelet adhesion properties which also contribute to cardiovascular protection.

3.7 Antimutagenic and anticancer effect: Many of the phenolic extracts from natural sources possess antioxidative and anti-inflammatory properties. These in turn contribute to chemoprotective or preventive activities. Many spices have proven to inhibit lipid peroxidation which plays an important role in protecting against cancer in many stages. Turmeric or curcumin in diet reduced the levels of mutagenic metabolites and carcinogenesis in mice and rats and it was also observed that there was a significant reduction of mutagens excreted in urine with in 30 days of curcumin administration (Srinivasan, 2010).

Turmeric, eugenol and mustard seeds have shown to possess antimutagenic effect. Turmeric/curcumin has been found to have chemoprotective effect against skin, liver, colon and oral cancer in mice. Curcumin can suppress the initiation as well as promotion of tumor. It is mentioned that inhibition of arachidonic acid metabolism, modulation of

cellular signal transduction pathway, inhibition of hormone and oncogene activity are few mechanisms by which curcumin may possess antitumor property. Curcumin was also a powerful inhibitor of proliferation of tumor cells (Srinivasan, 2010).

3.8 Fenugreek seeds known for hypocholesterolemic influence

Fenugreek seed is an extensively consumed spice. Fenugreek (*Trigonella foenum-graecum*) belongs to family: *Fabaceae* (Leguminosae). It is an annual herb, native of southern Europe and Asia, and widely grown all over India. Both plant and seeds are of medicinal use. Fenugreek has been traditionally used as a carminative, demulcent, expectorant, laxative and stomachic. Also used against bronchitis, fevers, sore throats, wounds, swollen glands, skin irritations, diabetes, ulcer, as lactagogue to promote lactation (Simon *et al.,* 1984).

Fenugreek seeds by nature have various components which posses medicinal properties. The proximate analysis of fenugreek seeds (Table-6 and Fig.7), shows that, it is rich in dietary fiber, also contains high amounts of saponins, diosgenin, trigonelline, 4-hydroxyisoleucine, *etc*. Fiber induces satiety (stomach fullness), replaces calories, increases chewing time, reduces appetite which intern reduces overeating, avoids over weight. The fenugreek favors the intraluminal binding increases fecal sterol and bile acid excretion. This leads to reduced circulation of lipoproteins apart from cholesterol which are the main agents of atherogenecity. The excretion of sterols depletes the storage cholesterol. Fenugreek possesses significant hypolipidemic or hypocholesterolemic property (Srinivasan *et al*., 2004) and thus qualifies for an investigation of an antilithogenic effect under lithogenic conditions.

Table 6: Proximate analysis of fenugreek seeds

Components	**Values**
Moisture (%)	11.44
Ash (%)	3.9
Protein (%	27.5
Fat (%)	6.71
Total fiber (%)	51.2
Soluble dietary fiber (%)	30.6
Insoluble dietary fiber (%)	20.6
Saponins (%)	5.12
Total phenols (mg of gallic acid equivalent/g of sample)	85.88

*All the values are in dry weight basis

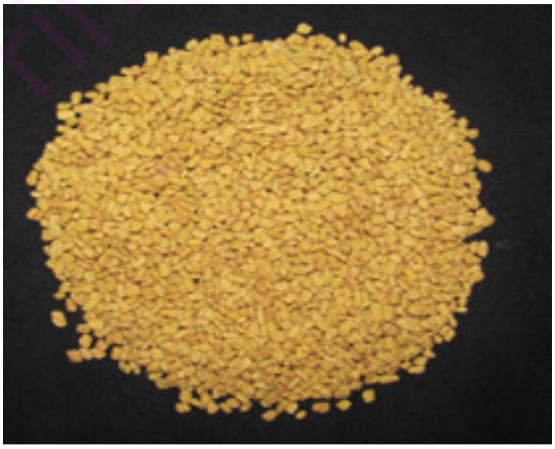


Fig.7 Fenugreek seeds used in the study

4.0 SCOPE OF PRESENT STUDY

CGS disease is one of the major contemporary health problems. CGSs are abnormal solid mass that were formed by the mixture of cholesterol crystals, mucin, calcium bilirubinate, and proteins. CGS is the result of a complex interaction of genetic and environmental risk factors. Discoveries linking gene transcription, protein function, lipid metabolism, and regulation of biliary lipid secretion in the formation of CGSs provide impetus to understand the disease. This disease is one of the most common with the prevalence of 10–15% in adults in Europe and the USA. Higher prevalence is seen in Mexican Americans, lower in Asians (overall ranging from 3% to 15%) and almost absent (less than 5%) in Africans. The prevalence is very high in some ethnic groups: 73% of female Pima Indians aged > 25 years, 35% in Chilean Mapuche Indians. The prevalence of gallstone disease is higher among females (4.2%) than males (1.88%) with total gallstone disease (3.1%) in India.

Several experimental animal model systems have been employed for understanding the pathogenesis and possible approach to prevention and cure of gallstones. Considerable research has gone into examining the influence of dietary components like proteins, carbohydrates, fiber, fat, particularly cholesterol and excess calorie intake on the pathogenesis of CGSs. It has been shown that experimental animals on diet containing casein are more prone to CGS formation than those on soya protein. Likewise, animals on diets containing polyunsaturated fat like fish oil have exhibited lower incidence of gall stone compared to those diets with saturated fat. It is very well known that dietary fiber is beneficial in preventing CGS formation. On the other hand, less attention has been paid towards the possible role of spices or their active principles in influencing CGS formation.

Among spices – turmeric, red pepper, fenugreek, garlic and onion were well documented for their hypolipidemic potential in various experimental animal models. The beneficial hypocholesterolemic property of the spices – turmeric and red pepper were attributable to their active principles - curcumin and capsaicin respectively. The hypocholesterolemic influence of dietary curcumin and capsaicin was found to be

mediated through a stimulation of the activity of liver CYP7A1, an enzyme having a regulatory role in cholesterol catabolism. Spice principles – curcumin and capsaicin, and spices fenugreek and onion have also been shown to be cholagoguic agents. It is believed that formation of CGS in the gall bladder is preceded by a supersaturation of bile with cholesterol. Hence, lowering of cholesterol concentration in the bile could prevent its supersaturation. A cholesterol lowering agent such as spices may therefore be able to reduce the incidence of CGS.

Studies in our laboratory on experimental induction of CGSs in mice and hamsters by feeding a lithogenic diet have revealed that the incidence of gallstones is 55-75% lower when the animals are maintained on 0.5% curcumin or 0.005% capsaicin containing diet. Biliary cholesterol concentration was also significantly reduced by spice principles feeding. The CSI which was 1.56 in animals fed atherogenic diet alone was considerably reduced to 0.54 and 0.35 in curcumin and capsaicin supplemented groups. The CSI of bile was also reduced significantly by curcumin feeding. Animal studies have also revealed significant regression of preformed CGS by these spice principles in a 10 week mice feeding trial. A 5 week feeding of curcumin and capsaicin resulted in 45 and 64% regression in preformed CGS, while the regression was still higher with 10 week feeding of these spice principles. The anti-lithogenicity of curcumin and capsaicin was considered to be due not merely to their ability to lower CSI, but also to their influence on biliary proteins. The investigation on the involvement of biliary proteins in cholesterol crystal nucleation revealed that in an *in vitro* bile model, LMW biliary proteins of the lithogenic diet fed animals have a pro-nucleating activity. On the contrary, LMW biliary proteins of the animals fed curcumin or capsaicin along with lithogenic diet showed a potent anti-nucleating activity.

The possibility of such a beneficial prevention and regression of CGSs by other known hypocholesterolemic spices remains to be examined. Among these potential agents fenugreek seeds were documented to have cholesterol lowering property. Fenugreek (*Trigonella foenum-graecum*) seed is one among the common spices which are esoteric food adjuncts being used to enhance the organoleptic properties of food. This seed spice

was also employed for medicinal purpose in many traditional systems as antibacterial, gastric stimulant, against anorexia, antidiabetic agent and as a galactogogue. In recent decades, several health beneficial physiological attributes of fenugreek seeds have been experimentally evidenced in animal studies as well as human trials. These include antidiabetic effect, hypocholesterolemic influence, antioxidant potency, digestive stimulant action, hepatoprotective effect, *etc*. Among these beneficial physiological effects, the antidiabetic and hypocholesterolemic property of fenugreek, both of which were mainly attributable to the intrinsic dietary fiber constituent, have promising nutraceutical value. It was proposed to investigate the antilithogenic property of dietary fenugreek seeds in experimental animal model both with respect to formation of CGSs and regression of CGSs.

Objectives of the proposed study

1. To evaluate the beneficial effect of dietary fenugreek seeds on CGS disease (CGS) with respect to:

a) Influence of fenugreek seeds on the incidence and severity of CGS
b) Influence of fenugreek seeds on regression of pre-established CGS

2. To study the mechanism of anti-lithogenic effect of fenugreek by examining the cholesterol nucleating factors such as proteins in bile, making use of *in vitro* models of cholesterol saturated bile.

3. To examine the synergistic / additive antilithogenic influence of a combination of fenugreek and another known hypocholesterolemic spice.

Reduction of atherogenic diet induced cholesterol gallstone formation in mice by dietary fenugreek (*Trigonella foenum-graecum*) seeds

Introduction

Fenugreek (*Trigonella foenum-graecum*) is a leguminous spice which is extensively cultivated as a food crop in India. It is a component of diet preparations in several Indian cuisines, often used to enhance the flavor and to stimulate appetite. It is also used as herbal medicine in many parts of the world. Seeds of fenugreek have many medicinal applications to derive antidiabetic (Srinivasan, 2006) and hypocholesterolemic effects (Srinivasan *et al*., 2004). Fenugreek seeds contain large amounts of dietary fiber (48%) which is responsible for the hypocholesterolemic and antidiabetic potential. This spice seeds also contain saponins (4.8%) and the alkaloid trigonelline (0.37%) which may also have a role in hypocholesterolemic activity (Srinivasan, 2006). An unusual amino acid 4-hydroxyisoleucine isolated from fenugreek possesses significant insulin secretagogue property (Narender *et al*., 2006).

Cholesterol gallstone (CGS) disease is a highly prevalent gastroenterological disorder resulting from alteration in hepatic and biliary cholesterol homeostasis. CGS is a multi-factorial disease involving both environmental as well as genetic factors (Johnston & Kaplan, 1993, Juvonen, 1994). The pathogenic conditions that generally precede the occurrence of CGS are lithogenic bile, gallbladder stasis and short nucleation time. Lithogenicity of bile is determined by relative concentrations of cholesterol, bile acids and phospholipid in bile. Generally, lithogenic bile occurs with disruption of cholesterol homeostasis leading to increased cholesterol secretion and subsequent supersaturation of bile with cholesterol (Apstein & Carey, 1996; Marzolo *et al*., 1990).

Several dietary components such as the nature of protein and fat are known to influence CGS (Ozben, 1989; Scobey *et al*., 1991); diets containing polyunsaturated fat show a lower incidence of CGS compared to those on diets with saturated fat (Scobey *et al*., 1991). It is very well known that dietary fiber is beneficial in preventing CGS formation

(Judd, 1985; Ebihara & Kiriyama, 1985). It has been shown that soluble dietary fiber from *konjac mannan* helps to reduce CGS incidence in mice (Ebihara & Kiriyama, 1985).

Among spices which are valued for their organoleptic as well as medicinal properties, fenugreek seeds, garlic, onion, red pepper or its pungent principle – capsaicin and turmeric or its yellow principle – curcumin have been widely documented for their hypocholesterolemic as well as lipid lowering properties (Srinivasan *et al*., 2004). Previously, it has been demonstrated that dietary curcumin (0.5%) and capsaicin (0.015%) are effective in inhibiting experimental CGS induction and also in regressing the pre-formed CGS in mice (Hussain & Chandrasekhara, 1993; 1994). The health beneficial antilithogenic influence of dietary garlic and onion has been reported (Vidyashankar *et al*., 2009). Since formation of CGS in the gallbladder is preceded by supersaturation of bile with cholesterol, the hypocholesterolemic spices may serve as potential dietary adjuncts to reduce the incidence of CGS and hence it is very relevant to study their effect during lithogenic diet induced CGS. With the aim of extending our knowledge on the effect of dietary fenugreek seeds on the possible anti-lithogenic influence mediated through hypocholesterolemic effect, an animal study was carried out with experimental induction of cholesterol gallstones in mice. In this context, the antilithogenic potential of fenugreek seeds was evaluated both in their raw and heat processed forms during lithogenic diet induced CGS formation in mice.

Materials and methods

Chemicals and Reagents

Cholesterol, bile salts, di-palmitoyl phosphatidyl choline, triolein, heparin, Bovine serum albumin (BSA), 3α-hydroxysteroid dehydrogenase**,** NAD**,** standard bile acids, and alpha cellulose were purchased from Sigma-Aldrich Chemicals Co., St. Louis, MO, USA. All other chemicals and solvents were obtained from SISCO Research Laboratory (Mumbai, India). Casein was purchased from Nimesh Corporation (Mumbai, India). All solvents used were of analytical grade and were distilled prior to use. Fenugreek seeds were purchased from local market and pulverized.

Animal diets

The animals were fed with AIN-76 semi-purified diet. The basal control diet consisted of: sucrose, 65%; casein, 20%; cellulose, 5%; AIN-76 mineral mix, 3.5%; AIN-76 vitamin mix, 1%; DL-methionine, 0.3%; choline chloride, 0.2% and refined peanut oil, 5%. Lithogenic diet was prepared by supplementing 0.5% cholesterol and 0.125% bile salts (1:1 mixture of sodium cholate and sodium deoxycholate) to the AIN-76 basal diet substituting same quantity of sucrose. Diets were prepared by mixing the ingredients in a mechanical mixer and pellets were prepared using hand-operated pelletizer. Diets were stored at 4°C in air-tight containers. The test diets were prepared by incorporating the fenugreek seed powder at three different levels namely, 5, 10 and 15 % (w/w) in the lithogenic diet. The incorporation of the fenugreek was at the expense of sucrose. In the second set of experiments, the test diets were prepared by incorporating the fenugreek seed powder, both raw and roasted at 12% levels (w/w) in the lithogenic diet. The incorporation of the fenugreek was at the expense of sucrose. Fenugreek seeds were roasted in an open pan for exactly 10 min under controlled conditions at a pan temperature of 60-80°C.

Animal treatment

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort and with due approval from the Institutional Animal Ethics Committee. Five week old male albino mice [OUTB / Swiss Albino / Ind / Cft(2c)] produced in our Experimental Animal Production Facility Unit, weighing 25 ± 2 g were grouped and housed 4-5 mice per cage with saw dust as bedding material. Animals were maintained in a room where the temperature was maintained at 22 ± 2^0 C with relative humidity of about 60 ± 5% and having regular 12 h cycle of day and night. The animals were given free access to food and water. Animal weights were recorded every week till the end of the experiment.

To study the effect of dietary fenugreek on the incidence of CGS as a function of dietary dose, seventy five mice were divided in to five groups with 15 animals per group. The animals were fed with different diets viz., Basal control diet, Lithogenic diet (HCD), and fenugreek (5, 10 and 15%) containing Lithogenic diets for a period of 10 weeks. In a

separate experiment, to study the effect of heat treatment on the anti-lithogenic potential of fenugreek seeds, eighty five mice were divided in to four groups, each group consisting of 20 animals and fed with different diets consisting of Basal control diet, Lithogenic diet (HCD), Lithogenic diet containing raw fenugreek seed powder (12%) and Lithogenic diet containing roasted fenugreek seed powder (12%) for 10 weeks.

Collection of gallbladder and scoring of CGS

At the end of the feeding duration, the animals were fasted overnight and sacrificed under ether anesthesia. Blood was drawn immediately by cardiac puncture and the serum was separated by centrifugation for further analysis. Cholecystectomy was performed and gallbladders were carefully collected and trimmed off any extraneous tissue. Liver was quickly excised, washed with ice-cold saline and blotted dry, weighed and stored at -20°C till further analysis. The volume of bile was noted and the weight of gallbladder along with stones was recorded. The gallbladders placed on an illuminator were evaluated for CGS under magnifying lens for the presence of gallstones by four individuals unaware of dietary treatments. The grading of stones was done on a five point scale (Akiyoshi *et al*., 1986). The bile from the gallbladders was appropriately pooled and stored at -20°C till further analysis.

Analysis of lipids

Biliary lipids were extracted by the method of Bligh and Dyer (1959). The upper methanolic phase was used for the estimation of total bile acids using 3α-hydroxysteroid dehydrogenase (Turley & Dietschy, 1970). The lower chloroform layer was used for the analysis of cholesterol and phospholipid. Serum and liver lipids were extracted by the method of Folch *et al*., (1957). Cholesterol levels were quantitated by the method of Searcy and Bergquist (1960). The HDL-cholesterol and non-HDL (LDL + VLDL) cholesterol in serum were estimated by adapting the protocol given by Warnick and Albers (1978). The method involved the precipitation and separation of HDL from LDL+VLDL by the use of heparin and manganese chloride. HDL-cholesterol in the supernatant was estimated as described before. Phospholipids were measured by ferrous ammonium thiocyanate method using di-palmitoyl phosphatidyl choline as reference standard as

described by Stewart (1980). Triglycerides were estimated according to the method described by Fletcher (1968), using triolein as standard. Cholesterol Saturation Index of the bile was calculated using the values of cholesterol, phospholipids, total lipids and bile acids in bile (both relative and total lipid concentrations) as described by Carey (1978).

Statistical analysis

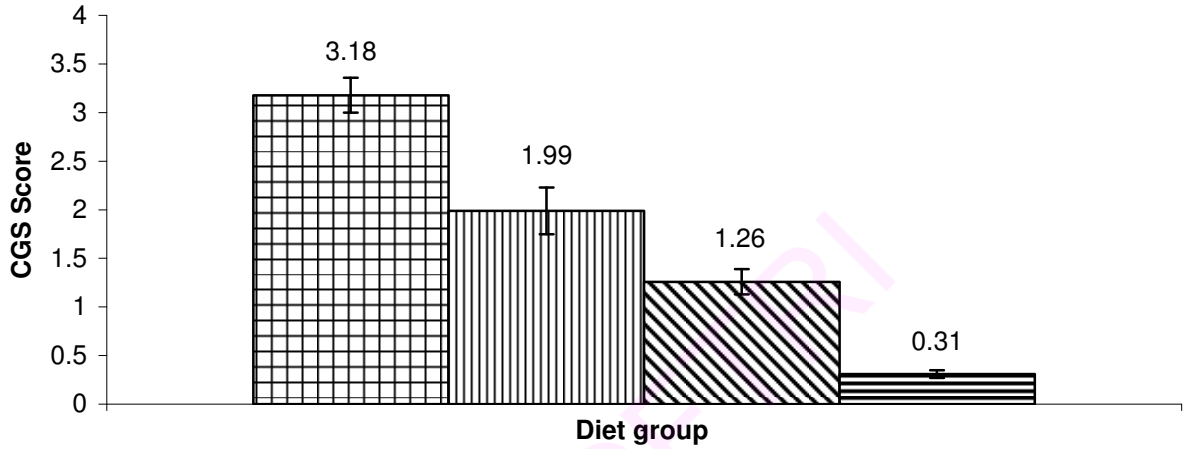
Statistical analysis was carried out using Graph pad prism statistical software. Results were analyzed by one way ANOVA and the significance level was calculated using Tukey Kramer multiple comparison test and differences were considered as significant at $P<0.05$.

Results

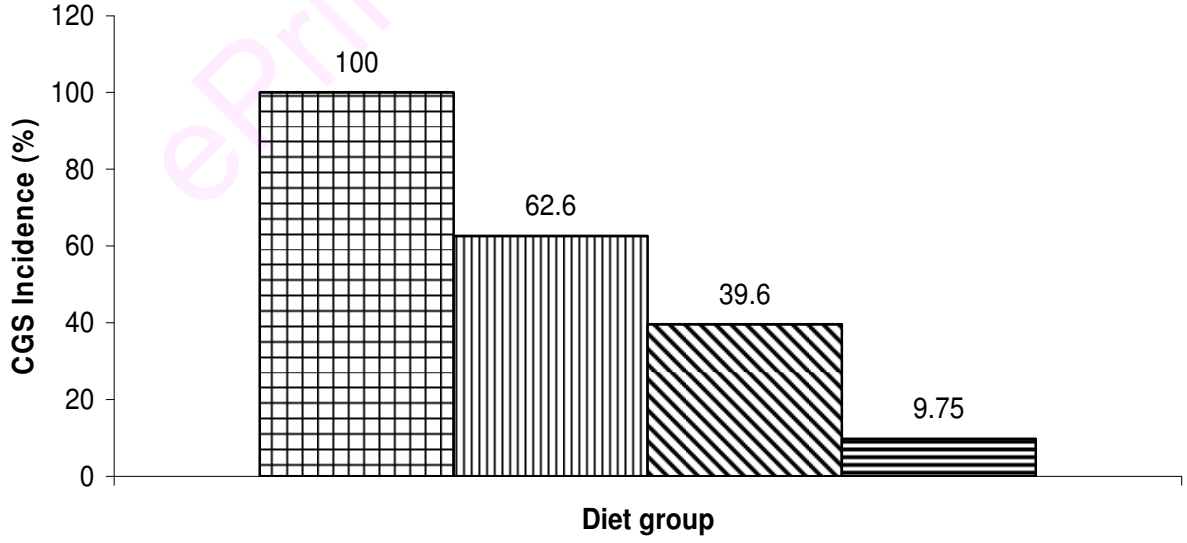
Effect of feeding fenugreek seeds on the incidence of CGS during experimental induction

Effect of dietary fenugreek seeds on CGS induction in mice was carried out by feeding lithogenic diet with and without fenugreek seed powder for a period of 10 weeks. Animals fed with basal control diet showed no sign of any cholesterol crystal or stone in the gall bladder, whereas all the animals in lithogenic diet (HCD) group showed CGS (Fig.1). Animals fed with lithogenic diet supplemented with fenugreek seed powder showed significant reduction in the incidence of CGS. The % incidence of CGS was calculated by taking the incidence in the lithogenic diet group as 100%. All the three dietary doses of fenugreek were effective in reducing the formation of CGS, the reduction in CGS formation being 37.4%, 60.4% and 90.2% in 5, 10 and 15% dietary fenugreek groups respectively. There were no significant differences in the body weight of animals between various diet groups at the end of feeding period of 10 weeks (Table-1). The liver weight was higher (by 65%) in lithogenic diet fed group as compared to basal control group. Dietary fenugreek significantly countered this increase in liver weight. The liver weights in these groups were 121–132% of the value in basal control group.

A



B



C

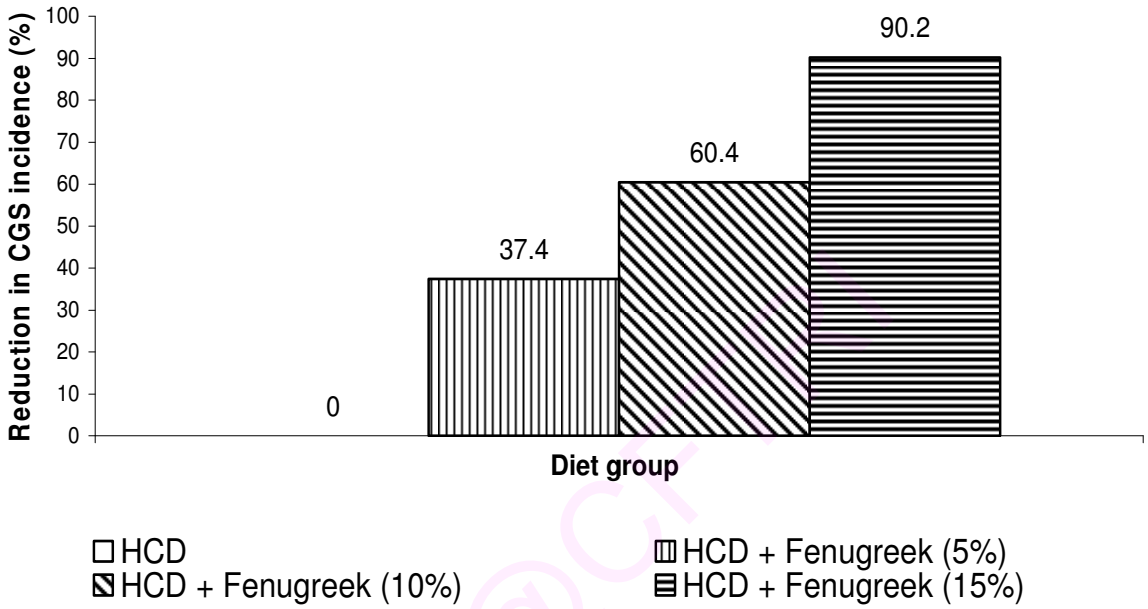


Fig.1. Effect of dietary fenugreek seeds on the incidence of CGS during experimental induction in mice

Values are mean ± SEM of 15 mice /group. HCD: High cholesterol diet.

Values in the three dietary fenugreek groups were statistically different when compared to HCD group ($P < 0.01$). CGS score: CGS grading was done by four individuals on a 5-point scale (0-4)

Table 1. Effect of dietary fenugreek seeds on liver weight during experimental induction in mice

Dietary group	Body weight (g)	Liver weight (g)	Liver weight (g/100g b.w.)
Basal Control	40.4 ± 1.92	1.60 ± 0.13	3.96 ± 0.32
HCD	42.5 ± 1.10	2.76 ± 0.12[a]	6.52 ± 0.31[a]
HCD + Fenugreek (5%)	43.3 ± 0.80	2.17 ± 0.07[b]	5.01 ± 0.18[b]
HCD + Fenugreek (10%)	43.4 ± 1.30	2.28 ± 0.08[b]	5.22 ± 0.16[b]
HCD + Fenugreek (15%)	41.3 ± 0.61	1.96 ± 0.05[b]	4.79 ± 0.12[b]

Values are mean ± SEM of 15 mice /group. HCD: High cholesterol diet.

a: Statistically significant compared to Basal control group ($P < 0.01$)

b: Statistically significant compared to HCD group ($P<0.01$).

Effect of feeding fenugreek on serum lipids during CGS induction

The serum lipid profile in animals fed fenugreek during experimental induction of CGS is presented in Table-2. The lithogenic diet feeding caused hypercholesterolemia where serum total cholesterol increased by 1.1-fold compared to basal control animals. Feeding of fenugreek along with lithogenic diet prevented the raise in serum cholesterol levels by 26, 36, and 31 % in 5% fenugreek, 10% fenugreek and 15% fenugreek groups respectively. The results revealed that LDL-cholesterol levels in particular were significantly reduced in all the fenugreek treatments. The reduction was higher at 10% level (48.1%) while it was 35 and 41% in 5 and 15% fenugreek respectively. The HDL-cholesterol levels slightly improved (by around 10%) in the fenugreek treated animals. Phospholipid content of serum was significantly reduced by lithogenic diet (by about 25%), whereas feeding of fenugreek at any of the three dietary levels completely countered this decrease in phospholipid content. The triglyceride content was 19% higher in the lithogenic diet group compared to basal control group. Feeding of fenugreek did not have any influence on the triglycerides. Feeding of lithogenic diet significantly increased the cholesterol: phospholipid ratio in serum (1.197 as compared to 0.47 in basal control) and dietary fenugreek significantly reduced the increased cholesterol: phospholipid ratio (0.686, 0.569 and 0.59 in the three dose levels).

Effect of feeding fenugreek on liver lipids during CGS induction

Liver lipid profile in animals fed fenugreek at three levels during experimental CGS induction is given in Table-3. Total cholesterol content was increased in lithogenic diet group by around 3-fold compared to basal control group. Feeding of fenugreek seed powder along with lithogenic diet significantly countered this increase in hepatic cholesterol content, the decrease compared to HCD group being 47, 61 and 64% in fenugreek 5%, 10% and 15% groups respectively. Hepatic phospholipid content was decreased in lithogenic diet group compared to basal control animals, the decrease being around 45%. This decrease in hepatic phospholipid concentration was significantly countered by dietary fenugreek seed powder (40 - 61% increase compared to HCD group). The elevated cholesterol: phospholipid ratio as a result of lithogenic diet was brought down significantly by fenugreek feeding. Feeding of lithogenic diet increased

Table 2. Effect of dietary fenugreek on serum lipid profile during CGS induction in mice

Dietary group	Cholesterol			Phospholipids	Triglycerides	C:P Ratio
	LDL	HDL	Total			
Basal Control	70.5 ± 5.3	50.3 ± 3.3	120.8 ± 5.21	256.9 ± 11.2	147.2 ± 7.2	0.470
HCD	190.1 ± 6.21[a]	43.6 ± 2.0	231.2 ± 4.70[a]	193.2 ± 10.8[a]	175.0 ± 9.0[a]	1.197[a]
HCD + Fenugreek (5%)	123.6 ± 5.42[b]	48.3 ± 1.96	171.8 ± 3.11[b]	250.4 ± 9.60[b]	177.6 ± 8.2	0.686[b]
HCD + Fenugreek (10%)	98.6 ± 4.43[b]	49.3 ± 2.0	148.8 ± 5.57[b]	261.3 ± 10.5[b]	179.2 ± 5.2	0.569[b]
HCD + Fenugreek (15%)	111.8 ± 4.99[b]	48.3 ± 1.71	160.6 ± 8.22[b]	272.2 ± 11.6[b]	181.3 ± 9.5	0.590[b]

Values (expressed as mg/dL) are mean ± SEM of 5 samples /group, each sample constituting 3 mice.

HCD: High cholesterol diet; C:P Ratio: Cholesterol: Phospholipid ratio

a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

Table 3. Effect of dietary fenugreek on liver lipid profile during CGS induction in mice

Dietary group	Total Cholesterol	Phospholipids	Triglycerides	Total lipids
Basal Control	14.9 ± 0.9	37.8 ± 2.2	25.5 ± 1.8	82.5 ± 4.5
HCD	45.9 ± 3.5	20.9 ± 1.25[a]	54.9 ± 3.96[a]	161.7 ± 11.1[a]
HCD + Fenugreek (5%)	24.3 ± 1.1[b]	29.3 ± 0.97[b]	51.3 ± 1.44	126.4 ± 4.3[b]
HCD + Fenugreek (10%)	18.1 ± 1.2[b]	33.7 ± 1.60[b]	51.7 ± 2.20	114.5 ± 6.6[b]
HCD + Fenugreek (15%)	16.4 ± 0.9[b]	31.5 ± 1.10[b]	44.3 ± 1.90[b]	105.8 ± 9.2[b]

Values (mg/g) are mean ± SEM of 15 mice /group.

HCD: High cholesterol diet

a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

hepatic triglyceride content and inclusion of fenugreek at 15% level in the diet significantly countered the raise in triglyceride content (19.3% decrease). Hepatic total lipid level which was elevated in lithogenic diet feeding was significantly countered by dietary fenugreek. The reduction in total lipid brought about by dietary fenugreek was 22, 29 and 35% in 5, 10 and 15% dietary doses, respectively.

Effect of feeding fenugreek on biliary lipid profile during CGS induction

Bile is the major route through which cholesterol and its metabolic products – bile acids are excreted. Biliary lipid profile in animals fed fenugreek in addition to lithogenic diet is given in Table-4. Lithogenic diet increased the cholesterol content of bile by 4.5-fold. Dietary fenugreek countered the increase in biliary cholesterol content significantly. The reduction was by 67, 74 and 73.5% in 5, 10 and 15% fenugreek levels respectively compared to lithogenic diet group. In other words, the lithogenic diet induced increase in biliary cholesterol was almost completely countered by dietary fenugreek at the two higher levels. Besides an increase in cholesterol concentration, any deleterious alteration in the phospholipid and bile acids in the bile may result in the precipitation of cholesterol in bile. Phospholipid concentration in the bile was increased in the lithogenic diet group by 1.9-fold compared to basal control animals. Parallel to the beneficial cholesterol lowering influence of dietary fenugreek, biliary phospholipid content was also decreased in fenugreek treatment along with lithogenic diet. This decrease was of the order of 22-33%. Bile salts form a major part of the bile solids and majority of the cholesterol is excreted from the body after converting into bile acids in liver and subsequent secretion into bile. Bile acid content in the bile remained essentially unaltered in the lithogenic diet group. Biliary bile acid levels were also not affected by dietary fenugreek under lithogenic condition.

In order to evaluate the relevance of the biliary lipid profile in CGS formation, cholesterol: phospholipid (C/PL) and cholesterol: bile acid (C/BA) ratios in bile were calculated (Fig.2). The higher ratios of C/PL and C/BA are the indicators of lithogenic bile and these ratios are very important in keeping the cholesterol in soluble form. Incorporation of fenugreek in the diet had a marked effect on the cholesterol:

Table 4. Effect of dietary fenugreek on biliary lipid profile during CGS induction in mice

Dietary group	Cholesterol	Phospholipid	Bile acids	Total Lipid
	mM			g/dL
Basal Control	7.42 ± 0.35	16.3 ± 1.21	155.0 ± 11.2	9.09 ± 0.36
HCD	33.6 ± 0.87[a]	39.4 ± 2.6[a]	160.2 ± 10.3	12.1 ± 0.72[a]
HCD + Fenugreek (5%)	11.2 ± 0.28[b]	32.6 ± 1.5[b]	141.1 ± 8.1	9.76 ± 0.68[b]
HCD + Fenugreek (10%)	8.73 ± 0.44[b]	31.9 ± 2.0[b]	153.2 ± 11.5	10.2 ± 0.46[b]
HCD + Fenugreek (15%)	8.91 ± 0.21[b]	31.1 ± 2.5[b]	137.0 ± 13.0	9.36 ± 0.52[b]

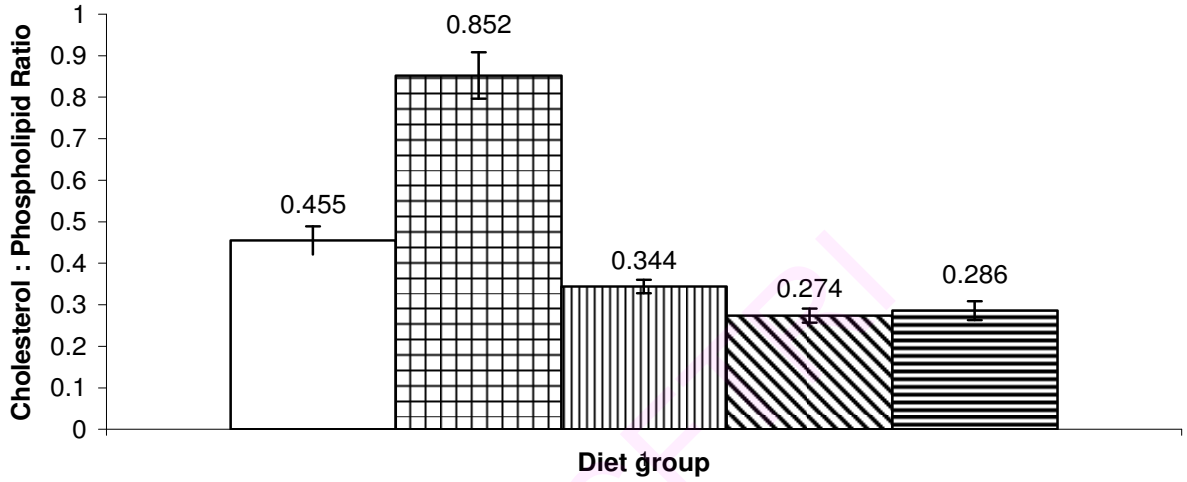
Values are mean ± SEM of 4 samples /group, each sample constituting 3 mice.

HCD: High cholesterol diet.

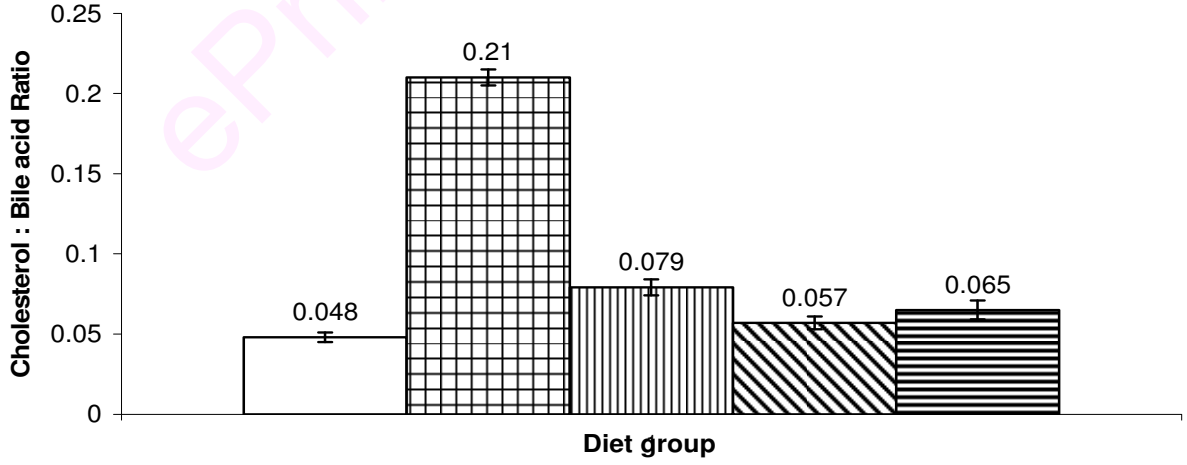
a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

A



B



C

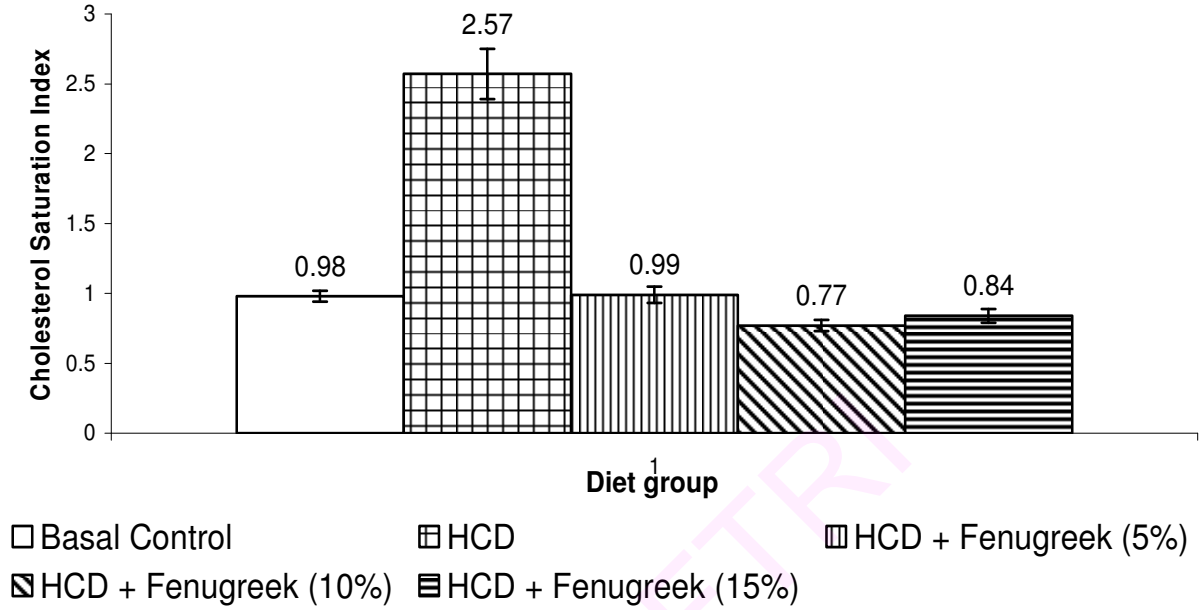


Fig. 2. Effect of dietary fenugreek on (A) Cholesterol: Phospholipid ratio, (B) Cholesterol: Bile acid ratio and (C) Cholesterol Saturation Index in bile during CGS induction in mice

Values are mean ± SEM of each group. HCD: High cholesterol diet. Values in the HCD group were statistically different when compared to Basal control group ($P < 0.01$). Values in each of the three dietary fenugreek groups were statistically different when compared to HCD group ($P < 0.01$)

phospholipid ratio in bile. As a result of a decreasing influence on biliary cholesterol concentration, the cholesterol: phospholipid ratio decreased significantly with the fenugreek treatment at all the three levels. Cholesterol: phospholipid ratio in lithogenic diet group was 0.85, where as it was 0.34, 0.27, and 0.29 (amounting to a decrease of 60%, 66% and 66%) in 5, 10 and 15% fenugreek levels respectively. Cholesterol: bile acid ratio which was 0.21 in the lithogenic group (as compared to 0.048 in basal control group), was considerably lowered in fenugreek fed group along with high cholesterol. The decreases in cholesterol: bile acid ratio was 62, 73 and 69% in 5%, 10% and 15% fenugreek groups respectively, as compared to HCD group. Cholesterol saturation index also decreased significantly with fenugreek treatment at the three dietary levels. The Cholesterol saturation index which was 2.57 in lithogenic diet group, decreased to 0.99, 0.77 and 0.84 in the fenugreek treatments with a decrease of 61, 70 and 67% in 5, 10 and 15% fenugreek levels, respectively.

Effect of heat processing on the antilithogenic potential of fenugreek seeds with respect to reducing the experimental induction of CGS.

In order to compare the antilithogenic potential of heat processed fenugreek seeds with that of unprocessed raw seeds, a separate animal study was conducted using one particular dietary dose of fenugreek seeds, namely 12%. There was a significant favorable effect as a result of incorporation of fenugreek seeds on the incidence of CGS during experimental induction by feeding high cholesterol diet for 10 weeks. The antilithogenic influence of heat processed fenugreek was comparable to that of raw fenugreek seeds. The incidence of CGS was reduced by 73-80% in fenugreek treatment (Fig.3). The increase in liver weight produced by lithogenic diet was effectively countered (by 33%) by inclusion of fenugreek in the lithogenic diet (Table-5). There was no marked difference in kidney, heart and spleen weights between the treatments.

The beneficial effect of dietary fenugreek on serum cholesterol was evident in both heat processed and raw fenugreek seeds and this was particularly seen in the LDL-cholesterol fraction where there was a 52 - 54% reduction compared to lithogenic diet group (Table-6). The effect on total serum cholesterol was around 37 - 39% in raw and roasted fenugreek treatments. Both heat processed and raw fenugreek treatment

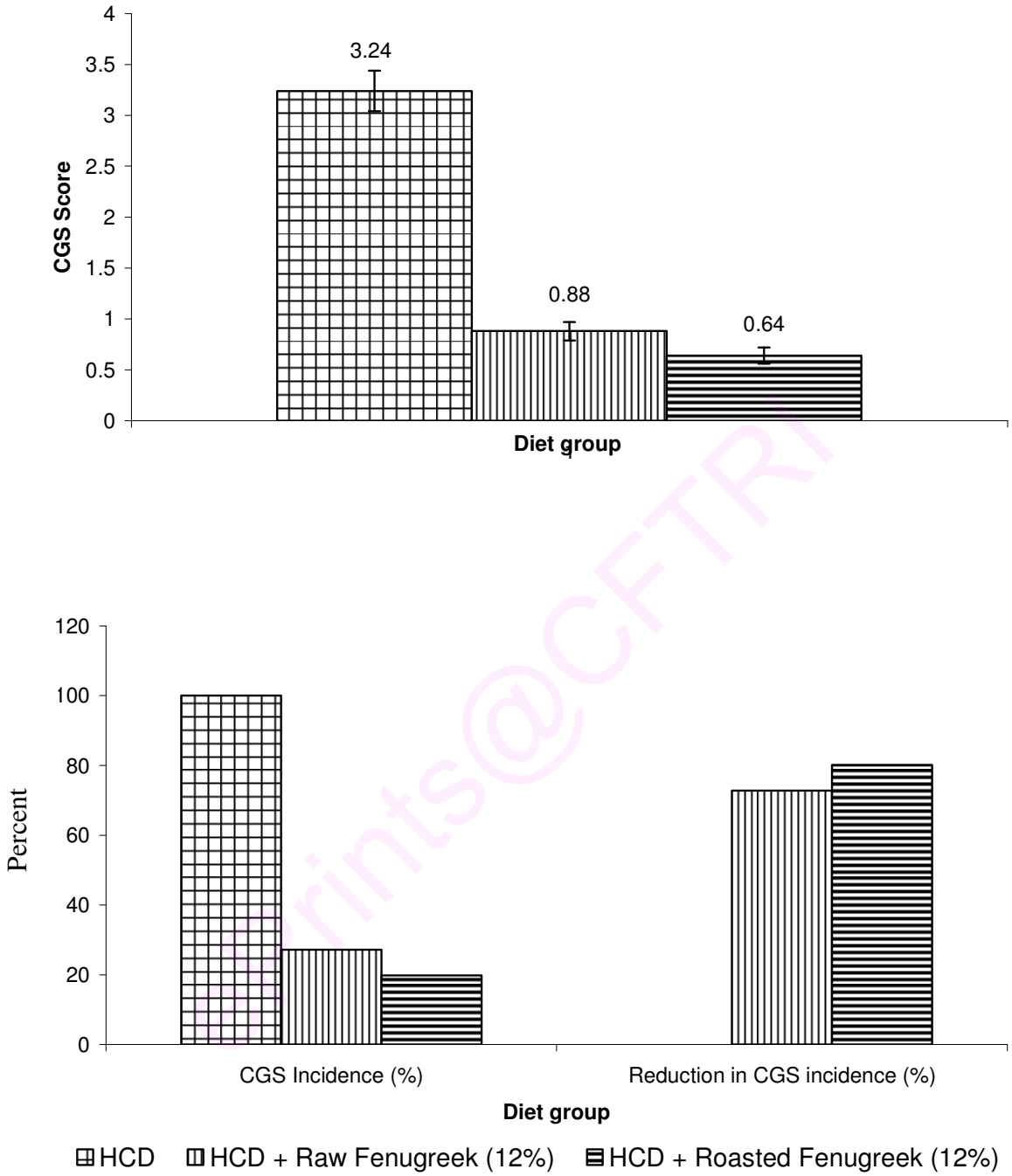


Fig. 3. Antilithogenic potential of dietary roasted fenugreek seeds during experimental induction of CGS in mice

Values are mean ± SEM of 25 animals /group. HCD: High cholesterol diet

Values in the two dietary fenugreek groups were statistically different when compared to HCD group ($P < 0.01$). CGS score: CGS grading was done by four individuals on a 5-point scale (0-4)

Table 5. Effect of dietary roasted fenugreek on body weight and organ weights during CGS induction in mice

Dietary group	Body Wt. (g)	Liver (g)	Liver g/100g b.w.	Kidney (mg)	Heart (mg)	Spleen (mg)
Basal Control	40.4 ± 1.92	1.60 ± 0.13	3.96 ± 0.32	409.8 ± 8.57	144.6 ± 3.86	118.7 ± 8.00
HCD	40.5 ± 0.63	2.47 ± 0.06[a]	6.09 ± 0.11[a]	428.7 ± 10.5	172.6 ± 3.5	126.3 ± 9.6
HCD + Raw Fenugreek (12%)	43.1 ± 0.6[b]	1.74 ± 0.02[b]	4.06 ± 0.06[b]	441.3 ± 10.3	183.5 ± 3.5	124.3 ± 8.9
HCD + Roasted Fenugreek (12%)	43.5 ± 0.6[b]	1.75 ± 0.03[b]	4.03 ± 0.07[b]	428.3 ± 8.37	184.3 ± 5.2	124.2± 8.10

Values are mean ± SEM of 25 animals /group. HCD: High cholesterol diet,

a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

Table 6. Effect of dietary roasted fenugreek on serum lipid profile during CGS induction in mice

Dietary group	Cholesterol			Phospholipids	Triglycerides	C:P Ratio
	LDL	HDL	Total			
Basal Control	76.4 ± 5.8	52.3 ± 4.3	128.7 ± 7.1	256.9 ± 11.2	177.8 ± 10.2	0.501
HCD	192.9 ± 8.6[a]	55.5 ± 3.1	248.4 ± 7.2[a]	161.4 ± 6.1[a]	238.8 ± 16.5[a]	1.539[a]
HCD + Raw Fenugreek (12%)	93.1 ± 5.1[b]	63.2 ± 4.8[b]	156.1 ± 4.7[b]	210.9 ± 7.1[b]	209.4 ± 10.5	0.740[b]
HCD + Roasted Fenugreek (12%)	88.9 ± 4.3[b]	63.0 ± 2.1[b]	152.1 ± 4.8[b]	228.7 ± 9.1[b]	222.0 ± 14.2	0.665[b]

Values (given as mg/dl) are mean ± SEM of 6 samples /group, each sample constituting of 4 mice.

HCD: High cholesterol diet

a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

markedly improved serum HDL-cholesterol, the increase being around 12%. Phospholipid content in serum which was decreased in high cholesterol treatment was significantly restored when the animals were fed with either raw or roasted fenugreek seeds. Serum phospholipid concentration was increased by 31-42% in dietary fenugreek treatment. Both heat processed and raw fenugreek in the diet significantly countered the changes in liver lipid profile brought about by the lithogenic diet (Table-7). There was a significant decrease in the content of hepatic cholesterol, triglycerides and total lipid by dietary fenugreek either heat processed or raw, while the hepatic phospholipid content increased. The decrease in hepatic cholesterol was 57-60%, triglycerides content decreased by 38-43% and the decrease in total lipids was around 42% and the phospholipid content increased by 22-27% with the addition of fenugreek to the diet.

Influence of fenugreek seeds either raw or roasted on biliary lipid profile is presented in Table-8. Dietary fenugreek seeds (either raw or roasted) decreased biliary cholesterol levels by 80%. The elevated phospholipid concentration produced in the bile by lithogenic diet was also significantly countered by inclusion of either raw or roasted fenugreek seeds. Total bile acid content in the bile was not affected either by the high cholesterol diet or by dietary fenugreek in association with high cholesterol. The cholesterol: phospholipid ratio, cholesterol: bile acids ratio, and cholesterol saturation index in bile, all of which had significantly elevated in high cholesterol treatment, was decreased almost equally in raw and roasted fenugreek treatments in association with cholesterol. Cholesterol: phospholipid ratio decreased to 0.33 and 0.32 in raw and roasted fenugreek treatments respectively from 1.25 in the lithogenic group which amounted to a decrease of around 75% compared to high cholesterol treatment (Fig.4). Cholesterol: bile acids ratio decreased to 0.073 and 0.067 in raw and roasted fenugreek treatments from 0.388 in the lithogenic group which amounted to a decrease of 81 and 83% compared to high cholesterol treatment. The CSI was reduced significantly from 2.88 to 1.12 and 0.98, with a percent decrease of 61 and 66% in raw and roasted fenugreek groups, respectively. Thus, the beneficial effect of roasted fenugreek seeds on the lipid profiles of animals during experimental induction of CGS was comparable to the effect produced by raw seeds.

Table 7. Effect of dietary roasted fenugreek on liver lipid profile during CGS induction in mice

Dietary group	Total Cholesterol	Phospholipids	Triglycerides	Total lipids
Basal Control	12.9 ± 0.7	40.8 ± 3.2	25.5 ± 1.8	84.5 ± 4.7
HCD	37.7 ± 3.1[a]	25.1 ± 2.58[a]	51.9 ± 4.7[a]	168.2 ± 12.1[a]
HCD + Raw Fenugreek (12%)	16.3 ± 1.3[b]	31.9 ± 1.86[b]	29.4 ± 3.2[b]	97.6 ± 5.2[b]
HCD + Roasted Fenugreek (12%)	14.9 ± 1.5[b]	30.5 ± 1.21[b]	32.1 ± 4.1[b]	95.5 ± 7.4[b]

Values (expressed as mg/g) are mean ± SEM of 12 animals /group.

HCD: High cholesterol diet

a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

Table 8. Effect of dietary roasted fenugreek on biliary lipid profile during CGS induction in mice

Dietary group	Cholesterol	Phospholipid	Total Bile acids	Total Lipids
	mM			g/dL
Basal Control	8.12 ± 0.46	19.2 ± 1.38	145.2 ± 9.7	8.85 ± 0.21
HCD	52.4 ± 8.5[a]	42.0 ± 3.0[a]	135.1 ± 8.6	11.8 ± 0.29[a]
HCD + Raw Fenugreek (12%)	10.2 ± 1.6[b]	30.6 ± 1.6[b]	139.3 ± 8.4	9.41 ± 0.40[b]
HCD + Roasted Fenugreek (12%)	10.3 ± 1.5[b]	32.5 ± 5.2[b]	154.2 ± 9.0	10.3 ± 0.52[b]

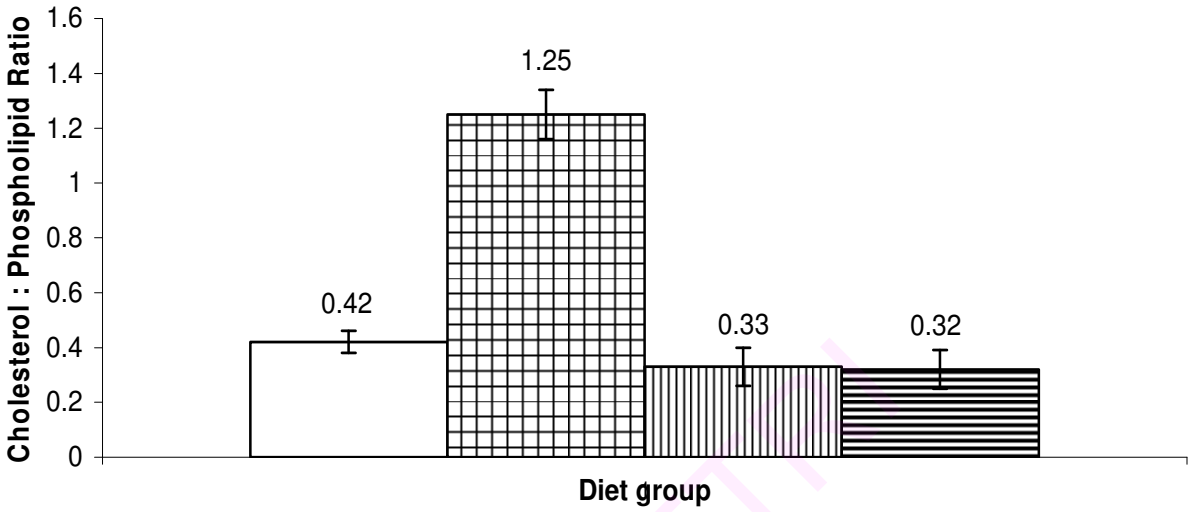
Values are mean ± SEM of 5 samples /group, each sample constituting of s animals.

HCD: High cholesterol diet

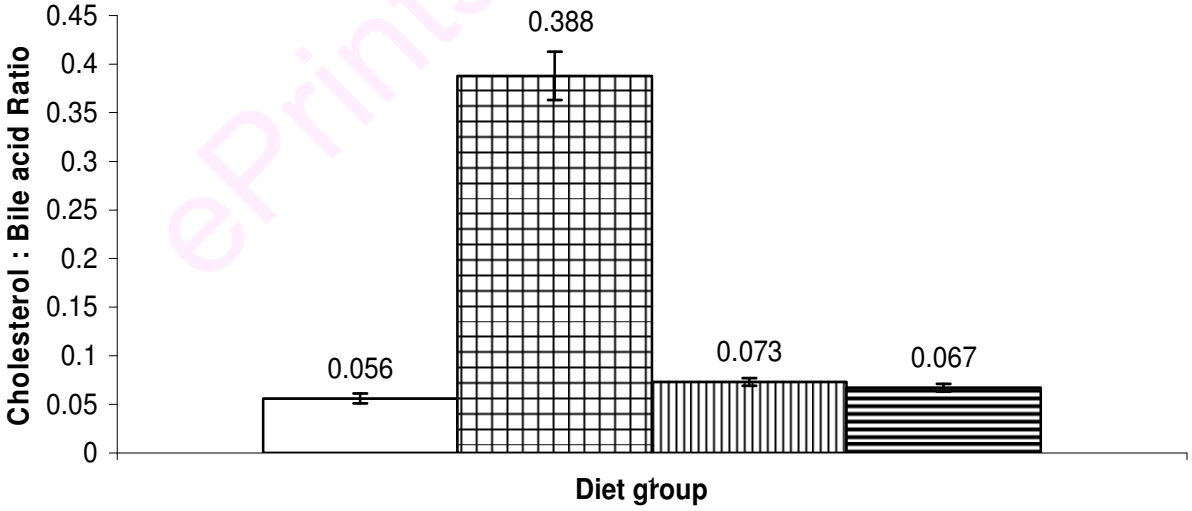
a: Statistically significant when compared to control group at $P < 0.01$

b: Statistically significant when compared to HCD group at $P < 0.01$

A



B



C

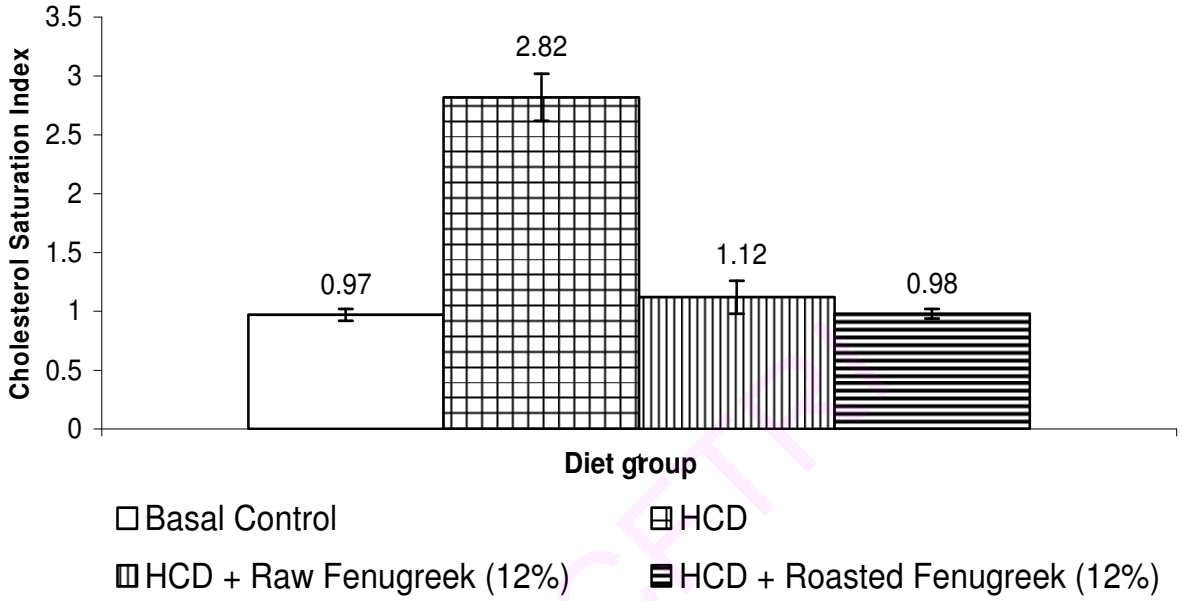


Fig.4. Effect of dietary roasted fenugreek on biliary (A) cholesterol: phospholipid ratio, (B) cholesterol: bile acid ratio and (C) cholesterol saturation index during CGS induction in mice

Values are mean ± SEM of each group. HCD: High cholesterol diet

Values in the HCD group were statistically different when compared to Basal control group ($P < 0.01$). Values in each of the two dietary fenugreek groups were statistically different when compared to HCD group ($P < 0.01$)

Discussion

The present study has documented that incorporation of fenugreek seeds in the diet has a favourable lowering effect on the incidence of CGS caused by a lithogenic diet. Fenugreek containing diet exerted a reduction in cholesterol, especially LDL-cholesterol besides increasing the contents of HDL-cholesterol and phospholipids in blood. The increase in liver cholesterol, triglycerides and hence total lipid content brought about by the lithogenic diet was markedly countered by dietary fenugreek in this animal model. Associated with such a beneficial influence on hepatic lipid profile, dietary fenugreek at all the three levels examined here countered the increase in liver weight brought about by the lithogenic diet during CGS induction, which was due to the accumulation of more lipids in this tissue.

Such a beneficial influence of dietary fenugreek on blood and liver lipid profile is in agreement with several similar observations reported earlier in other animal models as reviewed recently (Srinivasan, 2006). The hypocholesterolemic effect of fenugreek is due to fiber and saponin components of fenugreek. Defatted fraction of fenugreek seeds which essentially contains fiber and saponin is shown to exert hypocholesterolemic influence (Valette *et al*., 1984). Basch *et al.,* (2003) and Sauvaire *et al*., (1991) have related the hypocholesterolemic action to saponin content of the fenugreek seeds, which increases biliary cholesterol excretion. A marked decrease in blood cholesterol especially LDL-associated cholesterol has also been reported in human trials including diabetic subjects (Srinivasan, 2006). Fenugreek at 15, 30 and 60% dietary levels has been reported earlier to have anti-hypercholesterolemic effect in rats with attendant increase in bile acids excretion (Sharma, 1984). The present study has evidenced the anti-hyper-cholesterolemic potential of fenugreek seeds even at lower dietary levels, *viz*., 5 and 10% in experimental mice. Fenugreek is responsible for the intestinal drainage of bile acids and neutral sterols, thus favoring the reduction of serum cholesterol levels. This effect is due to fiber and saponin components of fenugreek seeds. The general mechanism of the ability of dietary fiber to lower plasma cholesterol is attributed to its physical adsorption (Trowell, 1975). The ability of the saponins to induce adsorption of bile acids by fiber may be related to its

surface activity. Saponins inhibit cholesterol absorption from intestine and helps in fecal excretion of bile acids (Sauvaire *et al*., 1991). Thus, the observed anti-lithogenic influence of dietary fenugreek seeds in the present study which is accompanied by beneficial modulation of cholesterol homeostasis in blood, liver and bile, is possibly exerted through the hypocholesterolemic action of soluble fiber and saponins present in them.

Fiber protects against the CGS formation through speeding intestinal transit and also reducing the generation of secondary bile acids (Marcus & Wheaton, 1986). Fiber supplementation to Prairie dogs maintained on lithogenic diet reduced biliary cholesterol saturation index, thus inhibiting CGS formation (Wayne *et al*., 1999). Supplementation of soluble dietary fiber promotes hepatic catabolism of cholesterol leading to an increased fecal bile acid excretion (Forman *et al*., 1968). Dietary soluble fiber of *Konjal mannom* is reported to be responsible for the inhibition of intestinal absorption of dietary cholesterol, which resulted in the improvement in biliary cholesterol supersaturation thus reducing CGS in mice (Ebihara & Kiriyama, 1985).

Dietary fenugreek is reported to increase total bile acid output which is due to an increased production of taurodeoxycholic and taurocholic acids caused by the stimulation of conversion of cholesterol to bile acids in liver (Bhat *et al*., 1985). Feeding of spice bioactive compounds having hypocholesterolemic potential, viz., curcumin and capsaicin was reported to reduce the incidence of CGS which was attendant with reduced biliary cholesterol concentration, cholesterol: phospholipid ratio and lithogenic index (Hussain & Chandrasekhara, 1993). The antilithogenic potential of fenugreek seeds was seen equally both in its raw as well as roasted form. In a recent study, rats fed with raw, roasted and sprouted fenugreek seeds for 40 days at 2.5 and 5% levels produced parallel results with respect to hypolipidemic influence (Joshi & Rajani, 2007). Our present results are in agreement with this that roasted fenugreek seeds were equally effective as their raw counterpart in exerting hypolipidemic influence and countering the CGS formation.

Thus, amalgamation of fenugreek seeds with a lithogenic diet significantly curtailed the formation of CGS by countering the elevation in blood and liver cholesterol. The elevated cholesterol: phospholipid ratio, cholesterol: bile acids ratio and the cholesterol saturation

index in the bile of lithogenic diet fed animals were effectively decreased by dietary fenugreek seeds. This study also revealed that the ability to exert anti-lithogenic influence was closely related to the hypocholesterolemic effect of the agent. This information highlights the potential of the possible pharmacological application of fenugreek seeds. This also reveals that cholesterol lowering effect of dietary ingredients such as fenugreek is not only cardio protective, but its advantage extends to possible prevention of CGS disease. Some of the components of the fenugreek especially fiber and saponins individually and in combination need to be investigated for further understanding the possible mechanism of action. The normal consumption of fenugreek seeds by population in India is reported to be 0.3 to 0.6 g/day/adult (Thimmayamma *et al*., 1983). The current animal study which has evidenced the health beneficial influence of fenugreek has employed 50 to 100 times this intake level. Such dietary levels are possible through particular dishes employing liberal amounts of fenugreek seeds and are actually in vogue in southern India. The liberal consumption of the same is proved to be safe, and may be easily implemented to derive health beneficial effects through its rich fibre content and other bioactive components.

Summary

Dietary hypocholesterolemic adjunts may have beneficial role in the prevention and treatment of cholesterol gallstones (CGS). In this investigation, fenugreek seed was evaluated for this potential on the experimental induction of CGS in experimental mice. CGS was induced in groups of mice by maintaining on a lithogenic diet (0.5% cholesterol) for 10 weeks. Fenugreek seed powder was included at 5, 10 and 15% of this lithogenic diet. Dietary fenugreek significantly lowered the incidence of CGS in these mice; the incidence was 63, 40 and 10% in 5, 10 and 15% fenugreek group respectively, as compared to 100% in lithogenic control. The antilithogenic influence of fenugreek is attributable to its hypocholesterolemic effect. Serum cholesterol level was decreased by 26–31% by dietary fenugreek, while hepatic cholesterol was lowered by 47–64% in these high cholesterol fed animals. Biliary cholesterol was 8.73–11.2 mM as a result of dietary fenugreek, as compared to 33.6 mM in high cholesterol feeding without fenugreek. Cholesterol saturation index in bile was reduced to 0.77–0.99 in fenugreek treatments as

compared to 2.57 in high-cholesterol group. Thus, fenugreek seeds offer the health beneficial antilithogenic potential by virtue of its beneficial influences on cholesterol metabolism.

oo O oo

Section A

Regression of pre-established cholesterol gallstones in mice by dietary fenugreek seed (*Trigonella foenum-graecum*)

Introduction

Cholesterol gallstone (CGS) disease is a highly prevalent gastroenterological disorder resulting from alteration in hepatic and biliary cholesterol homeostasis. The prevalence of this disease is very high in Europe and USA (10-15% of population), lesser in Asia (3-15%) and very low (< 5%) in Africa (Portincasa *et al.*, 2006). Pathologic conditions that generally precede the occurrence of CGS are lithogenic bile, gallbladder stasis and short nucleation time. The pathophysiology of CGS involves alteration in the delicate equilibrium between the three lipid components of bile, viz., cholesterol, bile acids and phospholipids. Increase in the concentration of cholesterol or decrease in the concentration of phospholipids and bile acids would push the cholesterol saturation index towards crystallization. CGS disease is multifactorial, involving the interaction of the genetic and environmental factors (Dowling, 2000), the environmental or exogenous factors affecting the disease most.

The treatment of this disease which makes use of bile salts like ursodeoxycholic acid and chenodeoxycholic acid, individually or in combination along with litholysis using extra corporal shock therapy suffers from a major drawback in the reoccurrence of ≈30-50% of cases, after the treatment or once the treatment is stopped (Portincasa *et al.,* 2006). Dietary management could be a viable alternative to the treatment of CGS or its reoccurrence. A number of studies have revealed that diet plays a significant role with respect to both incidence and also regression of preexisting gallstones. The reduction in the cholesterol content of blood, liver and bile is an important factor, which helps to reduce the CGS incidence. Several studies have demonstrated that dietary fiber has a marked influence on blood cholesterol levels (Kritchevsky, 1982; Dreher, 1987; Miettinen, 1987). Some of the legumes possess cholesterol lowering property in healthy humans and in

diabetic patients by virtue of their high fiber content (Sharma *et al.,* 1990a, Sharma *et al*., 1996).

Among the common spices, fenugreek seeds, garlic, onion, red pepper and turmeric have proven hypocholesterolemic potential (Srinivasan *et al*., 2004). Fenugreek seeds possess a significant hypocholesterolemic effect not only in various experimental animal models, but also in human subjects and this information has been exhaustively reviewed (Srinivasan, 2006).

Earlier, it has been shown that curcumin of turmeric and capsaicin of red pepper when included at 0.5 and 0.015% level respectively in the diet inhibited the experimental induction of cholesterol gallstones in mice (Hussain & Chandrasekhara, 1993). These two spice compounds have also been evidenced to regress preformed cholesterol gallstones in experimental mice when included in the diet after CGS induction (Hussain & Chandrasekhara, 1994). Recent studies in our laboratory have also evidenced that raw or heat-processed onion and garlic in the diet which showed anti-hypercholesterolemic effect during high cholesterol feeding, also exhibited the potential to prevent the induction of CGS (Vidyashankar *et al*., 2009) and also to regress the pre-established CGS in experimental mice (Vidyashankar *et al.,* 2010). Dietary fenugreek seeds have been shown to be a good cholagoguic agent (Bhat *et al*., 1985). We have also evidenced the beneficial antilithogenic effect of dietary fenugreek seeds in terms of reducing the incidence of CGS during experimental induction of the same in mice (Chapter-II). The beneficial effect was attributable to a favourable reversal of the altered lipid homeostasis in bile of these animals. The regression of pre-existing gallstones is a very important aspect, and in the absence of not much information on the dietary strategies to achieve regression of preexisting CGS, there is a need to explore hypocholesterolemic dietary components for this potential. Fenugreek seeds are a part of Indian dietary and also a constituent of traditional Indian and folk medicines since ancient times (Srinivasan, 2006). In the present investigation, we have investigated two dietary doses of fenugreek seeds in mice previously induced with CGS by high cholesterol feeding, for a possible antilithogenic influence with particular reference to regression of CGS.

Materials and methods

Materials

Cholesterol, dipalmitoyl phosphatidylcholine, bile salts, bovine serum albumin, ethylene diamine tetraacetic acid (EDTA), hydroxymethyl glutaryl Coenzyme A (HMG-CoA), dithiothreitol, triethanolamine, 3α-hydroxy steroid dehydrogenase, NAD, NADPH, standard bile acids kit (conjugated and unconjugated), Tris, triolein, EDTA, tetra methoxy propane, tertiary-butyl hydroperoxide, hydrazine hydrate and alpha cellulose were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Heparin and manganese chloride were obtained from SISCO Research Laboratory (Mumbai, India). Casein was purchased from Nimesh Corporation (Mumbai, India). All other chemicals and solvents used were of analytical grade and solvents were distilled prior to use. Fenugreek seeds were purchased from local market and pulverized.

Animal diets

The animals were fed with AIN-76 semi-purified diet. The basal control diet consisted of: sucrose, 65%; casein, 20%; cellulose, 5%; AIN-76 mineral mix, 3.5%; AIN-76 vitamin mix, 1%; DL-methionine, 0.3%; choline chloride, 0.2% and refined peanut oil, 5%. Lithogenic diet was prepared by supplementing 0.5% cholesterol and 0.25% bile salts (1:1 mixture of sodium cholate and sodium deoxycholate) to the AIN-76 basal control diet substituting equivalent quantity of sucrose. Diets were prepared by mixing the ingredients in a mechanical mixer and pellets were prepared using hand-operated pelletizer. Diets were stored at 4°C in air-tight containers. The test diets were prepared by incorporating the fenugreek seed powder at two different levels namely, 6 and 12 % (w/w) in the basal control diet. The incorporation of the fenugreek was at the expense of sucrose.

Animal treatment

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort with regard to the care and use of animals for experimental purposes and with due approval from the Institutional Animal Ethics Committee. Four weeks old male albino mice [OUTB- Swiss Albino/IND-CFT (2c)] procured from Experimental Animal

Production Facility of this Institute, weighing 22 ± 2 g were grouped and housed in polypropylene cages (3 mice per cage) with saw dust as bedding. Groups of animals were fed *ad libitum* with lithogenic diet and various test diets and had free access to water throughout the experimental period. Body weights were recorded at weekly intervals.

Groups of male mice (n = 108) were initially fed with lithogenic diet for 10 weeks to induce the CGS (Hussain & Chandrasekhara, 1994; Vidyashankar *et al*., 2009). Induction of CGS was confirmed by physical observation of the same in the gallbladder of 12 randomly selected mice which were sacrificed after fasting overnight. After confirming the induction of CGS, the remaining animals were regrouped (n= 24) based on their body weight. These four animal groups were imposed different dietary regimens viz., lithogenic diet, basal control diet, basal diet containing fenugreek (6%) and basal diet containing fenugreek (12%) for a further period of 5 or 10 weeks, as outlined in Fig.1.

Collection of gallbladder and scoring of CGS

Half the number of animals in each of these four diet regimens were fed with respective diets for 5 weeks while the remaining were fed for 10 weeks. At the end of the feeding duration, the animals were fasted overnight and sacrificed under ether anesthesia. Blood was drawn immediately by cardiac puncture and the serum was separated by centrifugation for further analysis. The liver was quickly excised, washed with ice-cold saline, blotted dry, weighed and stored at -20°C till further analysis. Cholecystectomy was performed and gallbladders were carefully excised. The volume of bile was noted and the weight of the gallbladder along with stones was recorded. The gallbladders were evaluated for CGS under magnifying lens for the presence of gallstones by four individuals unaware of dietary treatments. The grading of stones was done on a five point scale (Akiyoshi *et al.*, 1986). The bile from the gallbladders was pooled and stored at -20°C till further analysis.

Analysis of lipids

Biliary lipids were extracted by the method of Bligh and Dyer (1959) and the chloroform phase was used for lipid analysis. The upper methanolic phase was used for the estimation of total bile acids using 3α-hydroxysteroid dehydrogenase (Turley & Dietschy, 1970).

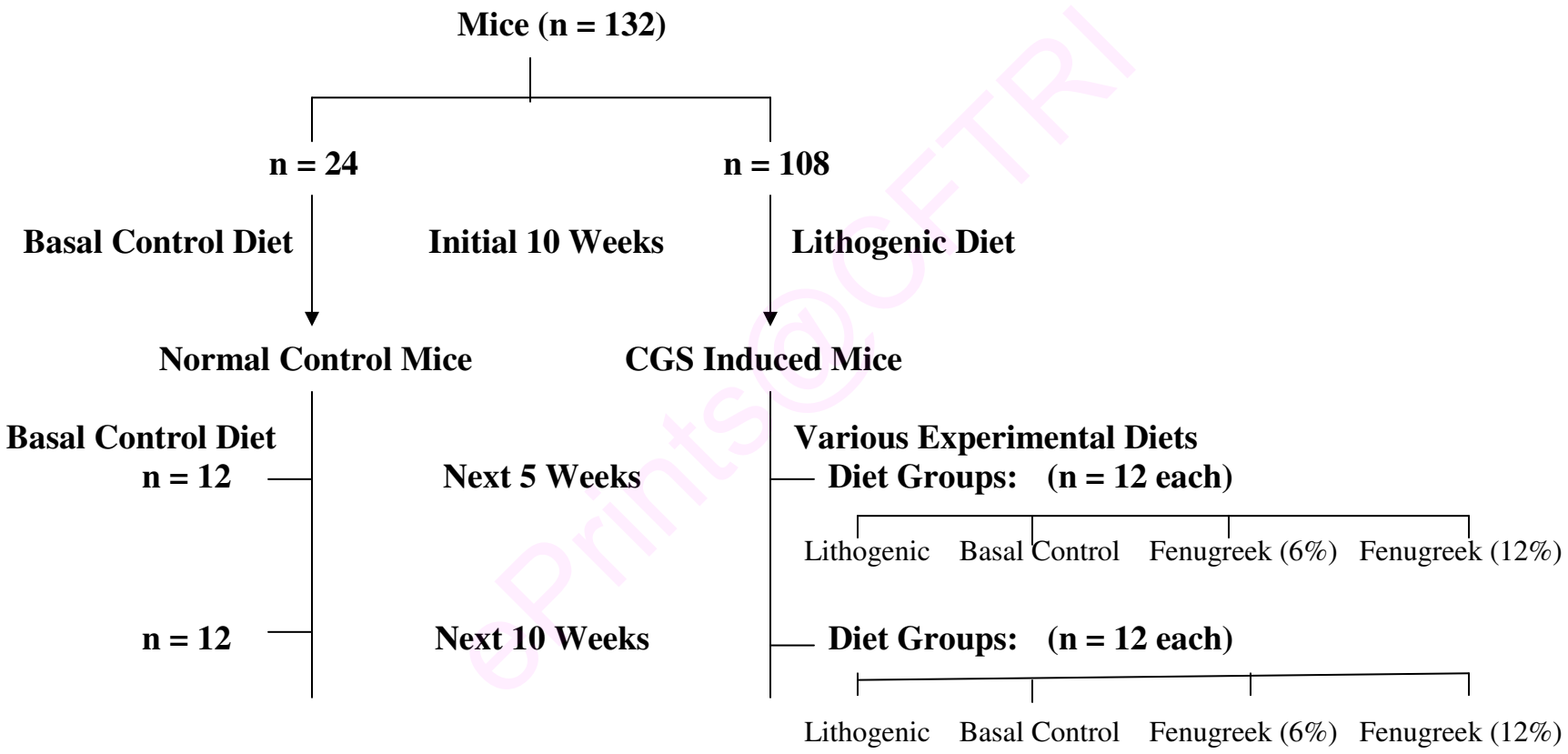


Fig.1 Experimental design for studying regression of CGS in mice by dietary fenugreek seeds.

Lithogenic diet: 0.5% cholesterol and 0.25% bile salts

The lower chloroform layer was used for the analysis of cholesterol and phospholipid. Serum and liver lipids were extracted by the method of Folch *et al*., (1957). Cholesterol was quantitated by the method of Searcy and Bergquist (1960). HDL-cholesterol and non-HDL (LDL+VLDL) cholesterol in serum were estimated by adapting the protocol given by Warnick and Albers (1978). The method involved the precipitation and separation of HDL from LDL+VLDL by the use of heparin and manganese chloride. HDL-cholesterol in the supernatant was estimated as described before. Phospholipids were measured by ferrous ammonium thiocyanate method using di-palmitoyl phosphatidylcholine as reference standard as described by Stewart (1980). Triglycerides were estimated according to the method prescribed by Fletcher (1968), using triolein as standard. CSI of the bile was calculated using the values of cholesterol, phospholipids, bile acids and total lipids in bile as described by Carey (1978).

Statistical analysis

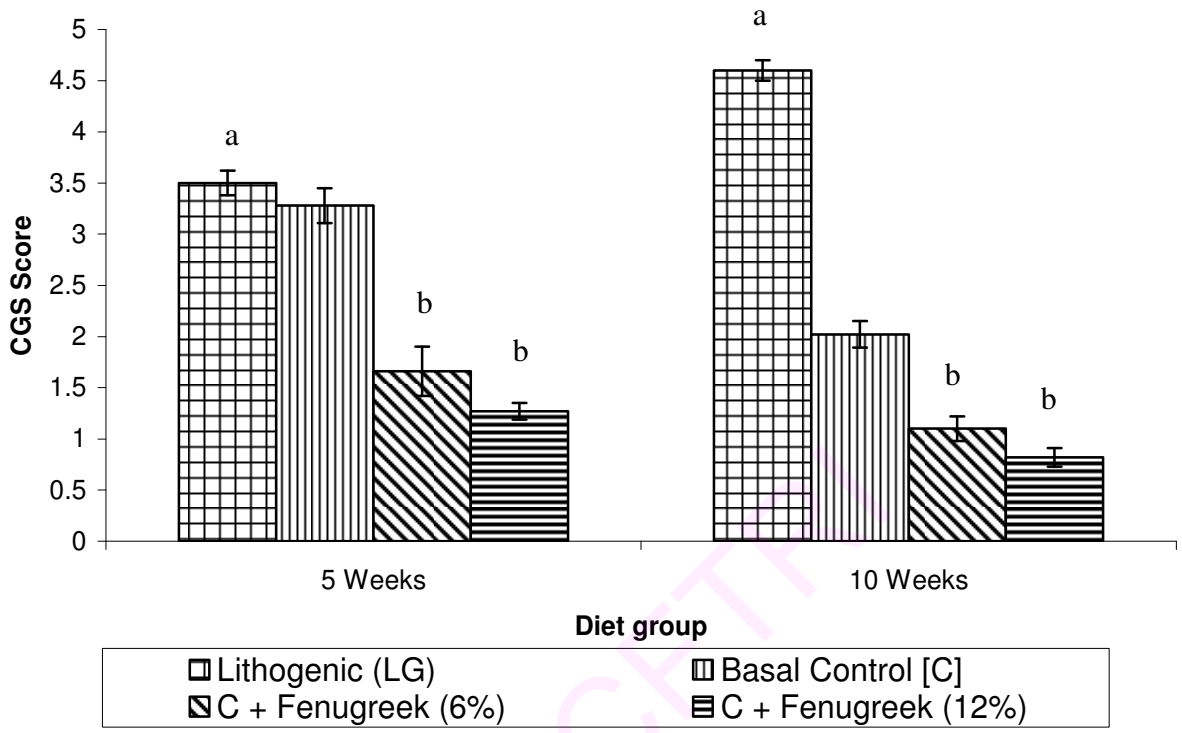
Statistical analysis was carried out using Graph pad prism statistical software. Results are analyzed by one way ANOVA and the significance level was calculated using Tukey Kramer multiple comparison test and results are considered as significant at $P<0.01$.

Results

After feeding a high cholesterol diet for 10 weeks to induce CGS, groups of animals were maintained on (1) High cholesterol diet, (2) Basal control diet, (3) Basal diet containing 6% fenugreek, and (4) Basal diet containing 12% fenugreek. Half the number of animals in each group was sacrificed at the end of 5 weeks, while the remainder half was sacrificed at the end of 10 weeks of post-CGS induction period.

Effect of feeding fenugreek for five and ten weeks on the regression of preformed CGS in mice

Feeding of lithogenic diet for 10 weeks had successfully induced CGS in mice, as confirmed by examining 12 animals randomly picked among the lot. The effect of feeding fenugreek for 5 and 10 weeks on CGS score in animals with induced CGS is presented in Fig.2 (A & B). The CGS score which was 3.50 in LG group and 3.28 in Basal control group was considerably reduced to 1.66 and 1.27 in fenugreek fed animals (6% and 12%) at 5 weeks.


CGS Score
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
b
b
b
5 Weeks
10 Weeks
Diet group
Lithogenic (LG)
Basal Control [C]
C + Fenugreek (6%)
C + Fenugreek (12%)

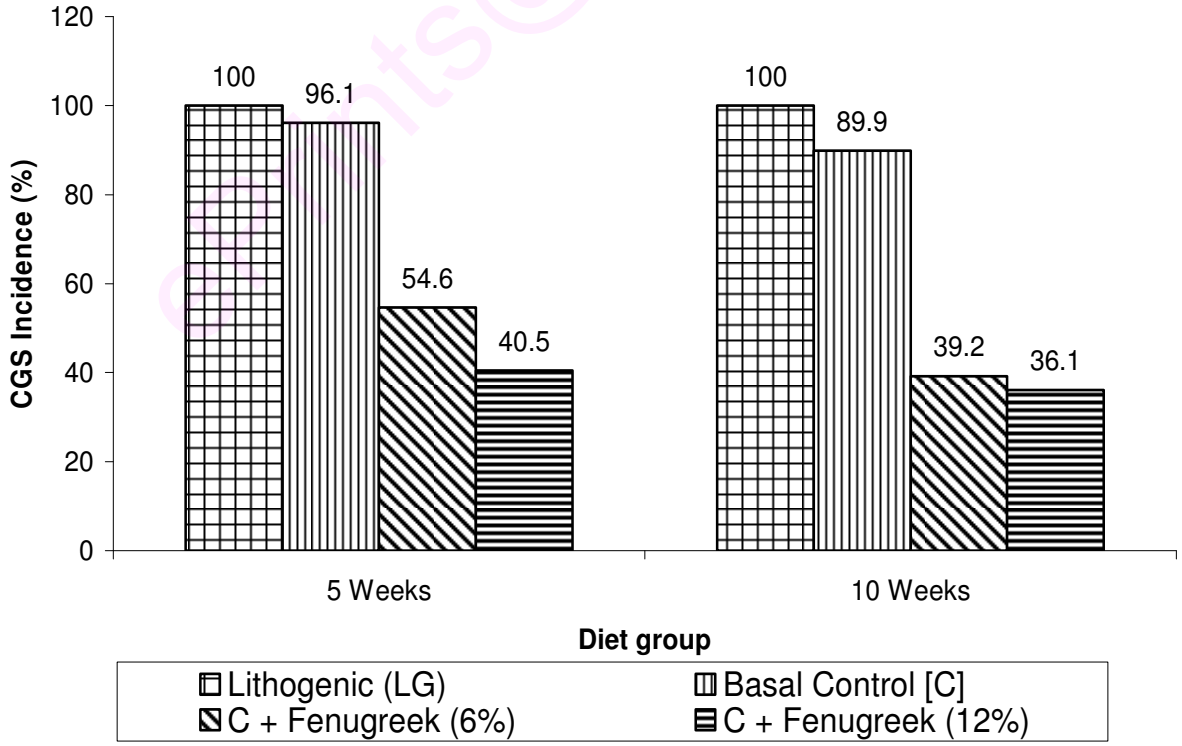

CGS Incidence (%)
120
100
80
60
40
20
0
100
96.1
54.6
40.5
100
89.9
39.2
36.1
5 Weeks
10 Weeks
Diet group
Lithogenic (LG)
Basal Control [C]
C + Fenugreek (6%)
C + Fenugreek (12%)

Fig.2A Effect of feeding fenugreek powder for 5 and 10 weeks on CGS score in CGS prevailing mice

Values are mean ± SEM of 12 mice/group. CGS score – Scored by 4 individuals and grading was done on a 5 point scale (0-4)

Animals in all these diet groups were fed initially with LG diet for 10 weeks to induce CGS.

Animals in Normal control group (maintained on basal control diet throughout) did not exhibit any CGS.

a: Statistically significant when compared to Normal control group at $P<0.01$
b: Statistically significant when compared to Basal control (C) group at $P<0.01$

Fig.2B Regresson of preestablished CGS in different animal groups

Top left to right; Basal control and HCD

Bottom left to right; Fenugreek 6% and Fenugreek 12%

The same was even lower at 10 weeks (1.10 and 0.82) in the case of fenugreek fed animals. Thus, regression of CGS brought about by dietary fenugreek was 45 and 60% after 5 weeks, while the regression of CGS brought about was 61 and 64% respectively after 10 weeks, as compared to a mere 4 and 10% in animals allowed to consume basal control diet (Fig.3).

The body weights of the animals were comparable among the four groups (Table-1). Liver weight was significantly increased in HCD group (by 68%) compared to normal control group. Fenugreek feeding during post-CGS induction significantly lowered the liver weights (14 - 18%) as compared to the animals maintained on basal control diet. There was no change in the weights of other organs heart, kidney and spleen as a result of fenugreek feeding (Data not shown).

Effect of feeding fenugreek for five and ten weeks on serum lipid profile in CGS prevailing mice

Influence of feeding fenugreek for 5 and 10 weeks during post-CGS induction period on serum cholesterol is presented in Table-2. Serum cholesterol content in the mice fed HCD during the post-CGS induction period remained higher compared to un-induced control group at both time intervals. Treatment with diets containing fenugreek during post-CGS induction period brought about decreases of the order of 36-48% in 6% fenugreek and 38-53% in 12% fenugreek respectively when compared to 18-28% in basal control group. There was a significant decrease in the content of LDL-cholesterol brought about by fenugreek treatment which was 58 and 63% as compared to 33% in basal control at 10 weeks; 44 and 48% as compared to 22% in basal control at 5 weeks. HDL-cholesterol essentially remained unaffected by the treatments.

Serum phospholipid of CGS-induced mice remained lowered when they were maintained on high cholesterol diet (Table-3). Fenugreek containing diets on the other hand significantly restored serum phospholipid content (14-27% increase in 5 weeks and 20-23% increase in 10 weeks compared to basal control group). As a result of this beneficial modification of both cholesterol and phospholipid concentrations, the

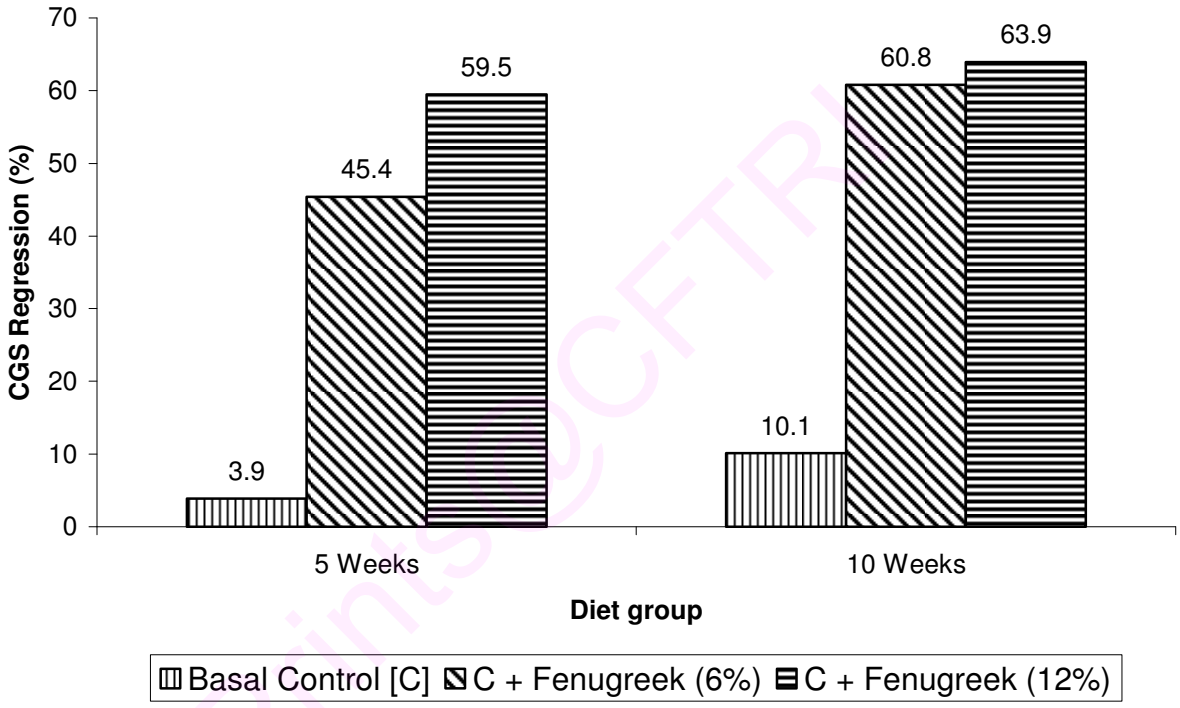


Fig.3 Higher regression of CGS by dietary fenugreek seeds.

Animals in these diet groups were fed initially with LG diet for 10 weeks to induce CGS.

Table 1. Effect of feeding fenugreek powder for 5 and 10 weeks on body weight and liver weight in CGS prevailing mice

Diet groups	Body weight (g)		Liver (g/100 g body weight)	
	5 week	10 week	5 week	10 week
Normal Control*	38.5 ± 0.63	41.8 ± 0.75	3.08 ± 0.12	3.15 ± 0.14
Lithogenic (LG)	40.2 ± 0.86	43.6 ± 1.97	5.17 ± 0.11[a]	5.69 ± 0.25[a]
Basal Control (C)	37.6 ± 0.74	42.1 ± 0.96	4.73 ± 0.18	4.35 ± 0.06
C + Fenugreek (6%)	38.1 ± 0.89	42.6 ± 1.10	3.94 ± 0.10[b]	3.76 ± 0.07[b]
C + Fenugreek (12%)	39.0 ± 0.75	42.9 ± 1.34	4.03 ± 0.06[b]	3.57 ± 0.06[b]

Values are mean ± SEM of 12 mice/group
Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS.
*Animals in normal control group were maintained on basal control diet throughout.
a: Statistically significant when compared to Normal control group at P<0.01
b: Statistically significant when compared to Basal control (C) group at P<0.01

Table 2. Effect of feeding fenugreek powder for 5 and 10 weeks on serum cholesterol in CGS prevailing mice

Diet group	Total cholesterol		LDL-cholesterol		HDL-cholesterol	
	5 week	10 week	5 week	10 week	5 week	10 week
Normal Control*	125.8 ± 5.00	133.2 ± 4.80	80.5 ± 3.20	86.2 ± 3.11	45.3 ± 3.10	47.0 ± 2.55
Lithogenic (LG)	320.3 ± 14.5[a]	352.0 ± 15.9[a]	276.3 ± 11.4[a]	309.1 ± 15.4[a]	44.0 ± 5.8	42.9 ± 4.88
Basal Control (C)	264.1 ± 12.7	252.1 ± 17.1	215.3 ± 10.7	206.1 ± 10.9	48.8 ± 2.8	46.0 ± 2.38
C + Fenugreek (6%)	206.0 ± 15.1[b]	178.5 ± 11.4[b]	154.4 ± 9.96[b]	128.6 ± 8.50[b]	51.6 ± 4.0	49.9 ± 2.07
C + Fenugreek (12%)	197.1 ± 16.5[b]	166.0 ± 8.30[b]	144.2 ± 8.38[b]	113.6 ± 8.60[b]	52.9 ± 3.5	52.4 ± 4.44

Values expressed as mg/dL are mean ± SEM of 12 mice/group
Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS.
*Animals in Normal control group were maintained on basal control diet throughout.
a: Statistically significant when compared to Normal control group at $P<0.01$
b: Statistically significant when compared to Basal control (C) group at $P<0.01$

Table 3. Effect of feeding fenugreek powder for 5 and 10 weeks on serum lipid profile in CGS prevailing mice

Diet group	Phospholipid		Triglycerides		C/PL Ratio	
	5 week	10 week	5 week	10 week	5 week	10 week
Normal Control*	298.7 ± 11.3	286.2 ± 14.2	155.0 ± 7.52	151.3 ± 6.40	0.42 ± 0.02	0.46 ± 0.02
Lithogenic (LG)	256.6 ± 12.3[a]	203.0 ± 6.35[a]	186.2 ± 13.2[a]	186.6 ± 11.6[a]	1.25 ± 0.06[a]	1.73 ± 0.08[a]
Basal Control (C)	287.3 ± 6.89	313.8 ± 26.4	170.8 ± 7.40	166.7 ± 12.9	0.92 ± 0.04	0.80 ± 0.05
C + Fenugreek (6%)	326.3 ± 14.6[b]	386.3 ± 11.9[b]	159.5 ± 13.7	147.5 ± 10.6	0.63 ± 0.05[b]	0.46 ± 0.03[b]
C + Fenugreek (12%)	365.8 ± 12.3[b]	376.0 ± 21.5[b]	165.0 ± 10.1	147.4 ± 9.89	0.54 ± .0.05[b]	0.44 ± 0.02[b]

Values expressed as mg/dL are mean ± SEM of 12 mice/group
Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS.
*Animals in Normal control group were maintained on basal control diet throughout.
C/PL – Cholesterol/phospholipid ratio
a: Statistically significant when compared to Normal control group at P<0.01
b: Statistically significant when compared to Basal control (C) group at P<0.01

cholesterol: phospholipid ratio was decreased by 32-41% in fenugreek fed animals compared to basal control group after 5 weeks, and by 43-45% after 10 weeks. It is significant to note that control diet feeding during post-CGS induction did produce partial restoration of the alterations in lipid parameters in serum, whereas dietary fenugreek brought about restoration to an even greater extent. Serum triglyceride content during the post-CGS induction period was higher in HCD group when compared to normal control group. In fenugreek diet fed groups, the same was not much different from the basal control diet fed animals. The same was modestly decreased upon feeding fenugreek diet as compared to the basal control group, although not statistically significant.

Effect of feeding fenugreek for five and ten weeks on liver lipid profile in CGS prevailing mice

Liver cholesterol content of mice continued on HCD during post-CGS induction period was increased by 5.3- and 6.1- times compared to normal controls at 5 and 10 weeks respectively (Table-4). The groups of mice fed fenugreek containing diets during post-CGS induction period had comparatively lesser hepatic cholesterol as compared to basal control group. The decreases brought about by fenugreek diets were 57 and 63% at 5 weeks and 53 and 54% at 10 weeks respectively. Similarly, hepatic triglyceride content was decreased by dietary fenugreek by 16 and 30% at 5 weeks as compared to basal control group. While the reduction in hepatic triglyceride in basal control group was 39% at 10 weeks as compared to lithogenic diet group, dietary fenugreek decreased it by 58 and 63% in 6% and 12% fenugreek groups, respectively. Total hepatic lipid content was lowered by 35-47% and 40-52% in fenugreek 6% and 12% fed groups respectively as compared to a decrease of 11 and 26% in basal control group.

Hepatic phospholipid content was significantly increased (by 21 and 29%) after feeding fenugreek for 5 weeks when compared to animals fed basal control diet. Similarly, dietary fenugreek increased the phospholipid content by 24-30% after feeding for 10 weeks when compared to the animals maintained on basal control diet. Hepatic cholesterol: phospholipid ratio (C/PL ratio) was increased in lithogenic diet group by 9- and 11.1- times (at 5 and 10 weeks, respectively) as a result of increased cholesterol content in liver compared to normal controls. In fenugreek fed groups, C/PL ratio was

Table 4. Effect of feeding fenugreek powder for 5 and 10 weeks on liver lipid profile in CGS prevailing mice

Diet groups	Cholesterol		Phospholipid		Triglycerides		Total Lipids	
	5 week	10 week	5 week	10 week	5 week	10 week	5 week	10 week
Normal Control*	9.50 ± 0.61	9.71 ± 0.68	34.2 ± 2.8	33.6 ± 1.7	30.0 ± 1.6	31.2 ± 2.0	91.5 ± 4.8	92.3 ± 5.6
Lithogenic (LG)	50.7 ± 3.9[a]	59.2 ± 4.02[a]	20.2 ± 0.8[a]	18.4 ± 1.0[a]	62.7 ± 4.9[a]	76.9 ± 4.2[a]	189.2 ± 8.5[a]	216.3 ± 9.3[a]
Basal Control (C)	29.1 ± 3.9	19.2 ± 0.57	21.5 ± 1.3	24.6 ± 2.0	55.3 ± 3.6	46.7 ± 2.4	167.5 ± 4.2	159.2 ± 4.9
C + Fenugreek (6%)	12.6 ± 1.7[b]	9.00 ± 0.71[b]	26.0 ± 1.2[b]	30.4 ± 0.7[b]	46.3 ± 2.2[b]	32.5 ± 1.8[b]	123.0 ± 5.8[b]	115.7 ± 6.0[b]
C + Fenugreek (12%)	10.8 ± 1.9[b]	8.84 ± 0.31[b]	27.7 ± 1.6[b]	31.9 ± 0.5[b]	38.7 ± 2.0[b]	28.6 ± 2.2[b]	113.5 ± 4.1[b]	104.1 ± 6.2[b]

Values given as mg/g fresh liver are mean ± SEM of 12 mice/group
Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS.
*Animals in Normal control group were maintained on basal control diet throughout.
a: Statistically significant when compared to Normal control group at P<0.01
b: Statistically significant when compared to Basal control (C) group at P<0.01

decreased by 64 and 71% after 5 weeks and by 62 and 64% after 10 weeks compared to basal control group. These results indicated that dietary fenugreek could effectively decrease liver cholesterol and elevate phospholipid levels, and consequently decrease the C: PL ratio in CGS prevailing conditions. It is important to note here that although basal control diet feeding during post-CGS induction significantly reversed the altered hepatic lipid profile, inclusion of fenugreek produced even more restoration.

Effect of feeding fenugreek for five and ten weeks on biliary lipid profile in CGS prevailing mice

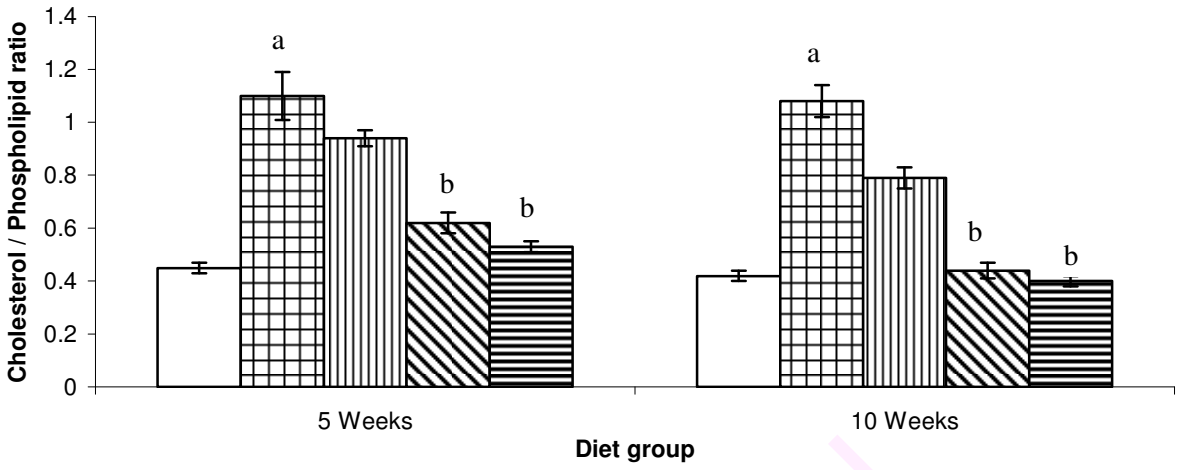
Biliary lipid profile of animals maintained for 5 and 10 weeks on fenugreek diets during post-CGS induction is presented in Table-5. Cholesterol content of bile was increased by 4.9- and 6.0- fold in lithogenic diet group compared to normal control group at 5 and 10 week time intervals. On the other hand, when compared to the animals maintained on basal control diet during post-CGS induction period, biliary cholesterol content was decreased by 47 and 58% by dietary fenugreek (6% and 12%) after 5 weeks. This decrease in biliary cholesterol caused by dietary fenugreek was 57 and 60% respectively, after 10 weeks. Total bile acid content was improved in fenugreek treatment for 10 weeks which was 28 and 47% in 6% and 12% fenugreek groups respectively, as compared to basal control group. The increase in biliary phospholipid content was countered by dietary fenugreek during post-CGS induction. The decrease in phospholipid content was 19-26% in fenugreek treatment compared to basal control group. Total biliary lipid was higher in lithogenic diet group compared to normal controls. While there was no significant change in the same by dietary fenugreek treatment for 5 weeks compared to basal control group, total biliary lipid was increased in 10 weeks in the 12% fenugreek group probably due to higher bile acid content.

The results revealed a significant decrease in the both C/PL ratio and CSI values with the dietary incorporation of fenugreek. Cholesterol: phospholipid ratio in the bile of lithogenic diet group was much higher (1.1), in view of the increased cholesterol content (Fig.4). On the other hand, this ratio in fenugreek diet groups was 0.62 and 0.53 in 6%

Table 5. Effect of feeding fenugreek powder for 5 and 10 weeks on biliary lipids in CGS prevailing mice

Dietary groups	Biliary lipids (mM)							
	Cholesterol		Phospholipid		Bile acids		Total lipid (g/dL)	
	5 week	10 week	5 week	10 week	5 week	10 week	5 week	10 week
Normal Control*	7.80 ± 0.30	7.68 ± 0.38	17.3 ± 1.20	18.5 ± 1.4	161.0 ± 10.0	165.4 ± 9.50	9.47 ± 0.35	9.78 ± 0.29
Lithogenic (LG)	38.1 ± 3.31[a]	45.8 ± 2.62[a]	34.5 ± 2.7[a]	42.4 ± 3.8[a]	185.7 ± 9.73	173.2 ± 11.7	13.2 ± 0.66[a]	13.3 ± 0.58[a]
Basal Control (C)	27.6 ± 0.81	20.5 ± 0.93	29.3 ± 1.5	25.9 ± 2.0	191.4 ± 8.14	179.1 ± 10.7	12.6 ± 0.42	11.6 ± 0.30
C + Fenugreek (6%)	14.7 ± 0.82[b]	8.80 ± 0.51[b]	23.8 ± 1.3[b]	20.1 ± 1.4[b]	180.2 ± 5.53	229.4 ± 16.3[b]	11.1 ± 0.59	12.5 ± 0.23
C + Fenugreek (12%)	11.5 ± 0.45[b]	8.15 ± 0.43[b]	21.7 ± 1.1[b]	20.2 ± 1.7[b]	185.3 ± 5.59	262.9 ± 24.3[b]	13.3 ± 0.52	14.6 ± 0.37[b]

Values are mean ± SEM of 12 mice/group
Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS.
*Animals in Normal control group were maintained on basal control diet throughout.
a: Statistically significant when compared to Normal control group at P<0.01
b: Statistically significant when compared to Basal control (C) group at P<0.01



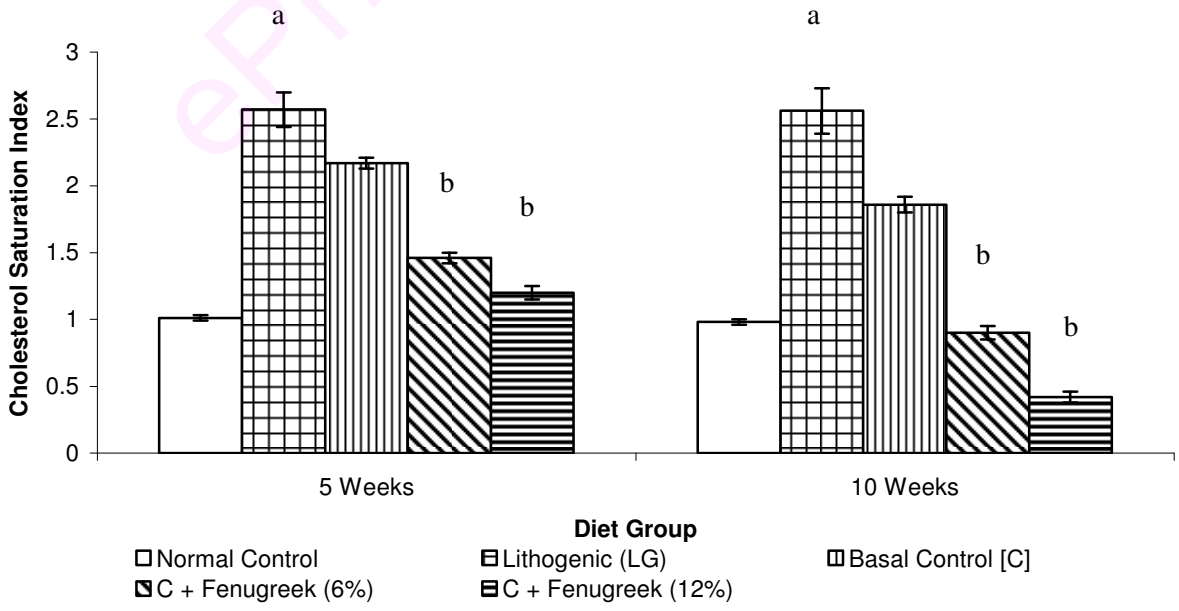


Fig.4 Effect of feeding fenugreek powder on cholesterol: phospholipid ratio, cholesterol: bile acid ratio and cholesterol saturation index in the bile in CGS prevailing mice

Values are mean ± SEM of 12 mice per group

Animals in all diet groups except Normal control were fed initially with LG diet for 10 weeks to induce CGS. Animals in Normal control group were maintained on basal control diet throughout.

a: Statistically significant when compared to Normal control group at $P<0.01$
b: Statistically significant when compared to Basal control (C) group at $P<0.01$

and 12% levels which amounted to 34 and 44% lower than in basal control group in 5 weeks. This ratio was 0.44 and 0.44, which amounted to 44 and 49% lower than in basal control group in 10 weeks. Similarly, cholesterol: bile acid ratio in the bile was higher (0.20 and 0.26) in lithogenic diet group (Fig.4). The same was significantly brought down upon feeding fenugreek (by 43 and 57% at 5 weeks, and by 67 and 73% at 10 weeks) which is more than that produced in the basal control group. Cholesterol saturation index (CSI) in the bile was 2.57 in animals maintained on high cholesterol diet during post-CGS induction period. CSI was slightly lower when the animals were maintained on basal control diet during 5 and 10 weeks post-CGS induction period (2.17 and 1.86). But CSI of bile was prominently lowered when the animals were maintained on fenugreek diets during the same period (1.46 and 1.20 at 5 weeks, and 0.90 and 0.42 at 10 weeks) (Fig.4).

Discussion

In pathological conditions, when the liver secretes higher amounts of cholesterol and lower amounts of bile acids and phospholipids into the bile, cholesterol: phospholipid ratio and cholesterol: bile acid ratio of bile increases. These events lead to supersaturation of bile resulting in the nucleation of cholesterol crystal. In conditions of continuous supply of cholesterol, the crystal grows into a big stone leading to the formation of cholesterol gallstone. CGS is usually treated by cholecystectomy or by the use of large doses of cholelitholytic drugs (Roslyn *et al*., 1993). The major drawback of these two types of treatment is the recurrence of stones upon discontinuation of the drug or some time after lithotripsy (Bouchier, 1990). An alternative to address CGS would be dietary intervention, which could help in the prevention of incidence, regression of existing CGS and prevention of the possible recurrence. Data on the effects of dietary components on gallbladder bile in patients or in animals with established cholelithiasis are limited.

Feeding of HCD for ten weeks effectively induced CGS in mice. The HCD provokes feedback mechanism, by inhibiting hepatic cholesterol synthesis by down regulating the HMG-CoA reductase, the rate-limiting enzyme in the synthesis of cholesterol. At the same time cholesterol-7α-hydroxylase, the rate-limiting enzyme in the synthesis of bile

acids is stimulated (Matheson *et al.*, 1995). The combined effect of these two processes will keep the concentration of biliary cholesterol within the critical limits. Continuous feeding of cholesterol-rich diet however shatters this equilibrium affecting the feedback mechanism, facilitating the deposition of more and more cholesterol in the solution than its carrying capacity (Carey, 1989).

To examine the hypothesis that dietary hypocholesterolemic constituents can possibly regress pre-formed CGS, this investigation was carried by inducing CGS in gallbladder of mice by feeding a lithogenic diet and later looking for its regression under the influence of diet. The results indicated that dietary fenugreek (6 and 12%) for 5 weeks after induction of CGS was sufficient to cause regression of CGS up to 45 and 60% and longer 10 week duration of feeding caused 61 and 64% regression. Substitution of lithogenic diet with basal control diet after CGS induction in mice caused only a moderate regression of CGS and a small reduction in CSI, due to partial restoration of the various lipid parameters in the bile. It is clear that more than the withdrawal of lithogenic diet, feeding fenugreek could beneficially influence both biliary lipid metabolism and CGS pathogenesis.

Similar to earlier reports, there was no marked change in the food consumption and body weight as a result of fenugreek feeding. The liver weight which was significantly higher in the HCD group was countered in fenugreek treatment as a result of reducing the lipid content. Supplementation of fenugreek up to 20% level has been reported to have no effect on the food intake of animals and the organ weights (Udayasekhara rao *et al.,* 1996).

The present animal study revealed that dietary fenugreek seed has a marked hypocholesterolemic effect, and also significantly regresses the preestablished CGS in mice. Many studies have reported significant reduction in serum cholesterol by the ingestion of various dietary constituents, especially fiber (Jenkins *et al.*, 1993, Anderson *et al.*, 1992), saponins (Petit *et al.,* 1995), turmeric and capsaicin (Hussain & Chandrasekhara*,* 1992, 1993, 1994), onion and garlic (Vidyashankar *et al.*, 2009), and fenugreek seeds (Srinivasan, 2006). The beneficial reduction of cholesterol concentration of tissues by dietary fenugreek or its defatted fraction has been evidenced in a number of

animal models as well as in human diabetic subjects (Srinivasan, 2006). The observed reduction in serum cholesterol was in the non-HDL fraction in fenugreek treated experimental mice, while HDL-cholesterol was not affected. Sowmya and Rajalakshmi (1999) have also reported similar reduced level of total cholesterol with concordant reduction in non-HDL-cholesterol upon feeding diet with fenugreek.

One of the mechanisms proposed for the hypocholesterolemic effect of fenugreek is increased excretion of bile acids and neutral steroids through faeces (Sharma, 1984). This results in the stimulation of the conversion of cholesterol to bile acids in liver (Bhat *et al.*, 1985). Fiber potentially reduces the rate of diffusion of cholesterol towards the absorptive mucosal surface of the intestine (Anderson & Chen, 1979) or inhibits cholesterol and bile acids uptake (Oakenfull & Sidhu, 1990). Hepatic cholesterol synthesis appears to be suppressed by the volatile fatty acids which enter in to blood after they are produced by colonic fermentation of unabsorbed soluble fiber. Fenugreek seeds contain rich amounts of soluble fiber galactomannan. It was also reported that diosgenin, a potentially hydrolyzed compound of fenugreek saponin in the intestinal tract will interfere with cholesterol absorption (Sauvaire *et al.*, 1991). Crude saponin fraction of fenugreek was reported to reduce serum cholesterol in rats (Sharma, 1986).

The ability of saponin to induce adsorption of bile salts was presumably related to its surface activity and bile acids adsorbed would be removed by excretion. This excretory loss would be balanced by metabolizing cholesterol to bile acids in the liver (Oakenfull, 1981). Sauvaire *et al.,* (1991) have reported that diosgenin of fenugreek in the intestine may contribute to the hypocholesterolemic effect. Cayen and Dvornik (1979) reported that diosgenin decreased serum LDL-cholesterol and increased HDL-cholesterol fraction, while the hypocholesterolemic activity of diosgenin is presumably mediated by increasing the excretion of neutral sterols. It also decreased liver cholesterol and triglycerides while phospholipids are unaltered.

The plant fibers may act similarly and adsorb bile acids and hence lower plasma cholesterol (Burkitt & Trowell, 1975). Fenugreek stimulates the hepatoenteric excretion of cholesterol thereby lowering its circulatory levels (Chaturvedi & Pant, 1988). Udayasekhara rao *et al.,* (1996) have suggested that the galactomannan present in

fenugreek seeds decreases both digestion and absorption of starch as well as uptake of bile salts in the intestine. Narender *et al.,* (2006) have reported that 4-hydroxyisoleucine, an unusual amino acid from fenugreek significantly decreases plasma total cholesterol and triglycerides levels, accompanied by increased HDL-cholesterol in dyslipidemic hamster. It was also suggested that HDL-cholesterol mediates the reverse transport of cholesterol from peripheral tissue to the liver for disposal by excretion in to bile. Thus, it is probable that foods rich in saponins and fiber are useful in reducing the risk of cholesterol and its related problems like CGS and heart diseases.

Diets supplemented with cholesterol have shown to produce lithogenic bile and cholesterol gallstones in experimental animals including Prairie dogs, squirrel monkeys, hamsters and mice (Denbesten *et al*., 1974, Osuga & Portman, 1971; Pearlman *et al.*, 1979; Tepperman *et al.*, 1964; Bergman & van der Linden, 1971; Hussain & Chandrasekhara, 1993). Feeding of the diet enriched with cholesterol markedly elevated the lithogenic index of bile. Decrease in the lithogenic index with the incorporation of fenugreek to diet reflects a favorable effect viz., reduced precipitation of cholesterol in bile. The structural and functional aspects of dietary fiber may explain to inhibit CGS and reduce the CSI by half with the incorporation of dietary fiber in to the cholesterol-rich diet (Wayne *et al.*, 1999). Hussain and Chandrasekara (1994) have earlier reported that dietary curcumin of turmeric and capsaicin of red pepper significantly regressed the pre-established CGS in mice. Similarly, fish oil feeding decreased the CSI by 25% after 5 weeks of treatment in cholelithiasis prevailing patients (Banerjee *et al*., 1992). Recently, dietary onion and garlic – the two hypocholesterolemic *Allium* spices have been evidenced to produce significant regression of pre-established cholesterol gallstones in experimental mice (Vidyashankar *et al*., 2010).

Fiber present in fenugreek may protect against gallstone formation also by speeding the intestinal food transit (Platel & Srinivasan, 2000) and reducing the generation of secondary bile acids by gut microflora (Marcus & Wheaton, 1986). It has been reported that fiber supplementation to Prairie dogs placed on a lithogenic diet inhibited cholesterol stone formation by reducing biliary cholesterol saturation (Wayne *et al.*, 1999).

Feeding of fenugreek resulted in a decrease in major part of the hepatic cholesterol pool, which is to be secreted in to bile by its conversion to bile acids resulting in an increased bile acid concentration. Phospholipid concentration was also increased upon feeding fenugreek. Increased bile acid and phospholipid concentration promote solubilization of biliary cholesterol in mixed micelles which are stable and hence reducing the possibility of nucleation of cholesterol crystals (Holan *et al*., 1979). These events affect the dynamic equilibrium of bile in such a way that the cholesterol supply to the growing CGS is cut-off and the CGS may degenerate or regress in the absence of continued supply of cholesterol. Dietary fenugreek reduced the total cholesterol pool, which is evident from the lowered cholesterol: phospholipid ratio in serum and liver. The current animal study re-demonstrates that dietary fenugreek has a profound effect on the lipid profile in blood, liver and bile. Considering the above facts it can be concluded that a consistent exchange and redistribution of biliary lipids between the carriers occur which is probably mediated by protein, thus inhibiting CGS formation or promoting the regression of CGS.

It may be concluded that while feeding of the control diet after induction of CGS regressed the pre-established CGS, the incorporation of fenugreek to the diet significantly enhanced the regression of CGS. This antilithogenic effect of dietary fenugreek was mediated through a favourable reduction in cholesterol in serum, liver and bile, and an increase in liver and biliary phospholipid content. As a result of modulation of lipid profile, there was a significant reduction in the cholesterol: phospholipid ratio and in the cholesterol saturation index with the incorporation of fenugreek in the diet. This study also reveals that the ability of fenugreek to exert anti-lithogenic influence is closely related to the hypocholesterolemic effect of this spice. This information enhances the potentiality of the pharmacological application of fenugreek seeds. This common food adjunct not only offers to protect from CGS formation, but can also markedly regress the pre-formed CGS. The study also reveals that cholesterol lowering dietary ingredient such as fenugreek is not only cardio protective, but its advantage extends to possible prevention of CGS disease. Some of the components of the fenugreek especially fiber and saponin, individually and in combination need to be investigated for further

understanding the mechanism of action. This finding is significant in the context of evolving an alternative to address CGS by dietary intervention, which could help in the prevention of incidence, regression of existing CGS and prevention of possible recurrence.

Summary

An animal study was carried out to evaluate the beneficial influence of dietary fenugreek seeds in terms of regression of pre-established cholesterol gallstones (CGS). CGS was induced by feeding a high cholesterol diet for a period of 10 weeks. After the CGS induction, groups of these animals were maintained for further 10 weeks on high cholesterol/ basal control diet/ 6% fenugreek powder / 12% fenugreek powder diets. Incidence of CGS and its severity were evaluated at the end of this feeding regimen. The incidence of CGS was significantly lowered as a result of dietary fenugreek seeds, the extent of regression being 61 and 64% in the lower and higher dose groups when compared to 10% regression in basal control group. The antilithogenic influence of dietary fenugreek was accompanied by significant reduction in serum cholesterol concentration which was more than 35%. Hepatic cholesterol concentration was also profoundly lowered by dietary fenugreek, the decrease being 53-63% compared to basal control diet. Biliary cholesterol concentration was significantly lower as a result of dietary fenugreek during post-CGS induction period resulting in decreased cholesterol: phospholipid ratio (0.44 and 0.40 as compared to 0.79 in the basal control group). Biliary cholesterol: bile acid ratio was lowered upon feeding fenugreek (by 67 and 73%) much more than in the basal control group. The cholesterol saturation index in the bile was also beneficially lowered by fenugreek treatment during post-CGS induction period (which was 0.90 and 0.42 as compared to 1.86 in the basal control diet group). The present study has evidenced the potency of hypolipidemic fenugreek seeds in regressing the pre-established CGS and this beneficial antilithogenic influence is attributable to its primary influence on cholesterol levels. This finding is significant in the context of evolving a dietary strategy to address CGS, which could help in the prevention of incidence, regression of existing CGS and preventing possible recurrence.

oo O oo

Section B

Hepatoprotective and antioxidant effect of fenugreek seeds in mice under lithogenic condition

Introduction

While fenugreek seeds have been in use for over 2500 years, this seed spice is also employed for medicinal purpose in many traditional systems of medicine, particularly as antibacterial, gastric stimulant, antidiabetic agent, galactogogue, and against anorexia (Chopra *et al*., 1986; Fillips & Foy, 1990). In recent decades, several health beneficial physiological attributes of fenugreek seeds have been experimentally evidenced in animal studies as well as human trials (Srinivasan, 2006). These include antidiabetic effect, hypocholesterolemic influence, antioxidant potency, digestive stimulant action, hepatoprotective effect, *etc*. Among these beneficial physiological effects, the antidiabetic and hypocholesterolemic property of fenugreek, both of which were mainly attributable to the intrinsic dietary fibre constituent, have promising nutraceutical value (Srinivasan, 2006).

Nearly 50% dry weight of fenugreek seeds is dietary fibre, making it the highest concentration among all natural sources of fibre. About 30% of fenugreek seed (w/w) is gel-forming soluble fibre similar to guar gum, oat bran, psyllium husk. The insoluble fibre which constitutes 20% of fenugreek seed is bulk-forming like wheat bran (Chatterjee & Prakashi, 1995). Adding fenugreek dietary fibre to refined flour helps to fortify with a balance of soluble and insoluble fibre. Flour fortified with 8-10% fenugreek dietary fibre has been used to prepare bakery foods like pizza, bread, muffins, and cakes (Chadha, 1985). Fenugreek seed is a source of steroidal saponin – diosgenin, which can be used to manufacture many pharmaceuticals, such as progesterone. The alkaloid trigonelline present in fenugreek seed is converted into niacin when the seed is roasted (Ambasta, 2000). Researchers have reported that fenugreek seeds contain substances that stimulate pancreas to release digestive enzymes, thereby helping in digestion (Platel & Srinivasan, 2004). The seeds' soothing effect makes them of value in treating gastritis and gastric ulcers (Ambasta, 2000).

Among common spices, fenugreek seeds, garlic, onion, red pepper and turmeric have proven hypocholesterolemic potential (Srinivasan *et al*., 2004). Fenugreek possesses a significant hypocholesterolemic effect not only in various experimental animal models, but also in human subjects (Srinivasan, 2006). Dietary fenugreek seed has been shown to be a good cholagogue (Bhat *et al*., 1985). We have also recently reported the beneficial antilithogenic effect of dietary fenugreek seeds in terms of reducing dietary cholesterol induced formation of cholesterol gallstones and also accelerating the regression of experimentally induced cholesterol gallstones in mice (Chapter-II and Chapter-IIIA). The beneficial effect was attributable to a favourable reversal of the altered lipid homeostasis in the bile of these animals. Fenugreek seeds are a part of Indian dietary and also a constituent of traditional Indian and folk medicine since ancient times (Srinivasan, 2006). In the present work, we have investigated two dietary doses of fenugreek seeds for a possible hepatoprotective effect in mice previously subjected to high cholesterol feeding so as to induce cholesterol gallstone disease. Attendant with a possible protective role on hepatotoxicity is the improvement in antioxidant status of liver tissue through beneficial modulation of antioxidant enzymes and hence the antioxidant influence of dietary fenugreek is also evaluated in this study.

Materials and methods

Materials

Cholesterol, bile salts, BSA, EDTA, DTT, TEA, NAD, NADPH, Tris, TMP, tertiary-butyl hydro peroxide, H_2O_2, GSSG, GSH, CDNB, xanthine, cytochrome C, xanthine oxidase, glutathione perioxidase and alpha cellulose were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Casein was purchased from Nimesh Corporation (Mumbai, India). AIN-76 mineral mix and vitamin mix were procured from SISCO Research Labs, (Mumbai, India). DL-Methionine and choline chloride were from Himedia (Mumbai, India). All other chemicals and solvents used were of analytical grade and solvents were distilled prior to use. Fenugreek seeds were purchased from local market, cleaned free of any stones or impurities and pulverized.

Animal diets

The basal control diet consisted of: AIN-76 semi-purified diet. Sucrose (cane sugar powder), 65%, casein, 20%, cellulose, 5%, mineral mix, 3.5%, vitamin mix, 1%, DL-methionine, 0.3% and choline chloride, 0.2% and refined peanut oil, 5%. Lithogenic diet was prepared by supplementing 0.5% cholesterol and 0.25% bile salts to the AIN-76 basal diet substituting the same quantity of sucrose. Diets were prepared by mixing the ingredients in a mechanical mixer and pellets were prepared using hand-operated pelletizer. Diets were stored at 4°C in air-tight containers. Fenugreek containing diets were prepared by incorporating the fenugreek seed powder at two different levels namely, 6 and 12 % (w/w) in the basal control diet. The incorporation of the fenugreek was at the expense of sucrose.

Animal treatment

This animal experiment was carried out taking appropriate measures to minimize pain or discomfort in accordance with the guidelines laid down regarding the care and use of animals for experimental procedures and with due approval from our Institutional Animal Ethics Committee. Male albino mice [OUTB - Swiss Albino / Ind-cft (2c)] procured from the Experimental Animal Production Facility of this Institute, 108 numbers in total and weighing 22 ± 2 g were housed in polypropylene cages with saw dust as bedding. All the animals were initially fed a lithogenic diet for 10 weeks so as to induce CGS in their gallbladder. In order to ensure formation of CGS, cholecystectomy was performed on 12 randomly selected mice and gallbladders were carefully collected. The gallbladders placed on an illuminator were evaluated for CGS under magnifying lens for the presence of gallstones.

During the post-CGS induction period, the remaining 96 animals were grouped into 8 groups of 12 animals each and were imposed different dietary regimens viz., lithogenic diet (Group 1a & 1b), basal control diet (Group 2a & 2b), basal diet containing 6% fenugreek seed powder (Group 3a & 3b) and basal diet containing 12% fenugreek seed powder (Group 4a & 4b) for a further period of 5 weeks (Groups 1a, 2a, 3a and 4a) and 10 weeks (Groups 1b, 2b, 3b and 4b). The animals were fed *ad libitum* the respective diets

and had free access to water throughout the experimental period. Body weights were recorded at weekly intervals. At the end of the feeding trial, the animals were fasted overnight and sacrificed under ether anesthesia. Blood was drawn immediately by cardiac puncture and the serum was separated by centrifugation for further analysis. Liver was quickly excised, washed with ice-cold saline, blotted dry, weighed and stored at -20°C till further analysis.

Liver function enzymes in serum

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase were assayed using appropriate kits and were expressed as Units/L. Liver protein was estimated according to Lowry *et al*., (1951) using bovine serum albumin as reference protein.

Lipid peroxides and antioxidant molecules in liver

Thiobarbituric acid reactive substances in the liver homogenate were fluorimetrically measured after extracting with butanol according to the method described by Ohkawa *et al*., (1979). The absorbance of the extracted butanol was measured at 515 nm as excitation wavelength and 553 nm as emission wavelength, and was compared with the standard tetramethoxy propane. Total thiols in liver homogenates were measured spectrometrically as described by Sedlak and Lindsay (1968). Glutathione was estimated according to the protocol described by Beutler *et al*., (1963). Ascorbic acid was determined spectrophotometrically according to the method given by Omaye *et al*., (1973).

Antioxidant enzymes in liver

Catalase activity was assayed by following the rate of decomposition of H_2O_2as described by Aebi (1984). Glutathione reductase activity was assayed by measuring the oxidation of NADPH by oxidized glutathione according to Carlberg and Mannervik (1985). Glutathione-S-transferase activity was assayed using the CDNB and by measuring the CDNB-glutathione complex formed as described by Warholm *et al*., (1985). Glutathione peroxidase activity was measured as described by Flohe and Gunzler (1984).

SOD activity was measured by quantifying the inhibition of cytochrome-C reduction in xanthine-xanthine oxidase system as described by Flohe and Otting (1984).

Statistical analysis

Statistical analysis was carried out using Graphpad prism statistical software. Results are analyzed by one way ANOVA and the significance level was calculated using Tukey Kramer multiple comparison test and results are considered as significant at $P<0.05$.

Results

At the end of 10 weeks of feeding a HCD, 10 out of 12 animals randomly selected, had developed CGS in the gallbladder, thus ensuring the lithogenic condition in the experimental animals for evaluating the beneficial influence of dietary fenugreek during the post-CGS induction period.

Hepatoprotective influence of dietary fenugreek

Activities of liver function enzymes in the serum of mice fed for 5 and 10 weeks fenugreek containing diets following an initial exposure to lithogenic diet for 10 weeks are presented in Table-1. The activities of ASAT, ALAT, LDH and alkaline phosphatase increased with the prolonged feeding of HCD. The feeding of basal control and fenugreek containing diets after the initial exposure to HCD decreased the activities of these enzymes, with the decrease more prominent in the fenugreek groups compared to the basal control group. The decrease in the activity of ASAT brought about by dietary fenugreek for 10 weeks was 19 and 23%, while ALAT activity was decreased by 41 and 50%. Reduction in the activity of LDH brought about in dietary fenugreek groups (10 weeks) was 31 and 48%, while the activity of alkaline phosphatase was decreased by 35% in these groups.

Beneficial influence of dietary fenugreek on antioxidant molecules and lipid peroxidation in liver

Data on the influence of dietary fenugreek in experimental mice after an initial exposure to HCD on the hepatic contents of vitamin-C, total thiols, glutathione and lipid

Table 1. Effect of dietary fenugreek powder on liver function enzymes in the serum of mice previously exposed to lithogenic diet

Treatment	Aspartate aminotransferase		Alanine aminotransferase		Lactate dehydrogenase		Alkaline phosphatase	
	5 Week	10 Week	5 Week	10 Week	5 Week	10 Week	5 Week	10 Week
Lithogenic Diet	258.1 ± 5.7	219.4 ± 7.93	77.0 ± 6.4	67.6 ± 4.80	1702.2 ± 75.3	2035.8 ± 136.3	314.0 ± 18.9	261.3 ± 8.4
Basal Control (C)	258.5 ± 11.9	216.0 ± 14.2	47.9 ± 1.3*	51.5 ± 3.93*	1595.0 ± 73.0	1843.5 ± 31.4	212.8 ± 13.9*	209.0 ± 6.9*
C + Fenugreek (6%)	247.7 ± 11.6	177.6 ± 4.53*	40.9 ± 4.1*	40.1 ± 2.57*	1432.0 ± 17.9*	1406.6 ± 49.6*	195.1 ± 10.3*	169.6 ± 7.8*
C + Fenugreek (12%)	211.5 ± 8.9*	169.3 ± 2.66*	41.9 ± 3.7*	34.0 ± 2.13*	1208.3 ± 44.5*	1050.0 ± 84.4*	200.3 ± 19.5*	170.5 ± 5.7*

Values (expressed as U/L) are mean ± SEM of 12 mice.

All groups of animals were initially maintained on lithogenic diet (High cholesterol containing) diet for 10 weeks.

* Significantly different from lithogenic diet group ($P<0.05$)

peroxides are given in Table-2. Lipid peroxide levels showed a reduction in the basal control and fenugreek fed groups compared to lithogenic diet group. The reductions were of the order of 42, 55 and 64% in the respective diet groups at the end of 10 weeks compared to lithogenic diet group. Both ascorbic acid and reduced glutathione showed improvement in their concentration with the addition of fenugreek to the diet. The increase in hepatic ascorbic acid content was 25 and 27% in the two fenugreek groups, respectively, at the end of 10 weeks. The increase in hepatic glutathione content was 33 and 40% in the two fenugreek groups, respectively, at the end of 10 weeks. Hepatic total thiol content was however not improved by fenugreek treatment as compared to lithogenic diet group. These results indicated that there is favorable effect of dietary fenugreek seeds on the hepatic antioxidant status.

Beneficial influence of dietary fenugreek on the activity of antioxidant enzymes in liver

Influence of dietary fenugreek in experimental mice after an initial exposure to HCD on the activity of hepatic antioxidant enzymes is given in Table-3 and Table-4. While there was no significant difference in the activity of catalase, the activities of glutathione reductase, glutathione-S-transferase and glutathione peroxidase were significantly improved as a result of dietary fenugreek. The increase in the activity of glutathione reductase was 24 and 45% in the dietary fenugreek groups at 5 weeks as compared to lithogenic diet group. The increase in the activity of glutathione-S-transferase in dietary fenugreek groups was 32 and 74% compared to lithogenic diet group. The activity of glutathione peroxidase was increased by 25% in both the dietary fenugreek groups. The activity of superoxide dismutase was lower in dietary fenugreek groups (29-34%) compared to the lithogenic diet group (Table-3).

Similarly, at the end of 10 weeks of dietary fenugreek treatment following an initial 10 weeks of lithogenic diet treatment, there was no marked difference in the activity of catalase (Table-4). There was a prominent increase in the activity of glutathione reductase by dietary fenugreek, the increase being 19 and 22%, respectively, over the lithogenic diet group. Glutathione-S-transferase activity was also increased in the

Table 2. Effect of dietary fenugreek powder on liver antioxidant molecules in mice previously exposed to lithogenic diet

Diet group	**Ascorbic acid** µg/mg protein		**Glutathione** nmol/mg protein		**Total thiols** nmol/mg protein		**Lipid peroxides** mmol MDA/mg protein	
	5 Week	10 Week	5 Week	10 Week	5 Week	10 Week	5 Week	10 Week
Lithogenic Diet	0.79 ± 0.04	0.95 ± 0.07	ND	26.7 ± 0.63	0.35 ± 0.01	0.45 ± 0.02	1.03 ± 0.06	1.38 ± 0.12
Basal Control (C)	0.89 ± 0.04	1.08 ± 0.08	ND	30.6 ± 0.86	0.34 ± 0.01	0.48 ± 0.02	0.89 ± 0.05	0.80 ± 0.02*
C + Fenugreek (6%)	0.85 ± 0.03	1.19 ± 0.07*	ND	35.5 ± 1.18*	0.38 ± 0.01	0.46 ± 0.01	0.72 ± 0.03*	0.62 ± 0.04*
C + Fenugreek (12%)	0.90 ± 0.03	1.21± 0.08*	ND	37.3 ± 1.44*	0.40 ± 0.01	0.46 ± 0.02	0.73 ± 0.04*	0.50 ± 0.10*

Values are mean ± SEM of 12 mice / group. ND: Not determined.

All groups of animals were initially maintained on lithogenic diet (High cholesterol containing) diet for 10 weeks.

*Significantly different from Lithogenic diet group ($P<0.05$)

Table 3. Effect of 5 weeks dietary fenugreek powder on the activities of antioxidant enzymes in the liver of mice previously exposed to lithogenic diet

Diet group	Catalase	GSH–reductase	GSH–transferase	GSH-peroxidase	SOD
	mmol/min/mg protein	µmol/ min/mg protein			U/min/mg protein
Lithogenic diet	79.6 ± 3.7	34.8 ± 4.1	0.267 ± 0.031	2.825 ± 0.128	15.3 ± 0.57
Basal Control (C)	74.3 ± 8.7	35.6 ± 2.6	0.322 ± 0.023	3.178 ± 0.096	14.9 ± 0.88
C + Fenugreek (6%)	69.4 ± 3.6	43.0 ± 2.5*	0.352 ± 0.024*	3.525 ± 0.201*	10.1 ± 0.74*
C + Fenugreek (12%)	71.6 ± 1.0	50.3 ± 1.9*	0.465 ± 0.054*	3.527 ± 0.283*	10.9 ± 0.85*

Values are mean ± SEM of 12 mice / group.

All groups of animals were initially maintained on lithogenic diet (High cholesterol containing) diet for 10 weeks.

*Significantly different from Lithogenic diet group ($P<0.05$)

Table 4. Effect of 10 weeks dietary fenugreek powder on the activities of antioxidant enzymes in the liver of mice previously exposed to lithogenic diet

Diet group	Catalase mmol/min/mg protein	GSH–reductase	GSH-transferase	GSH-peroxidase	SOD U/min/mg protein
		µmol/min/mg protein			
Lithogenic diet	63.4 ± 3.27	47.2 ± 3.56	0.343 ± 0.024	3.05 ± 0.19	22.5 ± 1.72
Basal Control (C)	67.8 ± 6.9	53.2 ± 4.45	0.348 ± 0.031	3.10 ± 0.18	14.8 ± 0.44*
C + Fenugreek (6%)	62.6 ± 3.25	56.4 ± 1.18*	0.394 ± 0.023*	3.47 ± 0.15*	13.1 ± 0.16*
C + Fenugreek (12%)	59.6 ± 3.48	57.4 ± 1.07*	. 0.426 ± 0.034*	3.99 ± 0.24*	12.5 ± 0.72*

Values are mean ± SEM of 12 mice /group.

All groups of animals were initially maintained on lithogenic diet for 10 weeks.

*Significantly different from Lithogenic diet group (P<0.05)

fenugreek treatment (by 12 and 17%). Glutathione perioxidase activity was increased by15 and 32% in the two dietary fenugreek groups. The activity of SOD was markedly decreased in basal control group as well in fenugreek treatment, the decrease being 34 - 44%.

Discussion

We have previously reported the health beneficial antilithogenic influence of dietary fenugreek seeds in experimental mice in terms of reducing the incidence of cholesterol gallstone formation under conditions of prolonged exposure to lithogenic diet (Chapter-II) and regressing the preformed cholesterol gallstones (Chapter-IIIA). In addition to causing hepatobiliary diseases, prolonged consumption of a HCD has resulted in an increase in the activity of liver function enzymes ALAT, ASAT and LDH in serum, indicative of liver toxicity. Our present study has indicated that dietary fenugreek possesses hepatoprotective activity, in view of the observed countering of the elevated activities of liver function enzymes – aminotransferases, LDH and alkaline phosphatase in blood, presumably by preventing the leakage of these enzymes from hepatic tissue. Rao *et al.,* (1996) have reported that there were no marked differences in the activity of some of the liver function enzymes like alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase with the addition of fenugreek into the diet. The observed beneficial effect of dietary fenugreek in the present study is under atherogenic conditions. This is also a novel report on the elevated activities of liver function enzymes as a result of continued cholesterol consumption.

It is to be noted that at any of the two particular time intervals studied here (5 weeks or 10 weeks), the beneficial effect of dietary fenugreek with respect to hepatoprotective and antioxidant influence as compared to the basal diet without fenugreek is discernible. The observed differences in the activities of the related enzymes in either lithogenic group or basal control group between the two time intervals (5 weeks and 10 weeks) could be attributable to the change in the age of the animal (5 additional weeks in the case of 10 week treatment). The increase in the activities of antioxidant enzymes after

the discontinuance of lithogenic diet for 5 or 10 weeks was higher in groups fed fenugreek containing basal diets as compared to the group fed basal diet without fenugreek. This study documents the recovery of liver health after a chronic exposure to cholesterol. The data also suggest that this recovery is accelerated by the presence of fenugreek in the diet.

Oxidative damage at the cellular or subcellular level is now considered to be a major event in disease processes like coronary vascular disease, inflammatory disease, diabetes, carcinogenesis, and aging (Janssen *et al*., 1993). Reactive oxygen radicals are detrimental to cells at both membrane and genetic levels. They induce lipid peroxidation in cellular membranes, generating lipid peroxides that cause extensive damage to membranes and membrane-mediated chromosomal damage (Janssen *et al*., 1993). The present animal study has also indicated the health beneficial antioxidant influence of dietary fenugreek seeds in mice have been previously chronically exposed to a HCD. Feeding of fenugreek containing diet lowered the lipid peroxidation in liver more than those maintained on basal control diet. This could be a consequence of enhancing the activities of antioxidant enzymes, especially glutathione reductase, glutathione-S-transferase and glutathione peroxidase and increasing the concentrations of ascorbic acid and reduced glutathione in the hepatic tissue. Both the dietary levels of fenugreek effectively increased hepatic levels of ascorbic acid and glutathione, while simultaneously reducing the amount of lipid peroxides.

Fenugreek exhibits high antioxidant activity in terms of scavenging of $H_2O_2$and DPPH radicals, inhibiting lipid peroxidation. The aqueous extract contains gallic acid which is known to possess antioxidant activity (Srinivasan, 2010). Trigonelline and diosgenin, active components of fenugreek have been tested for antioxidant activity (Soares *et al*., 2003). Administration of dietary fenugreek seeds resulted in increased GSH levels and activity of glutathione-S-transferase in the liver, but there was no appreciable change in superoxide dismutase and catalase (Choudhary *et al*., 2001). Our present observation of the antioxidant effect of dietary fenugreek after a period of high cholesterol treatment is similar to the above report with respect to hepatic GSH concentration and activities of glutathione-S-transferase and catalase. Dietary fenugreek seed has been shown to counter

the increased lipid peroxidation and alterations in the content of circulating antioxidant molecules – glutathione, β-carotene and α-tocopherol in alloxan diabetic rats (Ravikumar & Anuradha, 1999). The influence of fenugreek seed powder supplementation in the diet (for 30 days at a dosage of 2 g/kg body weight) on lipid peroxidation and antioxidant status has been studied in alloxan-diabetic rats (Anuradha & Ravikumar, 2001). The enhanced lipid peroxidation and increased susceptibility to oxidative stress associated with depletion of antioxidants in liver, kidney and pancreas observed in diabetic rats were normalised with fenugreek seed powder treatment (Anuradha and Ravikumar 2001). The protective effect of the aqueous extract of the seeds on the activity of Ca^{2+}-ATPase in liver homogenate in the presence of Fe^{2+}/ ascorbate *in vitro* was also investigated. Ca^{2+}-ATPase activity in liver was protected by the aqueous extract to nearly 80% of the initial activity (Anuradha & Ravikumar, 2001). The findings suggest that the soluble portion of fenugreek seeds could be responsible for the observed antioxidant property.

The fact that the activities of glutathione related enzymes – glutathione-S-transferase and glutathione peroxidase were higher in fenugreek groups as compared to the group that was fed basal diet without fenugreek especially at the 10 week duration can be interpreted as a demonstration that fenugreek seeds have antioxidant property *in vivo*. The activities of antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase have been examined in the tissues of diabetic rats treated with fenugreek (Genet *et al*., 2002). Fenugreek administration to diabetic animals considerably reversed the disturbed antioxidant levels and peroxidative damage in diabetic animals, thus suggesting that fenugreek seeds show an encouraging antioxidant property that can be exploited for the treatment / reversal of the complications of diabetes. Aqueous extract of fenugreek seeds was found to have protective effect in experimental ethanol toxicity in rats (Thirunavukkarasu *et al*., 2003). Simultaneous administration of aqueous extract of fenugreek seeds along with ethanol for 60 days prevented the leakage of enzyme activities aspartate transaminase, alanine transaminase and alkaline phosphatase into serum, and the rise in lipid peroxidation in liver as a result of ethanol toxicity. Fenugreek aqueous extract also enhanced the antioxidant potential in these animals by countering

the reduced activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase in liver and countering the depletion in glutathione, ascorbic acid and α-tocopherol concentrations (Thirunavukkarasu *et al*., 2003). Fenugreek seed powder in combination with vanadate was found to effectively counter alterations in the activities of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in alloxan diabetic rat brains (Siddiqui *et al*., 2005). Antioxidant properties of germinated fenugreek seeds *in vitro* have also been reported (Dixit *et al*., 2005).

While dietary fenugreek produced beneficial increase in the activities of GSH-related antioxidant enzymes in the liver, the same was not seen in the case of SOD. The relationship between the lipid effects and the antioxidative effects of an ethyl acetate extract of fenugreek in Wistar rats fed cholesterol-rich diet for 16 weeks has been recently reported (Belguith-Hadriche *et al*., 2010). Administration of fenugreek ethyl acetate extract significantly lowered the plasma levels of cholesterol. The content of TBARS and catalase and superoxide dismutase activities in liver, heart and kidney decreased significantly after oral administration of the extract compared with those of rats fed a cholesterol-rich diet. Our present study also showed decreased hepatic SOD activity by feeding fenugreek to mice similar to the observation of Belguith-Hadriche *et al*., (2010). There is no possible explanation for the observed decreased activity of hepatic SOD in fenugreek treatment.

The hepatoprotective and antioxidant influence of dietary fenugreek seeds presented here is the first report observed under lithogenic conditions. The observed hepatoprotective influence and improvement in antioxidant status of the animals as a result of dietary fenugreek treatment may have resulted as a consequence of correcting the disrupted cholesterol homeostasis by chronic cholesterol feeding which included hypercholesterolemia, higher levels of cholesterol in liver tissue and higher secretion of cholesterol into bile which eventually resulted in cholesterol gallstones in gallbladder. In gallstone induced mice, dietary fenugreek alleviated the severity of cholesterol gallstones by countering the cholesterol saturation in the bile (Chapter-IIIA). Beneficial influence of

dietary fenugreek seeds on cholesterol homeostasis has been attributed to their rich amounts of soluble fibre constituent (galactomannan) as well as to the saponins present in them (Srinivasan, 2006). It is also reported that diosgenin, a potentially hydrolyzed compound of fenugreek saponin in the intestinal tract will interfere with cholesterol absorption (Sauvaire *et al.*, 1991).

The present animal study has indicated the health beneficial hepatoprotective and antioxidant influence of dietary fenugreek seeds in mice that had been previously chronically exposed to a HCD. The antioxidant effect of dietary fenugreek observed here parallels the recently reported accelerated regression of cholesterol gallstones brought about by this spice through countering the altered cholesterol saturation index in bile through beneficial modification of biliary lipid profile (Chapter-IIIA). Incidentally, this is also the first report on the antioxidant influence of fenugreek seeds in mice under conditions of oxidative stress brought about in a hyper-cholesterolemic and lithogenic situation.

Summary

We have recently reported that dietary fenugreek seeds lower the incidence of CGS in HCD mice and also regress pre-established CGS. In this study, fenugreek was evaluated for hepatoprotective and antioxidant influence in mice fed HCD. After feeding HCD for 10 weeks, groups of animals were maintained for further 10 weeks on HCD / basal diet / basal diet containing 6 or 12% fenugreek. Activities of serum ASAT, ALAT, LDH and alkaline phosphatase increased with prolonged feeding of HCD. Activities of these enzymes were lower in animals fed basal control/ fenugreek containing diets after initial exposure to HCD, and were prominent in fenugreek groups. Hepatic lipid peroxides decreased and antioxidant molecules increased in fenugreek fed groups. Activities of hepatic antioxidant enzymes ─ glutathione reductase, glutathione-S-transferase and glutathione peroxidase were higher in fenugreek treatment. These results suggested hepatoprotective and antioxidant potential of fenugreek seeds under conditions of lithogenicity.

oo O oo

Effect of dietary fenugreek seeds on biliary proteins which influence nucleation of cholesterol crystals in bile

Introduction

Cholesterol gallstones (CGS) are most common among the gastrointestinal diseases with a prevalence of 10 - 15% in western population. CGS is the consequence of final stage of supersaturation of bile with cholesterol. The etiology / pathophysiology results from a failure of cholesterol homeostasis. The formation of cholesterol crystals and CGS can be observed in three stages, namely, supersaturation, initiation cum accelerated crystallization and conglomerisation of the crystals with the aid of various factors (Strasberg, 1998). When the cholesterol carrying capacity of bile is saturated, cholesterol will start to settle in various forms, and this is the foremost step in the precipitation of cholesterol crystals (Portincasa *et al.,* 2003).

It is now believed that supersaturation of bile with cholesterol is necessary but not sufficient for crystal formation. Crystallization of cholesterol from its supersaturated solution requires the presence of various other factors. These factors which influence cholesterol crystal and stone formation are classified as pronucleation and anti-nucleation factors. The critical balance between these factors will determine the predisposition of bile; i.e., higher concentration of pronucleating agents than the anti-nucleating agents will render the bile more susceptible to form cholesterol crystals and higher the concentration of anti-nucleating factors than the pronucleating factors prolongs the process of crystal formation and nucleation. In the last decade much work has been done in this field and various factors that affect crystallization or NT such as mucin, bilirubin, bile salt: lecithin ratio, calcium, fibronectin, biliary proteins especially low molecular weight (LMW) proteins. Immunoglobulins, apolipoproteins A1 and A2 and cholesterol saturation index (CSI) of bile are the most important factors (Busch & Matern, 1991).

Metabolic alteration in hepatic secretion and intestinal bacterial degradation of bile salts destabilize cholesterol carriers in bile to form cholesterol crystals. Existence and the quantity of various forms of the vesicular nucleating proteins play an important role in the aggregation and fusion. Crystal nucleation is an important step and assessment of NT is a

good indicator and predictor of cholelithiasis. A number of proteins have now been identified but understanding of the comparative role of all these proteins remains to be established because of confliction results. Recent studies have underscored that some of the proteins involved in hepatic cholesterol transport regulate the availability of cholesterol for biliary secretion.

We have shown that dietary fenugreek seeds are effective in reducing the formation of CGS under lithogenic conditions [Chapter-II] and also in the regression of pre-formed CGS [Chapter-III]. The antilithogenic influence of fenugreek seeds was attributable to their cholesterol-lowering effect in blood and liver, and the ability to lower cholesterol saturation index by altering the biliary lipid composition. Apart from beneficial modulation of biliary cholesterol saturation index, fenugreek may also influence cholesterol nucleating and antinucleating proteins that contribute to their antilithogenic potential. In view of this, the influence of dietary fenugreek on biliary proteins and glycoproteins in particular, was evaluated in an animal study. In order to understand the mechanism of cholesterol crystal nucleation and the probable effect of proteins present in biles of rats fed fenugreek, they were tested with supersaturated model biles for cholesterol nucleation.

Materials and Methods

Chemicals

Acrylamide, N,N-methylene-*bis*-acrylamide, BSA, bile acids, bile salts, cholesterol, commassie brilliant blue (CBB-G250), DPPC, EDTA, HEPES, 3α-HSD, hydrazine hydrate, H_2O_2, low molecular weight (LMW) protein markers, SDS, sodium azide (NaN_3), sodium metaperiodate, triglyceride purifier, TMP, TEMED, TBA, tripalmitin, Tris-HCl, ethyl urethane, glycine, Bio-gel A were purchased from Sigma Chemical Co. (St. Louis, USA). Intra-medic polyethylene tubing (PE-10) was purchased from Thomas Scientific Co. (New Jersey, USA). Choline chloride, agar-agar, cellulose, mercaptoethanol, sodium pyruvate were purchased from Himedia Laboratories (Mumbai, India). Casein was purchased from Nimesh Corporation (Mumbai, India). Fenugreek seeds were purchased locally and powdered. All other chemicals used were of analytical grade and the solvents were distilled before use.

Animal treatment

Animal experiment was carried out taking appropriate measures to minimize pain or discomfort, and with due approval from the Institutional Animal Ethics Committee. Male albino rats [OUT-Wistar, IND-cft (2c)] weighing about 120 g obtained from experimental animal production facility of this Institute were used. The animals were placed in individual cages in an approved animal house facility with 12 h light and dark cycles with temperature 25 ± 2ºC and fed fresh diets daily. Four groups of rats (n = 10 per group) were maintained on (1) Basal control (C), (2) C + 12% Fenugreek, (3) High cholesterol diet (HCD), and (4) HCD + 12% Fenugreek. The animals had free access to food and water. The AIN-76 basal control diet was prepared as mentioned earlier. HCD diet was prepared by adding 0.5% cholesterol and 0.25% bile salts, substituting same quantity of sucrose. The incorporation of the fenugreek was at the expense of sucrose.

Bile collection

At the end of the feeding duration i.e. eight weeks, rats were anaesthetized with ethyl urethane (1.2 g/kg body weight) by intra peritoneal injection. Laparatomy was performed and common bile duct was cannulated with PE-10tubing and bile was collected in sterile tubes for 3 h, during which the body temperature of the animals were maintained at 37 °C using incandescent lamps. The volume of the collected bile was noted and stored at -20°C until further analysis.

Biliary lipids

Biliary lipids were extracted by the method described by Bligh and Dyer (1959). The chloroform layer was used for the analysis of cholesterol and phospholipids. The methanol layer was used for the analysis of total bile acids. Cholesterol was estimated by the procedure of Searcy and Bergquist (1960). Phospholipid was assayed by ferrous ammonium thiocyanate method using DPPC as standard (Stewart, 1980). Total bile acid content was estimated using 3α-HSD enzyme as described by Turley and Dietschy (1970). CSI of the bile was calculated by using the values of cholesterol, phospholipids, total lipids and bile acids of bile (both relative and total lipid concentrations) as described by Carey (1978).

Cholesterol nucleation time

Hepatic bile of different diet groups individually as well as in combination in various proportions, viz., 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100 were incubated in a sterile screw capped air-tight vials for 21 days at 37 ºC with 0.03% NaN_3 as antimicrobial agent. Daily, an aliquot of bile was drawn and observed under polarized microscope for the appearance of crystals. Cholesterol crystal nucleation time (NT) is the time of first detection of at least three cholesterol crystals per microscopic field (100 X).

Preparation of model bile

Model bile of predetermined CSI was prepared according to the procedure as given by Kibe *et al.,* (1985). Cholesterol and phosphatidyl choline in chloroform and sodium taurocholate in methanol were mixed in different proportions to get desired CSI, flushed under nitrogen, shaken for 2 h at 37 °C, the mixture was evaporated under a stream of nitrogen and the sample was lyophilized for 6 h. The lyophilized powder was resuspended in Tris-buffer saline, the suspension was incubated with shaking at 100 rpm maintained at 55 °C for 6 h or until microscopically homogenous. The clear solution was filtered through a preheated 0.22µm Millipore filter, flushed with nitrogen. The solution was incubated at 37 °C for 15 min prior to use in the crystal growth assay.

Separation of biliary proteins by gel permeation chromatography

The rat bile was initially separated on a molecular sieving chromatography column as described by Busch and Matern (1991) using Biogel-A, with elution buffer containing 10mM Tris (pH 7.45), 150 mM sodium chloride, 4mM sodium taurodeoxycholate, and 3mM sodium azide to remove soluble mucin glycoproteins eluting in the void volume, as well as higher molecular weight (HMW) proteins (>200 kDa) present in the initial fraction (Fraction I). Thus, these were separated from LMW proteins (<200 kDa) present in the subsequently eluted main fraction (Fraction II) that also contained most of the biliary lipids. Both LMW and HMW fractions were pooled saperately and dialyzed against 10mM ammonium bicarbonate for 2 days at 4°C, lyophilized and stored at −20°C

Cholesterol crystal growth assay

Cholesterol crystal growth in the model bile was measured as described by Busch *et al.,* (1991). Aliquots of filtered (0.22 µm) aqueous solutions of the effectors of interest (both LMW and HMW) were inserted into vials equipped with Teflon-lined screw caps, lyophilized and resolubilised in 40 µL TBS, control vials contained equal volume of TBS and equilibrated at 37 °C. Aliquots (400 to 500 µL) of model bile equilibrated at 37 °C were distributed to each vial 15 min after model bile filtration. The crystal growth assay was carried out in two parallel sets: seeded and unseeded (spontaneous). Cholesterol monohydrate seed crystals were prepared according to Igimi and Carey (1981). Seeded crystals were prepared by sonication of cholesterol crystals that were grown in supersaturated solutions of cholesterol in ethanol and then filtered to retain only those crystals between 0.22 µm and 0.8 µm in diameter. At zero time, the growth of one set of test solutions was initiated by adding a small amount of seed crystals (10 to 25 µL, to achieve 0.5 to 5 g/mL the final test sample cholesterol). The unseeded set was adjusted by adding same volume of distilled water. The samples were flushed with nitrogen, incubated at 37°C and shaken twice daily at 100 rpm for minimum of 10 min each time. To determine the crystal concentration at a specific time, an aliquot (25 µL) of the model bile was sampled and diluted with Tris buffer saline containing 10 mM sodium taurodeoxycholate (dilution factor of 15 to 50). After 20 min and within 3 h, OD at single wavelength with in the visible range (400 ─ 900 nm) was measured and the measurements were made daily for a period of 21 days.

Protein and glycoprotein

Protein content was determined according to Lowry *et al.,* (1951) and glycoprotein estimation was done according to Mantle and Allen (1978) using mucin as a reference standard**.**

Separation of micelles and vesicles in bile by column chromatography

Cholesterol transportation forms i.e., micelles and vesicles were separated using gel filtration chromatography (Sepharose CL-4B-200) as outlined by Pattinson *et al.,* (1991). The column size of 70 cm × 1.7 cm equilibrated with phosphate-buffer saline containing

0.04% NaN_3 and 6 mM sodium taurocholate and flow rate adjusted to 17 mL/h. Aliquot of bile was labeled by incubation with radio label (0.05 µCi ^{14}C-cholesterol/ml) at 37^{o}C were loaded to the column and fractions of 1.7 mL were collected. Aliquot of 100 µL of fractions with the addition of 5 mL of scintillation fluid was counted in a liquid scintillation counter. Transportation forms of cholesterol in the bile of rats from each diet group were compared.

SDS-Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gels (12%) were developed in buffer system as described by Laemmli (1970). Lyophilized protein samples were resolubilized with sample buffer and applied to gels. Gels were fixed overnight and stained with CBB-G250.

Statistical analysis

Statistical analysis was carried out using Windows 98 Microsoft excel statistical software and Prism graphpad statistical software. Results were analyzed and the significance level was calculated using Tukey Kramer multiple comparison test and the results were considered significant at $P<0.05$.

Results

Biliary lipid profile

Dietary fenugreek showed a significant effect on biliary lipid profile, the effect being more distinct when fed along with HCD (Table-1). Incorporation of fenugreek into HCD had a favorable effect, cholesterol content being reduced by 70.5%, phospholipids content increased by 33.5% and bile acids increased by 49%. Addition of fenugreek to HCD reduced both C: P and C: BA ratio to a level comparable to basal control. C: P and C: BA ratio of HCD group was 1.12 and 0.125 while in the other groups it was in the range of 0.22 - 0.32 and 0.031 – 0.047, respectively. CSI decreased significantly by dietary fenugreek along with HCD (0.75) compared to HCD (1.98).

Table 1. Biliary lipid profile of rats fed with diets containing fenugreek

Diet group	Lipids (mM)			Total lipids (g/dL)	C:P ratio	C:BA ratio	CSI
	Cholesterol	Phospholipids	Bile acids				
Control (C)	4.74 ± 0.22	14.9 ± 0.8	100.0 ± 2.5	10.2 ± 0.25	0.318 ± 0.020	0.047 ± 0.004	0.70 ± 0.07
C + Fenugreek	4.06 ± 0.25[a]	18.6 ± 0.7[a]	132.9 ± 5.6[a]	10.1 ± 0.66	0.218 ± 0.013[a]	0.031 ± 0.002[a]	0.60 ± 0.04
HCD	17.0 ± 0.75[a]	15.2 ± 0.8	136.5 ± 5.3[a]	11.7 ± 0.18[a]	1.118 ± 0.049[a]	0.125 ± 0.005[a]	1.98 ± 0.05[a]
HCD+ Fenugreek	5.78 ± 0.45[b]	20.9 ± 1.5[b]	150.4 ± 2.1[b]	10.2 ± 0.22[b]	0.277 ± 0.022[b]	0.038 ± 0.002[b]	0.75 ± 0.07[b]

Values are mean ± SEM of 6 animals per group. HCD: High cholesterol diet

a: Significantly different from Control group ($p<0.05$)

b: Significantly different from HCD group ($p<0.05$)

Biliary proteins and glycoproteins

Addition of fenugreek affected the volume of bile secreted, total biliary solids, total protein content, glycoprotein content and lipid peroxides in bile (Table-2). The effect of fenugreek was more prominent when fed along with HCD than with control diet. Addition of fenugreek increased the bile flow by 19.5%, while total solids were comparable to basal control where as total protein content was reduced to half and comparable to basal control. There was a significant decrease in the glycoprotein content (27.5%) and lipid peroxides (13.5%) with the addition of fenugreek to HCD.

Vesicle and micelle forms of biliary cholesterol

The gel filtration profile of bile of different groups on Sepharose CL-4B-200 is given in Fig.1. While there was not much change in the elution profile of control (C) and C + Fenugreek group (Fig.1A), the elusion profile of the bile of HCD group indicated a bigger void volume peak suggesting an increase in the vesicular form (Fig.1B). The increase in vesicles with HCD feeding was partially countered in the case of the bile of HCD + Fenugreek group (Fig.1C), the reduction in the vesicle being 65.4% with concomitant increase in the smaller vesicles (94%) and with little change in the micelle form of cholesterol (Table-3). The control (C) and C + Fenugreek group had a smaller vesicular peak, a moderately shouldered smaller vesicular and a major micellar peak, while HCD and HCD + Fenugreek groups had a distinct vesicular and micellar peaks. Dietary fenugreek increased the smaller vesicular peak which is clearly shouldered in between the two peaks (Fig.1C).

Nucleation of cholesterol crystals in the bile of various diet groups

Observation of the nucleation of cholesterol crystals in the bile of HCD group of rats indicated that it nucleated on the 6^{th} day (Table-4). Addition of bile from other groups to that of HCD group in increasing proportions prolonged the cholesterol NT. When mixed with bile of other diet groups such as control (C), C + fenugreek and HCD + fenugreek, in different proportions ranging from 80:20 to 20:80 v/v, the same bile required 8─19 days for cholesterol nucleation. Thus, dietary fenugreek is indicated to prolong the cholesterol NT of the bile, probably by modulation of factors that have a role in cholesterol nucleation.

Table 2. Effect of dietary fenugreek on bile flow rate, total solids, biliary protein and lipid peroxides

Dietary group	Volume of bile (mL/h)	Total solids (g %)	Protein (mg/mL)	Glycoprotein (mg/mL)	Lipid peroxides (nmol/dL)
Control (C)	0.64 ± 0.02	2.29 ± 0.14	2.17 ± 0.04	0.32 ± 0.02	186.7 ± 10.0
C + Fenugreek	0.70 ± 0.03	2.43 ± 0.16	2.04 ± 0.04	0.31 ± 0.04	187.7 ± 15.4
HCD	0.65 ± 0.04	2.73 ± 0.19[a]	5.81 ± 0.10[a]	0.41 ± 0.01[a]	215.6 ± 14.2[a]
HCD + Fenugreek	0.78 ± 0.02[b]	2.33 ± 0.14	2.43 ± 0.06[b]	0.29 ± 0.02[b]	188.6 ± 10.2[b]

Values are mean ± SEM of 6 animals per group.

a: Significantly different from Control group ($p<0.05$);

b: Significantly different from HCD group ($p<0.05$)

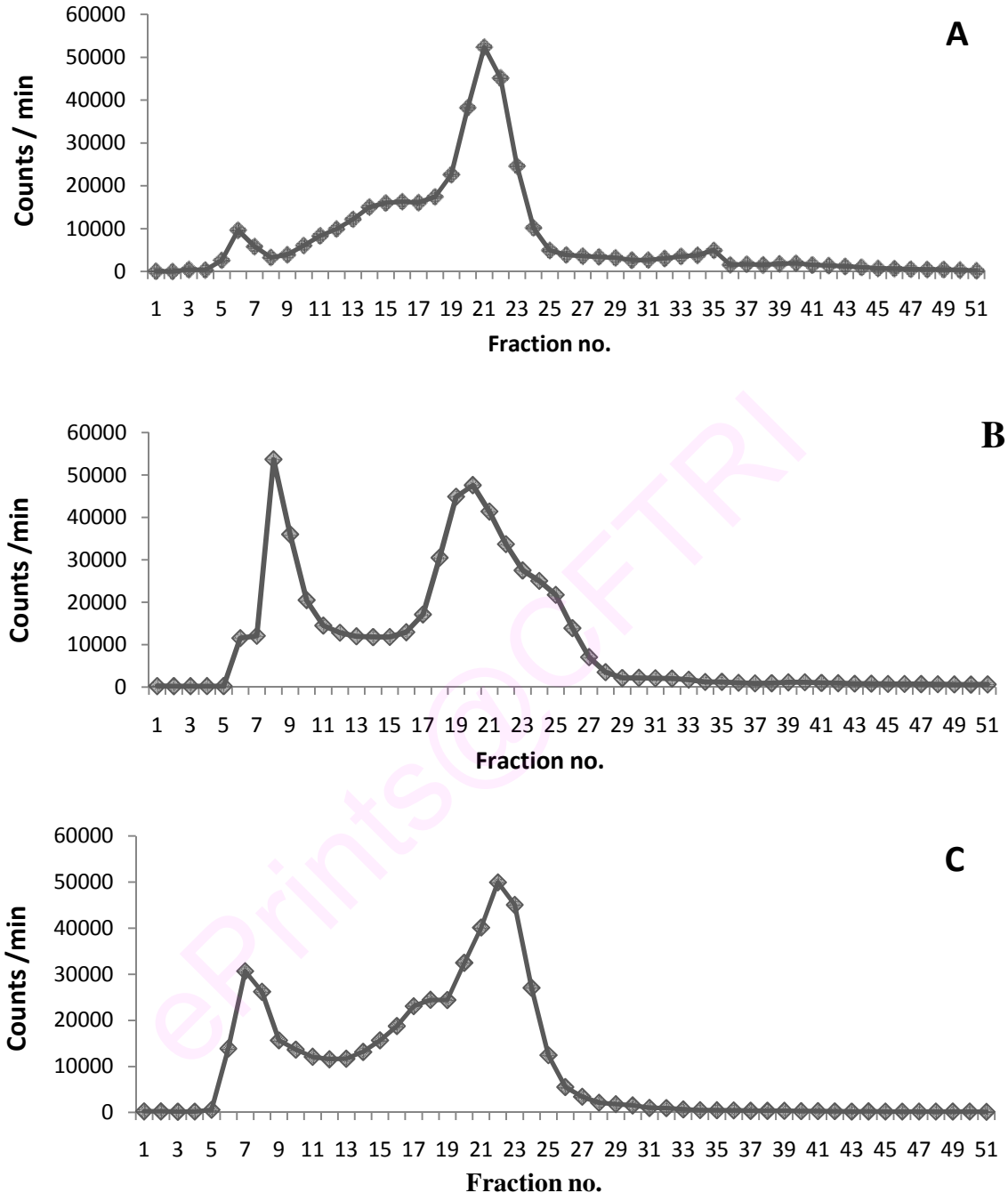


Fig.1. Gel filtration profile of rat bile on Sepharose CL-4B-200

(A) Basal Control and Control + Fenugreek groups showing clear smaller vesicular shoulder leading to micellar peak. (B) HCD group showing a distinct bigger void volume vesicular peak and a broad smaller vesicular zone. (C) HCD + Fenugreek group showing a distinct void volume vesicular peak and micellar peak.

Table 3. Distribution of cholesterol between its transport forms in the bile of rats fed with fenugreek

Diet group	% Total cholesterol		
	Vesicle	**Small vesicle**	**Micelle**
Control (C)	5.67	26.3	68.0
C + Fenugreek	5.68	30.7	63.6
HCD	24.3	16.7	59.0
HCD + Fenugreek	15.9	32.4	51.8

*Values are mean of 6 animals per group

Effect of biliary protein fraction on cholesterol crystal growth in model bile

Biliary protein fractions of different diet groups were eluted on a gel permeation column to separate high molecular weight and low molecular weight fractions (Fig.2). There was a clear separation of biliary proteins into two peaks. These fractions were independently pooled, concentrated and used for studying their influence in cholesterol crystal growth assay studies.

Effect of LMW protein fraction of bile from different groups on cholesterol crystal growth is given in Fig.3 and Table-5. The effect of LMW protein fraction was more pronounced than that of HMW protein fraction. Addition of LMW protein fraction from the bile of HCD group had the NT as early as 24 h, whereas the addition of biliary proteins from other groups prolonged this NT. This shows the significant beneficial effect of dietary fenugreek on cholesterol NT and cholesterol crystal concentration. The crystal growth rate (I*g*) and final crystal concentration (I*c*) of the model bile to which biliary LMW protein fraction from HCD group was added were highest (I*g*=2.3, I*c*=1.56) compared to others (I*g* was around 1.5 and I*c* <1.5. Similar results were observed in unseeded sets also where both I*g* and I*c* were highest (1.8 and 1.47, respectively) in the model bile with added biliary proteins from HCD group as compared to all other treatments. The crystal concentration showed that HCD + Fenugreek had a crystal concentration less than that of HCD.

The effect of HMW protein fraction of bile from different diet groups on cholesterol crystal growth is given in Fig.4 and Table 6. The crystal growth assay was carried in two separate sets i.e., unseeded and seeded (in which the seeded cholesterol crystals (10 µg) were added as a starter material for the initiation of crystallization). Addition of HMW protein fraction obtained from the bile of HCD group shortened the cholesterol crystal growth time by increasing the cholesterol crystal concentration. In the case of HCD group, the NT was around 24 h, whereas the addition of biliary proteins from other groups prolonged this NT. The maximum crystal growth rate (I*g*), final crystal concentration (I*c*) and onset time of crystal detection (I_t) were determined. Addition of biliary protein from

Table 4. Nucleation of cholesterol crystals in the bile of lithogenic rats mixed with bile of rats fed diets containing fenugreek

Diet group	**NT (days)**							**Diet group**
	100:0	**80:20**	**60:40**	**50:50**	**40:60**	**20:80**	**0:100**	
HCD	6	10	13	15	17	ND	ND	**Control (C)**
HCD	6	10	14	15	18	ND	ND	**C + Fenugreek**
HCD	6	8	12	13	15	19	20	**HCD + Fenugreek**

ND- Cholesterol crystals not detected even on 21 days; HCD: High cholesterol diet

Each value is the mean of 6 samples

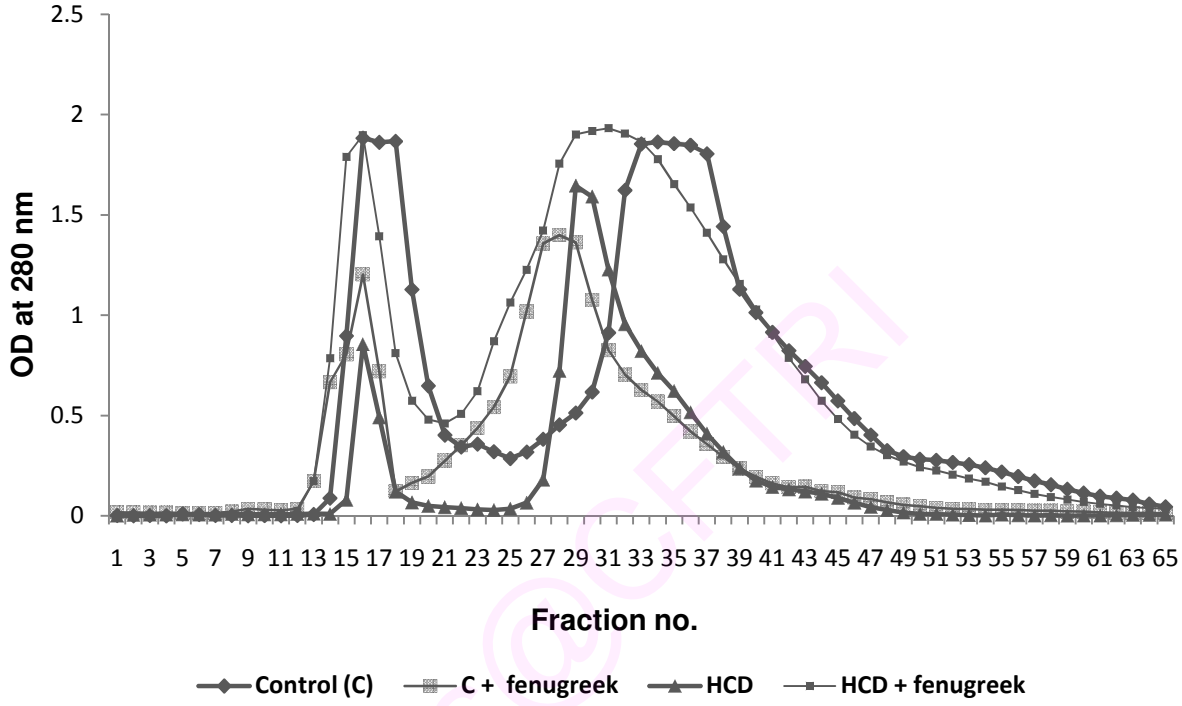


Fig.2. Biliary Protein fractions of different diet groups eluted by gel permeation chromatography

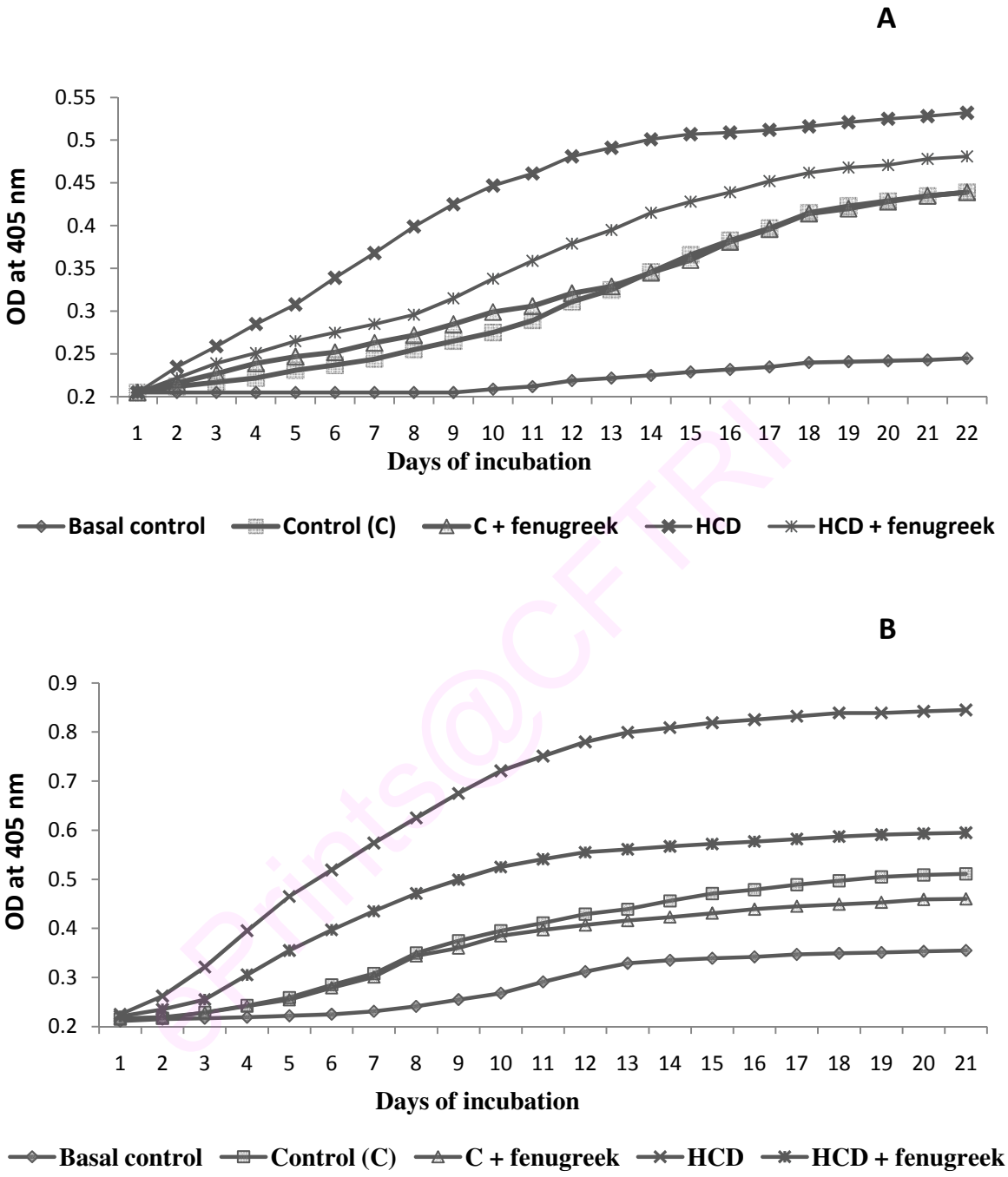


Fig.3. Effect of biliary LMW protein fraction from different groups on cholesterol crystal growth in model bile

(A) Unseeded; (B) Seeded

Table 5. Influence of LMW protein fractions from bile of rats fed different diets on cholesterol crystal growth parameters tested in model bile

Diet group	Protein (µg)	Seeded		Unseeded		
		I_g	I_c	I_g	I_c	I_t
Control (C)	250	1.2	1.43	1.2	0.99	2.30
C + Fenugreek	250	1.3	1.60	1.6	1.11	2.50
HCD	250	2.3	1.65	1.8	1.47	1.00
HCD + Fenugreek	250	1.4	1.55	1.6	1.37	2.20

Data calculated from Fig.1; Each value is the mean of 6 samples.
I_g-Growth Index, I_c- Crystal Index, I_t- Onset time of crystal detection

the lithogenic group to supersaturated model bile shortened the NT and increased the crystal growth rate and crystal concentration. The crystal growth rate (I*g*) and final crystal concentration (I*c*) of the model bile to which HMW protein fraction of bile from lithogenic group were highest (I*g*=1.78, I*c*=1.21) compared to others (I*g* was 1.08 – 1.43 and I*c* was < 1.5). Similar results were observed in unseeded sets also where both I*g* and I*c* of HCD group recorded highest (1.45 and 1.11, respectively) in the model bile with added biliary proteins from HCD group compared to other treatments where it recorded I*g* of around 1.0 – 1.21 and I*c* of around 0.9 – 0.92 respectively.

SDS-PAGE profile of biliary LMW and HMW protein fractions

Biliary HMW and LMW proteins obtained from different groups were resolved on SDS-PAGE (Fig.5). There was a clear difference in the proteins and their band intensity especially in the LMW protein fraction. The SDS-PAGE profile indicated that important biliary proteins which distinguish between the HCD group and other groups are the proteins which are having a molecular weight around 14 kDa and around 28 kDa. Intensity of the band indicated that these two proteins are present at very lower concentration in HCD group compared to other groups. The 28 kDa protein might be responsible for retarding cholesterol crystallization in model bile.

Discussion

Health beneficial anti-lithogenic potential of dietary fenugreek seeds in experimental mice in terms of preventing the incidence of CGS and regression of preformed CGS has been observed [Chapter-II and Chapter-III]. Such an influence although has been attributable primarily to their influence on cholesterol metabolism so as to relieve supersaturation of biliary cholesterol, additional influences on cholesterol nucleation and antinucleation factors present at the site of CGS cannot be ruled out. The present study has revealed that dietary fenugreek seeds have a marked influence on factors that affect cholesterol crystallization and as such showed a positive effect with respect to cholesterol crystal NT, crystal growth and composition of cholesterol crystals.

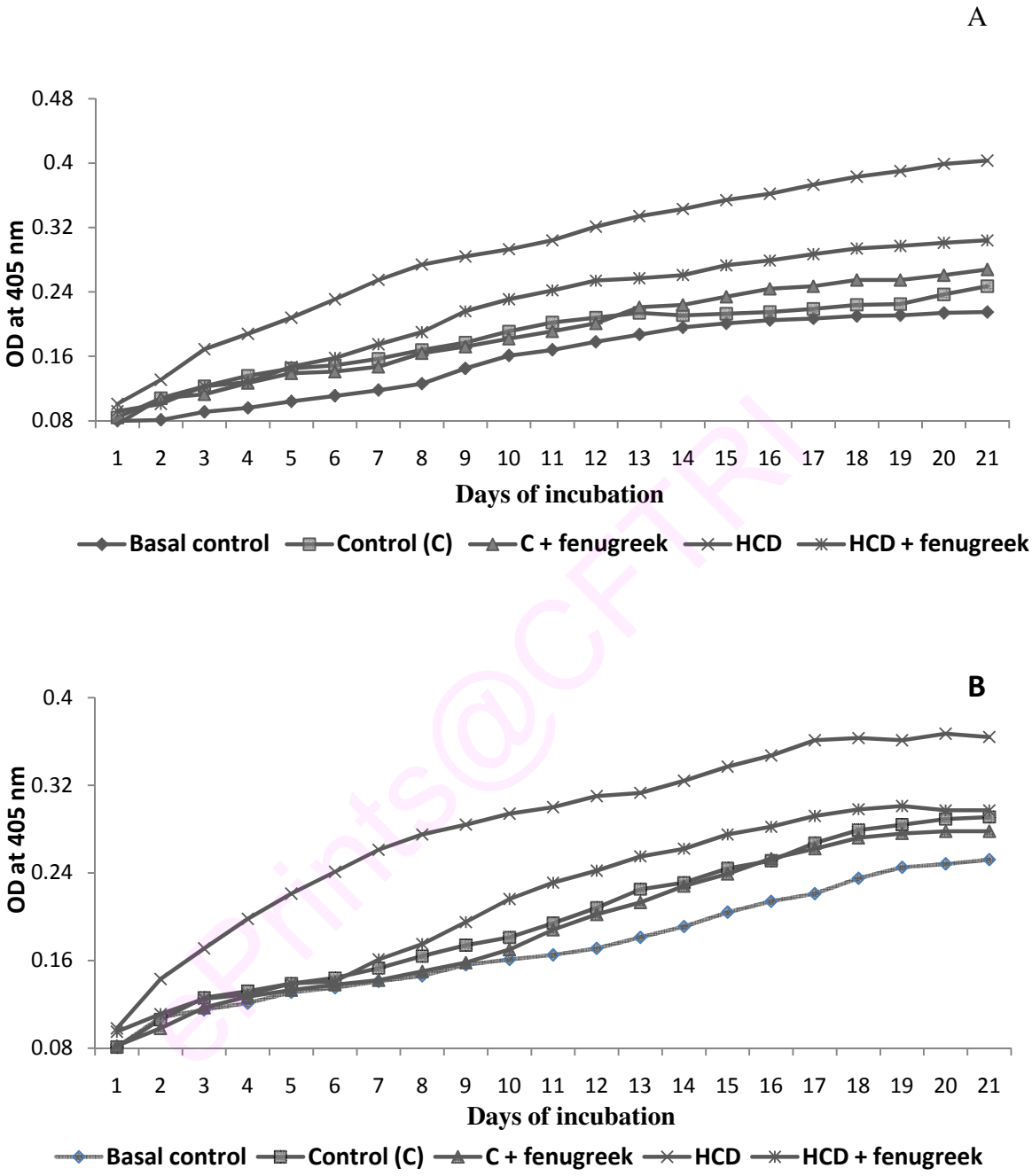


Fig.4. Effect of biliary HMW protein fraction from different groups on cholesterol crystal growth in model bile

(A) Unseeded; (B) Seeded

Table 6. Influence of HMW protein fractions from bile of rats fed different diets on cholesterol crystal growth parameters tested in model bile

Diet group	Protein (µg)	Seeded		Unseeded		
		I_g	I_c	I_g	I_c	I_t
Control (C)	250	1.08	1.13	1.0	0.92	2.50
C + Fenugreek	250	1.08	1.13	1.02	0.9	2.60
HCD	250	1.78	1.21	1.45	1.11	1.00
HCD + Fenugreek	250	1.43	1.1	1.2	0.92	2.20

Data calculated from Fig.2; Each value is the mean of 6 samples.
I_g-Growth Index, I_c- Crystal Index, I_t- Onset time of crystal detection

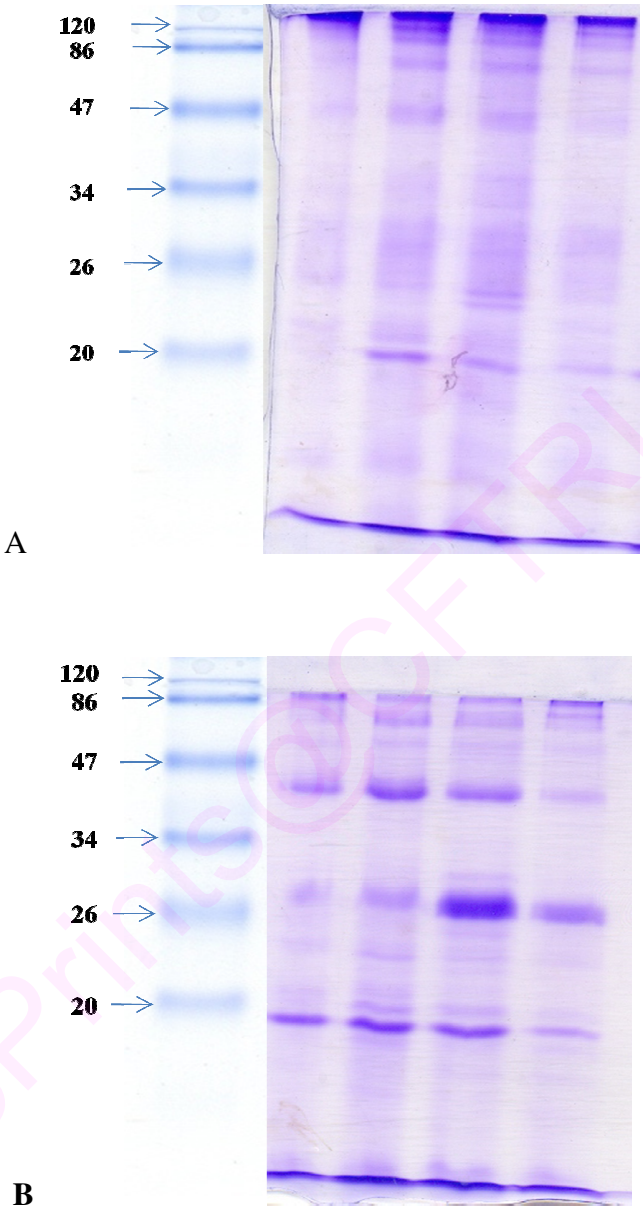


Fig.5. SDS-PAGE profile of (A) High molecular weight protein fraction of rat bile of different groups and (B) Low molecular weight protein fraction of rat bile of different groups

(Lanes from left to right): 1: Marker proteins, 2: Basal control (C), 3: C + Fenugreek. 4: HCD + Fenugreek, 5: HCD

Incorporation of fenugreek in the diet of rats had a favourable influence on the lipid profile of hepatic bile. While cholesterol: phospholipid ratio in HCD group was >1, the same was <1 in all other treatments. Dietary fenugreek was reported to increase bile acid secretion into bile (Bhat *et al.,* 1985) which is attributable to its increased production due to an increase in the activity of hepatic CYP7A1, the regulatory enzyme in bile acid synthesis (Srinivasan & Sambaiah, 1991). Several earlier studies have reported hypocholesterolemic effect of curcumin (yellow principle of turmeric), capsaicin (pungent principle of red pepper), onion and garlic which have a favourable influence on biliary cholesterol: phospholipid ratio, cholesterol: bile acid ratio and CSI, was also accompanied by antilithogenic potential (Hussain & Chandrasekhara, 1992; 1994; Vidyashankar *et al.,* 2009; 2010). *i.e*., these spices lowered the incidence of cholesterol gallstones (CGS) and also regressed preformed CGS in mice.

Cholesterol crystal nucleation in bile is reported to be influenced by biliary proteins but the crystallization sequence due to proteins is not completely clear. Pronucleating proteins such as mucin (Lee & Smith, 1989) and other glycoproteins (Lee & Smith, 1989; Burnstein *et al.,* 1983) are thought to provide a nucleating matrix for cholesterol crystal formation. IgM and IgA can serve as pronucleating agents (Teramen *et al.,* 1995). The antinucleation proteins such as apolipoproteins (Apo-AI, Apo-AII) were also found in bile. It was established that many glycoproteins play an important role in the process of cholesterol crystallization. The present study has revealed that biliary total protein and glycoprotein increased in the HCD group while dietary fenugreek countered these increases. Biliary glycoproteins were reported to affect both crystal growth as well as NT and consist of 6 sub-classes having molecular weight of 40, 50, 58, 80, 98 and 143 kDa (Teramen *et al.,* 1995). Gallstone inducing diet increased total cholesterol and mucin content, which acted as pronucleating factor, while phospholipids and total bile acid content decreased which act as anti-nucleating factors (Wu *et al.,* 2007). The size of the crystal vesicles increased with the duration of feeding.

Mucin promotes nucleation and its hypersecretion precedes the stone formation. It was reported that the concentration of biliary mucin was elevated in gallstone patients. Higher

total protein content in bile is associated with shorter NT. Mucin shares with other epithelial mucin the ability to bind lipids and bile pigments. The hydrophobic binding site in the polypeptide core of mucin may provide a congenial environment for nucleation. Apo A-I stabilizes non-micellar fraction by forming an Apo A-I and lipid complex particle, resulting in prolonged cholesterol crystal nucleation. A some glycoproteins and IgA have shown antinucleating activity (Tazuma *et al.,* 1991). Apo-AI and Apo-AII inhibit the rate of crystallization possibly in metastable bile. The second major factor which influences crystallization is the deficiency of Apo-AI and Apo-AII (Kibe *et al.,* 1984).

The difference between bile of patients with and without gallstones for their disposition to form crystals may lie in the presence of antinucleating factors in bile. Hahm *et al.,* (1992) have reported that cholesterol nucleation and crystal growth requires mucin/glycoprotein and higher content of it was found in stoned patients. Swobodnik *et al.,* (1991) reported that biliary proteins play a key role in the nucleation of crystals. Significant increase was reported in the amount of intracellular mucin as early as 18 h of feeding lithogenic diet (Premsingh *et al.,* 1987). Cholesterol crystals were detected as early as 7 days in cholesterol fed groups with increase in the content of glycoproteins before the saturation and crystallization (Zak *et al.,* 2007). Biliary proteins directly modify crystallization process by protein-lipid interaction (Secknus & Holzbach, 1997). Six proteins with molecular mass between 52 - 200 kDa were reported to be common in all the stone patients (Miquel *et al.,* 1992). It was understood that glycoproteins contribute to physicochemistry of bile by modifying the nucleation and growth of formed crystals. Fibronectin, a disulfide bonded dimer is a α_2-surface binding glycoprotein, which helps in the aggregation of phospholipid vesicles; fusion increases size of vesicles, which were vulnerable for nucleation (Chijiiwa *et al.,* 1991).

An increase in the concentration of biliary lipids and deoxycholic acid favored cholesterol in vesicular form, with increase in vesicular cholesterol: phospholipid ratio and decreased cholesterol crystal NT (Hussaini *et al.,* 1995). Supersaturation of bile with cholesterol favoured crystal formation, which tends to form unstable vesicles and onset of cholelithiasis (Zak *et al.,* 2007). Concentration of bile is a principal factor in CSI and

cholesterol NT. NT was prolonged in gallstone patients having dilute bile despite having high CSI and NT was shorter if bile is concentrated despite having low CSI (van Erpecum *et al.,* 1990).

The gel filtration chromatography of bile revealed the presence of various forms of cholesterol *viz*., vesicles, which eluted in the void volume, smaller vesicles and mixed micelles. Bile of both basal control (C) and C + fenugreek groups showed distinct portion of smaller and mixed vesicles compared to bigger vesicular peak, while the HCD group had a well-defined void volume vesicular peak, small vesicular zone and distinct micellar zone. In HCD + fenugreek group, there was a reduction in the void volume vesicular peak with concomitant increase in small vesicular and micelle zone.

Not all supersaturated bile form gallstone and additional factors must also be present, since only 10% of the population having supersaturated bile were said to form gallstones. An inhibitor type of protein may help to explain this (Holzbach, 1995). Biliary proteins act as main crystal promoters by binding non-covalently to lipid molecules and by the formation of lipid-protein complexes. Electrophoretic separation of biliary LMW proteins revealed the presence of high concentration of a protein having a molecular weight of around 28 kDa. Busch *et al.,* (1991) have reported that individual crystal binding proteins having MW of 28, 63 and 74 KDa inhibit crystallization and their effect is dose dependent. The 74 kDa protein has a heavy chain (63 kDa) and a light chain (28 kDa), which show potent inhibitory effect at less than physiological concentrations.

Cholesterol NT of the combination of biles from various diet groups revealed that mixing of the bile of HCD group with those of other groups prolonged the cholesterol NT, while bile of HCD group by itself nucleated very early (6^{th} day). This is supported by the report that bile of HCD group which nucleated in ≤ 4 days showed higher content of mucin than the bile which showed prolonged crystal observation time (> 4days). Wilhelmi *et al.,* (2004), who observed higher quantity of newly formed crystals in bile supplemented with mucin but not in controls (absence of mucin). Bile of HCD group showed higher response to the mucin (LMW protein fraction) added at 250 µg/mL of bile compared to other treatments. While feeding fenugreek along with HCD showed lesser response to the added

mucin compared to HCD group. Even a lower concentration of mucin (100 µg/mL) was reported to significantly reduce NT in supersaturated model bile (Yamasaki *et al.,* 1993).

In the present study, hepatic biliary proteins from fenugreek fed rats were examined for their influence on cholesterol crystal nucleation. The results indicated that bile from HCD group nucleated in 7 days, while the NT was prolonged in mixtures of bile of different groups with the bile of HCD group. There was a prolonged NT in the bile of HCD + fenugreek group. Biliary LMW as well as HMW protein fractions prolonged cholesterol nucleation in model bile, but the effect was more prominent in LMW protein fraction. Dietary fenugreek also countered the increase in crystal concentration in HCD group. These results suggested role of distinct antinucleating proteins which might be responsible for the prolonged NT brought about by fenugreek when included in the HCD. Eder *et al*., (1995) have reported that generation of lipid peroxidation products in model bile significantly reduced cholesterol crystal formation time indicating that lipid peroxidation may play a role in CGS pathogenesis. In vivo study also reports increased levels of MDA in the bile samples of patients with cholesterol gallstones as compared to stone free patients (Eder *et al.,* 1995). Our results are in agreement with the earlier reports, where the bile of HCD group showed higher levels of MDA. It was also observed that there was a significant decrease in the level of lipid peroxides formed in the fenugreek containing groups, leading to delayed onset of cholesterol crystallization. Thus, dietary fenugreek not only reduced cholesterol levels of bile but also increased the bile volume, bile acid content, decreased CSI and had a favorable effect with respect to glycoproteins in general, antinucleating factors in particular, and vesicular form of cholesterol in bile, all of which delayed cholesterol NT. Dietary fenugreek might have evinced both an increase in antinucleating proteins and a decrease in pronucleating factors. The increased concentration of antinucleating proteins might have probably reduced the effect of pronucleating proteins on crystallization.

In summary, feeding lithogenic HCD increased the CSI of the bile, which is the foremost factor for cholesterol crystallization, while feeding fenugreek along with HCD had a favorable effect. Fenugreek supplementation to HCD reduced the cholesterol content

of the bile, increased bile secretion, biliary phospholipids and bile acids, reduced the biliary protein content, prolonged the cholesterol NT, decreased the vesicular form of cholesterol and increased the small vesicular and micellar form of cholesterol. The present study is the first to report a novel antinucleating activity present in the hepatic bile of rats fed fenugreek seeds. This study provides evidence that the anti-lithogenicity of fenugreek seeds is not only controlled by reducing hepatic and biliary cholesterol but perhaps also by causing the secretion of antinucleating proteins in the bile and by suppressing the pronucleating activity.

Summary

Formation of cholesterol gallstones in gallbladder is controlled by procrystallizing and anticrystallizing factors present in bile. Dietary fenugreek seed has been recently observed to possess antilithogenic potential in experimental mice. An animal experiment was carried out to evaluate the effect of dietary fenugreek on the compositional changes in the bile, particularly effect on glycoproteins, low molecular weight (LMW) and high molecular weight (HMW) proteins, cholesterol NT, and cholesterol crystal growth. Groups of Wistar rats were fed for 10 weeks with diets: (1) Basal control, (2) Basal control + Fenugreek (12%), (3) High cholesterol diet (HCD), and (4) HCD + Fenugreek (12%). Feeding of HCD containing 0.5% cholesterol for 10 weeks rendered the bile lithogenic. Incorporation of fenugreek into HCD decreased the cholesterol content (70.5%), total protein (58.3%), glycoprotein (27.5%), lipid peroxides (13.6%) and cholesterol saturation index (from 1.98 to 0.75), increased the bile flow rate (19.5%), prolonged the cholesterol NT, reduced the vesicular form of cholesterol (65%) accompanied with an increase in the smaller vesicular form (94%). There was an improvement in the phospholipid content (33%) and total bile acid content (49%) in HCD + fenugreek group as compared to HCD group. Electrophoretic separation of LMW proteins showed the presence of high concentration of 28 kDa protein which might be responsible for the prolongation of cholesterol NT in the fenugreek fed groups. These findings indicate that the beneficial anti-lithogenic effect of fenugreek which is primarily by reducing the cholesterol content in the bile is also affected

through a modulation of the nucleating and anti-nucleating proteins which in turn affect the cholesterol crystallization.

oo O oo

Study on the antilithogenic influence of a combination of fenugreek seeds and onion

Introduction

Cholesterol gallstone (CGS) pathogenesis is a primary disorder arising out of altered hepatic and biliary chlolesterol homeostasis. Gallstones are formed in the gallbladder due to precipitation of cholesterol, bilirubin and calcium salts in bile. Majority of gallstones were contributed by cholesterol and very small numbers of gallstones were primarily composed of calcium salts of bilirubin and phosphate. Pathologic conditions that generally precede the occurrence of CGS were lithogenic bile, gallbladder stasis and short nucleation time. Lithogenicity of bile was determined by relative concentration of three main components viz., bile acids, phospholipids and cholesterol. Generally, lithogenic bile occurs with disruption of cholesterol homeostasis, leading to increased cholesterol secretion and subsequent supersaturation of cholesterol in bile (Apstein & Carey, 1996; Marzolo *et al.,* 1990). Gallbladder stasis increases the opportunity for concentration of supersaturated bile in the gallbladder to form gallstones. It is universally accepted that cholesterol supersaturation in bile was the pre-requisite step in the onset of CGSs. The onset of CGS was associated with various physicochemical disturbances in bile, which includes excess secretion of cholesterol and reduced secretion of bile acids by the liver into bile and increased absorption of water from the gallbladder thus creating a hydrophobic environment. These series of events lead to supersaturation of bile with cholesterol, a pre-requisite for CGS formation (Von Erpecum & Von Berge-Henegouwen, 1989).

Several animal studies have evaluated the role of dietary components in preventing CGS. It has been shown that dietary proteins, carbohydrates, fibre, and fat play a role in the induction of CGSs (Thornton *et al.,* 1983; Ebihara & Kiriyama, 1985; Mott *et al.,* 1992). A few instances of dietary factors playing a role in the regression or amelioration of already existing CGS were also reported. Spices: garlic, onion, fenugreek, red pepper and turmeric have been documented to be effective as hypocholesterolemic agents under conditions of experimentally induced hypercholesterolemia and hyperlipidemia (Srinivasan *et al.,* 2004). It has been shown that curcumin of turmeric, capsaicin of red pepper, onion,

and garlic when included in the diet reduced the induction of CGSs in mice (Hussain & Chandrasekhara, 1992; Vidyashankar *et al.,* 2009). Dietary fenugreek seeds were also found to exert excellent anti-lithogenic influence by reducing the incidence of lithogenic diet induced CGS formation (Chapter-II). Dietary spice bioactive compounds (Hussain & Chandrasekhara, 1994), *Allium* spices (Vidyashankar *et al.,* 2010) and fenugreek seeds (Chapter-IIIA), have also been evidenced to regress preformed CGSs in experimental mice when included in the diet after CGS induction.

Spices are an integral part of Indian diet and they are included in the diet to enhance the sensory quality of food. In the context of *Allium* spices (onion and garlic) and fenugreek being already reported to have an antilithogenic influence individually, and that spices are generally consumed in combination as spice mixes, it would be relevant to evaluate the combination of such spices for a health beneficial effect. The present study has hence evaluated the antilithogenic influence of two hypocholesterolemic spices, namely fenugreek seeds and onion when consumed together for a possible additive or synergistic effect. These two test spices were included in the diets both individually at the respective doses that were previously observed to produce the desired effect and in combination during the induction of CGS by HCD feeding.

Materials and methods

Chemicals

Cholesterol, DPPC, triolein, heparin, ammonium thiosulphate, ferrous chloride, bile acids, 3α-HSD**,** iso-citrate dehydrogenase, glucose-6-phosphate dehydrogenase, cholesterol oxidase, TBA, TMP, *t*-butyl hydroperoxide, acrylamide, N,N-methylene-bis-acrylamide, SDS, triethanolamine hydrochloride, tris-(hydroxymethyl)aminomethane, NAD**,** NADP, NADPH, glucose-6-phosphate, DTT, sodium arsenite, coenzyme-A, 3-HMGCoA, 7β-hydroxycholesterol, Triton X-100, CBB, hydrazine hydrate, protein standard kit for electophorosis, sodium metaperiodate, BSA, EDTA, TEMED and HEPES were purchased from Sigma-Aldrich Chemicals, (St.Louis, USA). All other chemicals and solvents used were of analytical grade and solvents were distilled before use. Edible grade casein was procured from Nimesh Corporation (Mumbai, India). AIN-76 mineral mix was from

SISCO Research Labs (Mumbai, India). All vitamins, DL-methionine and choline chloride were from Himedia (Mumbai, India). Cane sugar, fenugreek seeds, refined groundnut oil and onion were purchased from the local market. Sugar and fenugreek were pulverized. Onions were shredded and dehydrated in a freeze-dryer.

Animal diets

The animal diets were AIN-76 semi-purified diet. The basal control diet consisted of: sucrose, 65%; casein, 20%; cellulose, 5%; AIN-76 mineral mix, 3.5%; AIN-76 vitamin mix, 1%; DL-methionine, 0.3%; choline chloride, 0.2% and refined groundnut oil, 5%. Lithogenic diet was prepared by supplementing 0.5% cholesterol and 0.25% bile salts (1:1 mixture of sodium cholate and sodium deoxycholate) to the basal diet substituting same quantity of sucrose. The test diets were prepared by incorporating the fenugreek seed powder (12%) / onion powder at 2% / fenugreek powder at 12% and onion at 2% incorporated into HCD at the expense of sucrose. All these diets were prepared by mixing the ingredients in a mechanical mixer and pellets were prepared using hand-operated pelletizer. Diets were stored at 4°C in air-tight containers.

Animal treatment

Animal experiment was carried out taking appropriate measures to minimize pain or discomfort in accordance with the guidelines laid down and with due approval from the Institutional Animal Ethics Committee. Six week old male albino mice [OUTB / Swiss Albino / Ind / Cft(2c)] produced in our Experimental Animal Production Facility Unit, weighing 27±1.0 g g were grouped and housed in polypropylene cages 4-5 mice per cage with saw dust as bedding material. Animals were maintained in a room where the temperature was maintained at 22 ± 2^0 C, with relative humidity of about 60±5% and having regular 12 h cycle of day and night. The animals were given free access to food and water. Animal weights were recorded every week till the end of the experiment.

Collection of gallbladder and scoring of CGS

At the end of the feeding duration, the animals were fasted overnight and sacrificed under ether anesthesia. Blood was drawn immediately by cardiac puncture and the serum

was separated by centrifugation for further analysis. Cholecystectomy was performed and gallbladders were carefully collected and trimmed off any extraneous tissue. Liver was quickly excised, washed with ice-cold saline and blotted dry, weighed and stored at -20^{0}C till further analysis. The volume of bile was noted and the weight of gallbladder along with stones was recorded. The gallbladders placed on an illuminator were evaluated for CGS under magnifying lens for the presence of gallstones by four individuals unaware of dietary treatments. The grading of stones was done on a five point scale (Akiyoshi *et al*., 1986). The bile from the gallbladders was appropriately pooled and stored at −20^0 C till further analysis.

Lipid profile

Serum and liver lipids were extracted by the method of Folch *et al*., (1957). Biliary lipids were extracted by the method of Bligh and Dyer (1959). The upper methanolic phase was used for the estimation of total bile acids using 3α-HSD (Turley & Dietschy, 1970). The lower chloroform layer was used for the analysis of cholesterol and phospholipid. Cholesterol levels were quantitated by the method of Searcy and Bergquist (1960). The HDL-cholesterol and non-HDL (LDL + VLDL) cholesterol in serum were estimated by adapting the protocol given by Warnick and Albers (1978). The method involved the precipitation and separation of HDL from LDL+VLDL by the use of heparin and manganese chloride. HDL-cholesterol in the supernatant was estimated as described before. The HDL2 and HDL3 fractions of the HDL were estimated as described by Hirano *et al.,* (2008). Phospholipids were measured by ferrous ammonium thiocyanate method using di-palmitoyl phosphatidyl choline as reference standard as described by Stewart (1980). Triglycerides were estimated according to the method described by Fletcher (1968), using triolein as standard. Cholesterol saturation index of the bile was calculated using the values of cholesterol, phospholipids, total lipids and bile acids in bile (both relative and total lipid concentrations) as described by Carey (1978).

Reactive oxygen species (ROS), lipid peroxides and antioxidant molecules in liver

Thiobarbituric acid reactive substances in the liver homogenate were measured fluorimetrically after extracting with butanol and were compared with the standard TMP as

described by Ohkawa *et al*., (1979). ROS was estimated in the liver homogenate as described by Driver *et al.,* (2000). Total thiols in liver homogenates were measured as described by Sedlak and Lindsay (1968). Glutathione was estimated according to the protocol described by Beutler *et al*., (1963). Ascorbic acid was determined according to the method given by Omaye *et al*., (1973).

Antioxidant enzymes in serum and liver

Catalase activity was assayed by following the rate of decomposition of H_2O_2 as described by Aebi (1984). Glutathione reductase activity was assayed by measuring the oxidation of NADPH by oxidized glutathione according to Carlberg and Mannervik (1985). Glutathione-S-transferase activity was measured as described by Warholm *et al.,* (1985) using the CDNB and by measuring the CDNB-glutathione complex formed. Glutathione peroxidase activity was measured as described by Flohe and Gunzler (1984). SOD activity was measured by quantifying the inhibition of cytochrome-C reduction in xanthine-xanthine oxidase system as described by Flohe and Otting (1984).

Liver function enzymes in serum

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase were assayed using appropriate kits and were expressed as Units/L. Liver protein was analyzed according to Lowry *et al.,* using BSA as reference protein (1951).

Activities of enzymes of cholesterol metabolism

HMG-CoA reductase activity in liver was assayed by following the formation of CoA as out lined by Hulcher and Oleson (1973). Activities of CYP7A1 and CYP27A1 in liver were assayed as described by Petrack and Latario (1993) and Li *et al.,* (2006) using HPLC.

Microscopic observation of CGSs and Histopathological evaluation

Some of the randomly selected gallstones in each group were taken and observed under the scanning electron microscope to study the size and their surface crystals of the formed gallstones. Sections of fresh liver and gallbladder were taken for microscopic examination

of the histopathological changes that occur in the liver and inflammation of the gallbladder wall.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels (12%) were developed in buffer system as described by Laemmli (1970). Lyophilized biliary protein samples were resolubilized with sample buffer and applied to gels. Gels were stained with comassive brilliant blue.

Statistical analysis

Statistical analysis was carried out using GraphPad prism statistical software. Results were analyzed by one way ANOVA and the significance level was calculated using Tukey Kramer multiple comparison test and results were considered as significant at $P<0.05$.

Results

Effect of dietary fenugreek seeds, onion and their combination on HCD *induced CGS incidence*

Table-1 presents data on food intake, body weight, liver weight and gallbladder weights in various groups of experimental animals. There was no difference in body weights and food intake between the groups. There was a significant difference in the liver and gallbladder weights as a result of addition of spices individually as well as in combination. The increases in liver and gallbladder weights produced by HCD feeding were significantly countered by dietary fenugreek as well as dietary onion. This effect on liver weight was more in the case of combination of fenugreek and onion as compared to the individual spices. The reduction in liver weight in HCD+combination of fenugreek and onion was 29% compared to the individual spices: fenugreek (26%) and onion (12.5%). The reduction in gallbladder weights was 56%, 53% and 56% respectively. The incidence of HCD induced CGS in animal groups with simultaneous feeding of fenugreek, onion and their combination is given in **Fig.1**. There was a significant decrease in the incidence of CGS in all spice fed groups, the percent incidence being 25% in HCD+Fenugreek, 75% in HCD+Onion, and 23.8% in HCD+combination, as compared to 100% HCD.

Table 1. Effect of dietary fenugreek and onion combination on animal weight, diet intake, liver weight and gallbladder weight in high cholesterol diet fed mice.

Animal group	Initial animal weight (g)	Final animal weight (g)	Food intake (g/day/mice)	Gallbladder weight (mg)	Liver weight (g/per 100 g body weight)
Basal control	26.9 ± 0.06	38.6 ± 0.75	4.62 ± 0.03	17.5 ± 2.8[a]	3.58 ± 0.08[b]
HCD	26.9 ± 0.06	38.3 ± 0.78	4.49 ± 0.12	39.4 ± 0.7	6.40 ± 0.14
HCD + F	26.9 ± 0.06	36.1 ± 0.44	4.95 ± 0.12	18.4 ± 1.26[b]	4.75 ± 0.14[b]
HCD + O	27.0 ± 0.10	37.6 ± 0.74	5.06 ± 0.17[a]	29.2 ± 2.1[a]	5.60 ±0.12[b]
HCD + F + O	27.0 ± 0.15	39.0 ± 0.92	4.98 ± 0.12	17.4 ± 3.0[b]	4.54 ± 0.06[b]

Values are mean ± SEM of 18 animals in each group
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

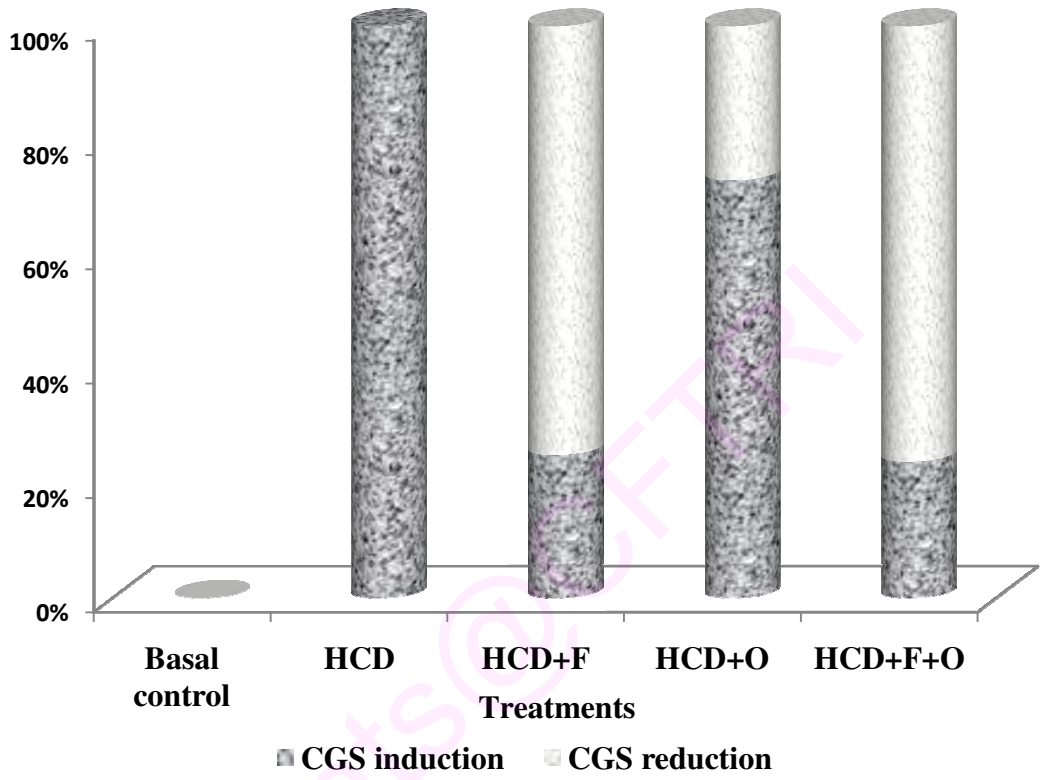


Fig.1. Effect of dietary fenugreek and onion combination on the incidence CGS (both incidence and extent of reduction) in mice maintained under lithogenic condition.

HCD had a gallstone of about 1mm in size, while others had gallstones of comparitively lesser size (**Fig.2**). The morphological observation of gallstones from different groups showed the presence of flakes with clear cut edges in HCD group, while in the spice fed groups apart from reduction in size of gallstone, there was no clear cut edges (**Fig.3**).

Effect of dietary fenugreek, onion and their combination on serum lipids during CGS induction

Serum lipid profile of animals fed spices during the induction of CGS is presented in **Table-2**. Feeding of HCD caused hypercholesterolemia where there was an increase in serum cholesterol concentration (300.5 mg/dL) which was 1.8-fold compared to basal control (168.3 mg/dL). Addition of fenugreek, onion and their combination to HCD significantly resisted the increase in serum cholesterol concentration. The decrease in serum total cholesterol brought about in HCD+combination of spices was 42.2% compared to 38.3% in HCD+Fenugreek and 39.6% in HCD+Onion. The decreases in the LDL-cholesterol concentration among the treatments were about the same, viz., 46% in HCD+Onion group, 44% in HCD+Fenugreek and 41% in HCD+combination. Fenugreek and onion addition to HCD increased the total HDL content by 10 - 15% compared to HCD group. At the same time dietary fenugreek and onion decreased the HDL_3 fraction by 40-80% with proportionate increase in the HDL_2 (15-22%) fraction. The decrease in serum phospholipids brought about by HCD feeding was significantly countered by inclusion of fenugreek, onion or their combination in the diet, the extent of reversal being highest in the case of combination spices. Serum phospholipid content was 40.6% higher in HCD+combination, while the same was 18.7% and 12.3% higher in HCD+Fenugreek group and HCD+Onion group, respectively, as compared to HCD group. Serum triglyceride content was beneficially decreased *i.e.* by 15-20% in all the three spice fed groups, as compared to HCD group.

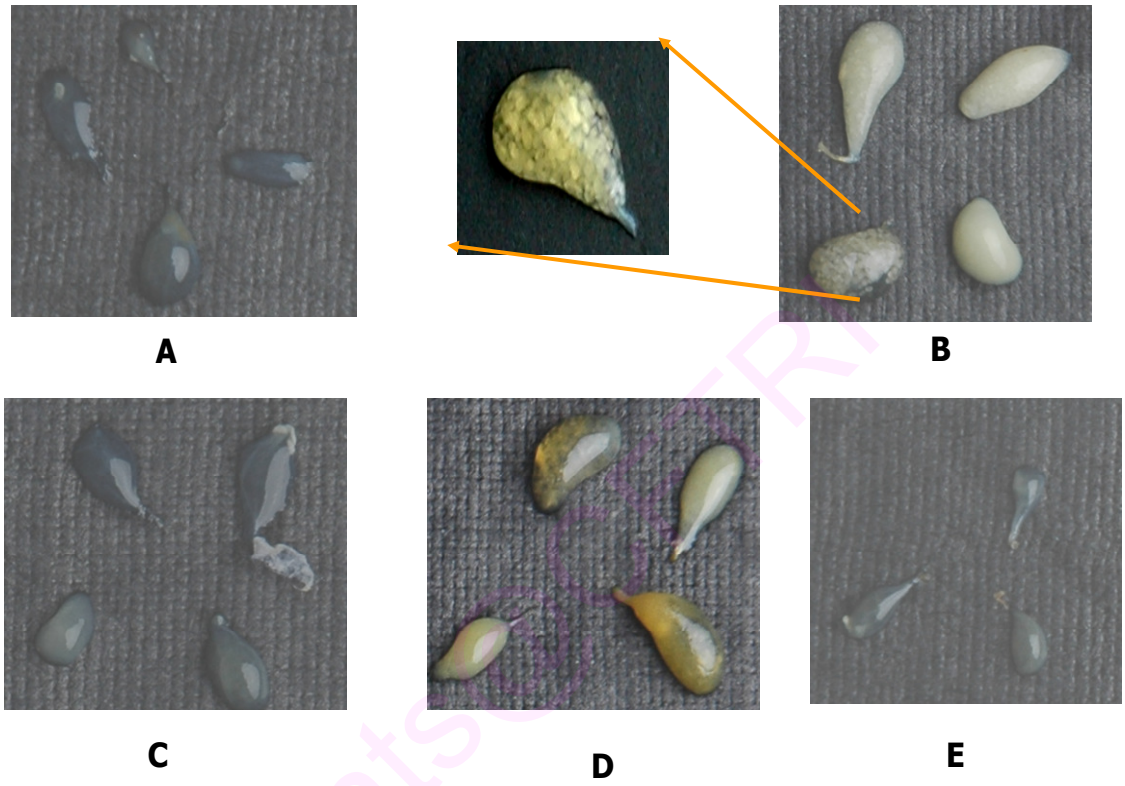


Fig.2. Incidence of CGS in different animal groups

A: Basal control, B: HCD, C: HCD+Fenugreek, D: HCD+Onion,
E: HCD+Fenugreek+Onion

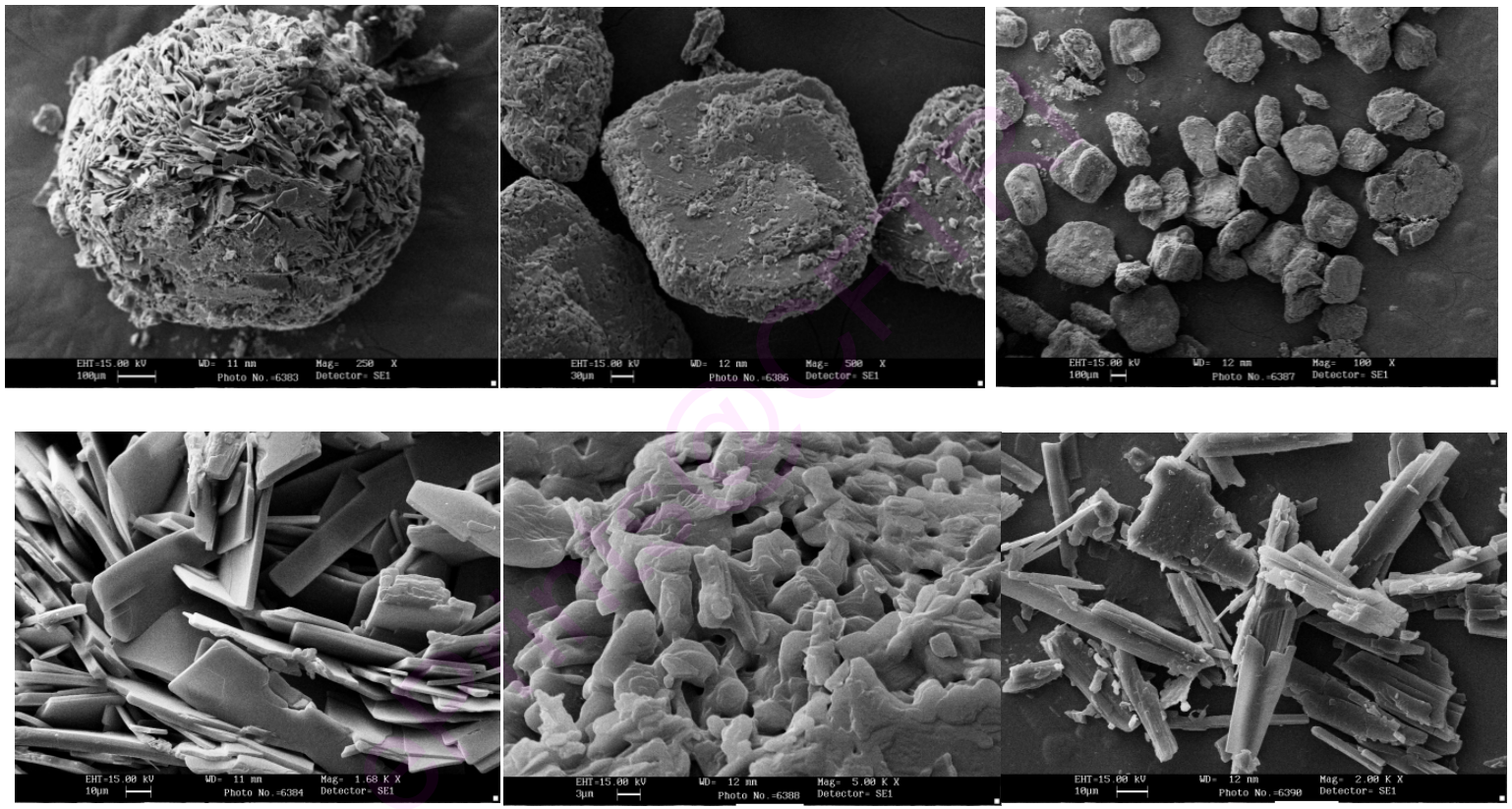


Fig.3. SEM of gallstones and gallstone surface crystals in different dietary groups

A: HCD, B: HCD+Fenugreek, C: HCD+Onion (Top row: gallstones and Bottom row: their respective surface crystals).

Table 2. Effect of dietary fenugreek and onion combination on serum lipid profile in high cholesterol diet fed mice.

Animal group	Cholesterol					Phospholipids	Triglycerides	C:P ratio
	HDL_2	HDL_3	Total HDL	LDL	Total			
Basal control	33.3 ± 3.4[b]	15.3 ± 1.7[b]	48.7 ± 4.2	122.4 ± 4.6[b]	168.3 ± 4.5[b]	343.7 ± 18.7[b]	143.3 ± 5.6[b]	0.49 ± 0.02[b]
HCD	43.6 ± 2.7	5.76 ± 0.6	52.5 ± 3.1	263.9 ± 12.8	300.5 ± 12.6	220.4 ± 10.6	197.6 ± 4.2	1.32 ± 0.08
HCD + F	36.8 ± 3.9	8.48 ± 1.6	45.3 ± 3.9	147.9 ± 11.5[b]	185.4 ± 7.3[b]	261.6 ± 11.9[b]	158.3 ± 4.6[b]	0.71 ± 0.04[b]
HCD + O	34.0 ± 3.2[a]	10.5 ± 1.1[a]	44.5 ± 3.0	140.5 ± 5.6[b]	181.5 ± 4.2[b]	247.4 ± 10.8[b]	168.9 ± 6.2[b]	0.75 ± 0.05[b]
HCD + F + O	37.7 ± 1.6	9.18 ± 0.5[b]	46.9 ± 1.9	157.3 ± 16.3[a]	173.7 ± 7.0[b]	309.8 ± 12.0[b]	161.9 ± 9.2[b]	0.62 ± 0.06[b]

Values (mg/dL) are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

Effect of dietary fenugreek, onion and their combination on hepatic lipid profile during CGS induction

Liver lipid profile of different groups of animals during CGS induction is given in **Table-3**. Hepatic cholesterol content was significantly reduced with the addition of fenugreek, onion or their combination to HCD diet. The extent of decrease in hepatic cholesterol brought about by inclusion of the combination of spices was 72%, while it was 70.5% in fenugreek fed group and 50% in onion fed group. The decrease in hepatic phospholipid content was significantly reversed by dietary fenugreek (26%) and combination of fenugreek and onion (32%). Triglyceride content of the liver was significantly reduced with the dietary incorporation of fenugreek, onion and fenugreek+onion, the reduction being 39.5%, 10% and 54.0% respectively. Thus, the combination of fenugreek and onion produced greater reduction than the individual spices. Total lipid in the liver was reduced by 56.6%, 16% and 61.3%, respectively in fenugreek, onion and fenugreek+onion treatments, respectively. C: P ratio in the liver was decreased to 0.69 (fenugreek), 1.5 (onion) and 0.64 (combination), while it was 2.81 in the case of HCD.

Effect of dietary fenugreek, onion and their combination on biliary lipid profile during CGS induction

Biliary lipid profile of various treatment groups is tabulated in the **Table-4**. Biliary Cholesterol, total lipids, C: P ratio, C: BA ratio and CSI decreased significantly with dietary incorporation of fenugreek, onion and fenugreek+onion to HCD diet, as a result of improved contents of phospholipids and total bile acids. A decrease of 78%, 61% and 81% in cholesterol content, 66.5%, 43.7% and 67% in CSI value was noticed with addition of fenugreek, onion and their combination to HCD, respectively. There was a significant favorable effect on bile acid content with the incorporation of fenugreek, onion and their combination. Bile acid levels were improved by 12%, 27%, and 12% in HCD+fenugreek, HCD+onion and HCD+spice combination, respectively, as compared to HCD group. The ratios of C: P and C: BA which were elevated by HCD, were significantly countered by the incorporation of fenugreek, onion or their combination to the HCD. The decreases in C: P ratio were 71.5%, 41.5% and 66.5%,

Table 3. Influence of fenugreek and onion combination on liver lipid profile in high cholesterol diet fed mice.

Animal group	Cholesterol	Phospholipids	Triglycerides	Total lipids	C: P ratio
Basal control	5.17 ± 0.16[b]	24.3 ± 0.68[b]	40.8 ± 3.2[b]	75.1 ± 2.50[b]	0.22 ± 0.01[b]
HCD	50.6 ± 1.60	17.0 ± 1.11	112.4 ± 2.8	243.8 ± 12.9	2.81 ± 0.24
HCD + F	14.8 ± 0.90[b]	21.4 ± 0.89[b]	68.0 ± 6.9[b]	105.7 ± 6.24[b]	0.69 ± 0.04[b]
HCD + O	25.1 ± 0.71[b]	17.4 ± 0.48	100.1 ± 3.2[a]	205.9 ± 9.16[b]	1.50 ± 0.06[b]
HCD + F + O	14.2 ± 1.23[b]	22.4 ± 0.80[b]	51.7 ± 5.0[b]	94.4 ± 6.57[b]	0.64 ± 0.06[b]

Values (mg/g) are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

Table 4. Influence of fenugreek and onion combination on biliary lipid profile in mice fed high cholesterol diet.

Animal group	Cholesterol	Phospholipids	Bile acids	Total lipids (g/dL)	CSI	C: P ratio	C : BA ratio
	mM						
Basal control	7.88 ± 0.44[b]	16.9 ± 0.47[b]	165.6 ± 1.43[b]	8.69 ± 0.45[b]	0.91 ± 0.03[b]	0.47 ± 0.03[b]	0.045 ± 0.003[b]
HCD	59.6 ± 0.62	29.8 ± 0.41	162.4 ± 2.18	11.7 ± 0.21	4.14 ± 0.02	2.00 ± 0.02	0.365 ± 0.004
HCD + F	13.2 ± 0.37[b]	23.0 ± 0.40[b]	182.3 ± 4.10[a]	10.7 ± 0.20	1.39 ± 0.37[b]	0.57 ± 0.02[b]	0.073 ± 0.003[b]
HCD + O	23.0 ± 0.48[b]	19.6 ± 0.14[b]	206.7 ± 3.50[b]	11.3 ± 0.25	2.33 ± 0.06[b]	1.17 ± 0.03[b]	0.111 ± 0.003[b]
HCD + F + O	11.5 ± 0.37[b]	17.4 ± 0.68[b]	182.8 ± 2.98[a]	10.3 ± 0.40[a]	1.37 ± 0.04[b]	0.67 ± 0.02[b]	0.063 ± 0.002[b]

Values are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion; CSI: Cholesterol saturation index
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

respectively, while the decreases in C: BA ratio were 80%, 70% and 83%, respectively in HCD+fenugreek, HCD+onion and HCD+fenugreek+onion groups as compared to HCD group.

Effect of dietary fenugreek, onion and their combination on hepatic cholesterol metabolizing enzymes during CGS induction

The activity of hepatic HMG-CoA reductase in different groups of animals is presented in **Fig.4**, while the activities of hepatic CYP7A1 and CYP27A1 are given in **Fig.5**. With addition of fenugreek, onion or their combination to HCD, there was a significant increase in the activity of all these three enzymes involved in cholesterol metabolism. The activity of HMG-CoA was three-fold higher in HCD+fenugreek group, while it was 1.2-fold higher in HCD+onion group, as compared to HCD group. The activity of hepatic CYP7A1 was improved by 17%, 12%, and 11.5% as a result of including fenugreek, onion and fenugreek+onion in HCD, respectively. The activity of hepatic and CYP27A1 was increased by 41.8% and 21.7% in fenugreek and onion treatments, respectively.

Effect of dietary fenugreek, onion and their combination on serum antioxidant enzymes during CGS induction

The activity of antioxidant enzymes in the serum of various treatment groups are given in **Table-5**. There was an improvement in the activity of glutathione-S-transferase by 24%, 24% and 19%, respectively, compared to HCD, while the activity of glutathione reductase was improved by 34% by the combination. There was also a significant reduction in lipid peroxide levels with the addition of spices to HCD, which was 23-33.7% in the spice incorporated groups.

Effect of dietary fenugreek, onion and their combination on hepatic antioxidant molecules, antioxidant enzymes and lipid perioxides during CGS induction

Antioxidant molecules, lipid peroxides, ROS in the liver of various treatment groups are given in **Table-6**. There was a significant improvement in total thiols, glutathione and ascorbic acid content which was accompanied by a significant decrease in the ROS and lipid peroxides with incorporation of fenugreek, onion and fenugreek+onion to HCD.

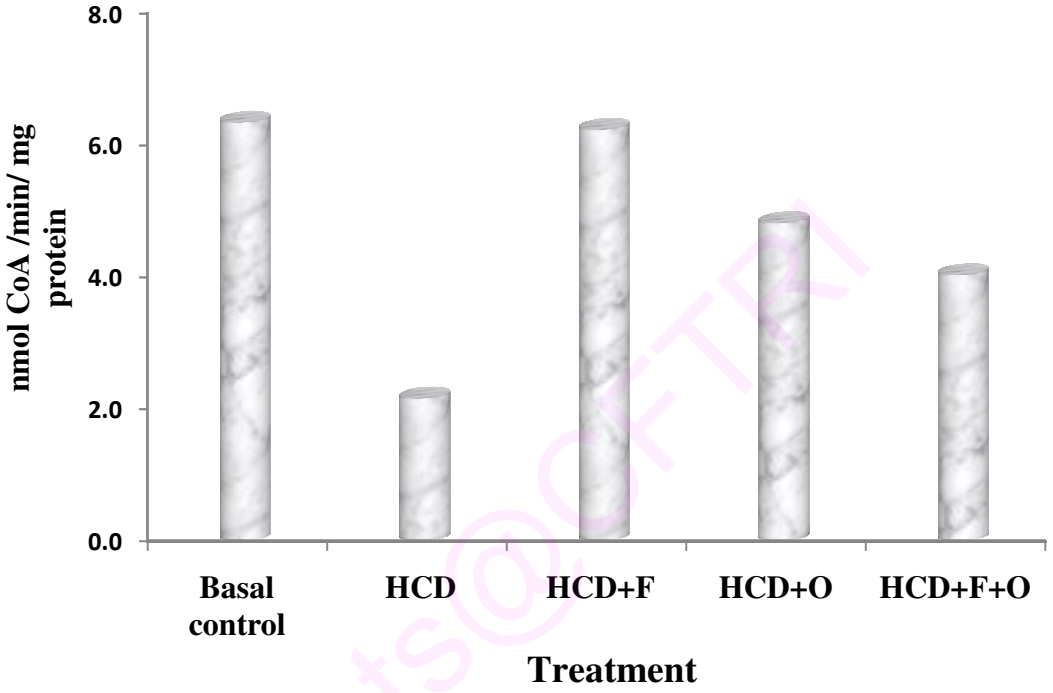


Fig.4. Effect of dietary fenugreek and onion combination on the activity of hepatic HMG-Co-A reductase in mice under lithogenic diet.

HCD: High cholesterol diet; F: Fenugreek; O: Onion

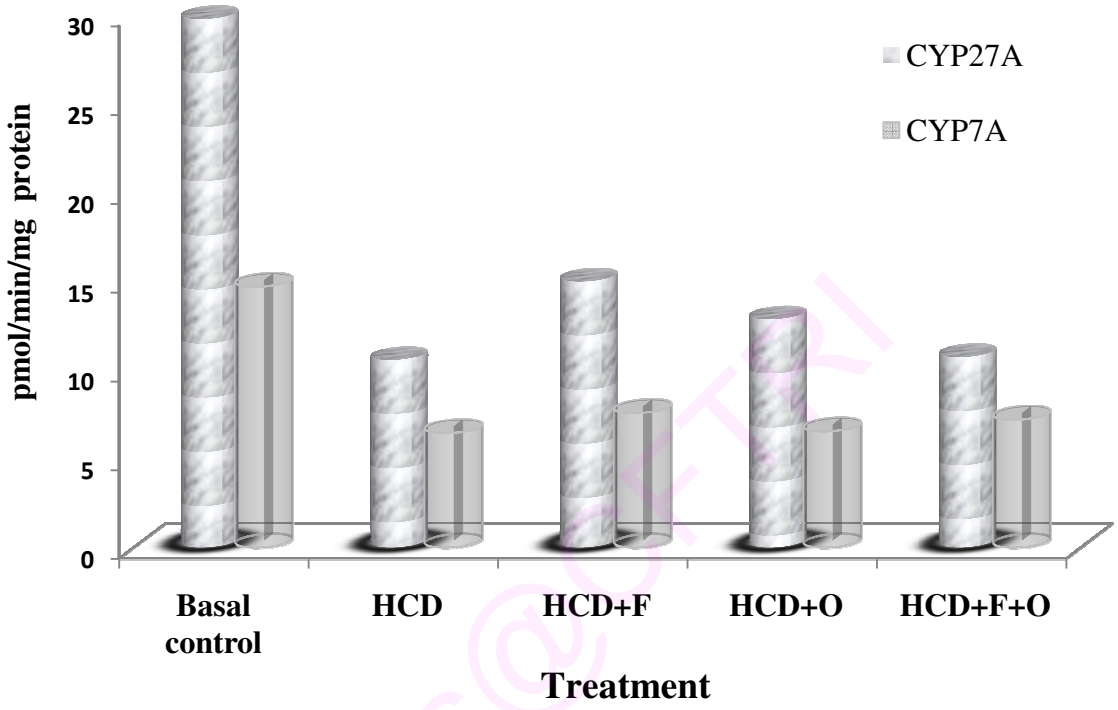


Fig.5. Effect of dietary fenugreek and onion combination on the activity of cholesterol 7α-hyderoxylase (CYP7A) and sterol 27α-hyderoxylase (CYP27A) in the liver of mice under lithogenic diet.

HCD: High cholesterol diet; F: Fenugreek; O: Onion

Table 5. Effect of dietary fenugreek and onion combination on the activity of serum antioxidant enzymes in HCD fed mice.

Animal group	Catalase	Glutathione-S-transferase	Glutathione reductase	Glutathione peroxidase	Superoxide dismutase	Lipid peroxides (mmol MDA per mg protein)
	micro mol/min/mg protein				U/min/mg protein	
Basal control	0.35 ± 0.03	4.38 ± 0.35	2.24 ± 0.22	23.2 ± 0.78	0.42 ± 0.04	171.6 ± 5.5[b]
HCD	0.48 ± 0.04	3.76 ± 0.22	1.66 ± 0.12	20.9 ± 0.31	0.45 ± 0.04	220.0 ± 8.4
HCD + F	0.38 ± 0.03	4.66 ± 0.27[a]	1.86 ± 0.15	23.8 ± 0.89	0.37 ± 0.04	169.9 ± 8.3[b]
HCD + O	0.44 ± 0.03	4.66 ± 0.21[a]	1.85 ± 0.15	22.7 ± 0.95	0.50 ± 0.05	145.9 ± 5.2[b]
HCD + F + O	0.38 ± 0.04	4.48 ± 0.30[a]	2.16 ± 0.16[a]	23.2 ± 0.92	0.46 ± 0.03	167.7 ± 5.7[b]

Values are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

Table 6. Influence of fenugreek and onion combination on antioxidant molecules in the liver of high cholesterol diet fed mice.

Animal group	ROS	LPO	Glutathione	Total thiols	Ascorbic acid
	mmol MDA/ mg protein	µmol MDA/mg protein	nmol/mg protein	nmol/mg protein	µg/mg protein
Basal control	0.55 ± 0.06[b]	166.3 ± 22.9[b]	53.4 ± 3.0[b]	0.36 ± 0.02[b]	9.26 ± 0.58[b]
HCD	1.20 ± 0.09	264.9 ± 13.1	30.2 ± 1.9	0.28 ± 0.01	7.35 ± 0.25
HCD + F	0.67 ± 0.04[b]	212.3 ± 10.5[b]	43.3 ± 3.0[a]	0.48 ± 0.01[b]	8.91 ± 0.32[b]
HCD + O	0.73 ± 0.02[b]	207.2 ± 10.9[b]	50.2 ± 3.7[b]	0.41 ± 0.01[b]	9.08 ± 0.28[b]
HCD + F + O	0.66 ± 0.08[b]	214.5 ± 10.6[b]	49.3 ± 2.2[b]	0.45 ± 0.01[b]	8.78 ± 0.21[b]

Values are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

The decrease in ROS brought about by dietary spices was 39 - 45% and the decrease in lipid peroxides was 19 - 22%. There was a favorable increase of 43 - 66% in glutathione content, 46 - 71% in total thiols and 19 - 23.5% in ascorbic acid content, but there was no additive effect when fenugreek and onion were given as combination.

Activities of hepatic antioxidant enzymes in various treatment groups are presented in **Table-7**. There was a favorable effect on the activity of these enzymes by the inclusion of fenugreek, onion or their combination into HCD. The activity of hepatic glutathione-S-transferase was increased by 64% and 45% by dietary fenugreek and combination of fenugreek and onion, respectively when compared to HCD group. The activity of hepatic glutathione reductase was increased by 39% and 32% by dietary onion and combination of fenugreek and onion, respectively. The activity of glutathione perioxidase was increased by 51% and 64% by dietary onion and combination of fenugreek and onion, respectively. Incorporation of fenugreek, onion and fenugreek+onion to HCD did not affect the activities of catalase and SOD in liver.

Effect of dietary fenugreek, onion and their combination on liver function enzymes in serum during CGS induction

There was a significant decrease in the activities of liver function enzymes in the serum of animal groups with the additions of fenugreek, onion or their combination to HCD (**Table-8**). The combination of the two spices with HCD did not have any additive effect when compared to the individual effects of fenugreek and onion alone. SGPT activity was decreased by 54.7%, 42.9%, and 60.1% in HCD+F, HCD+O, and HCD+F+O group compared to HCD group. Similarly SGOT was decreased by 42.3%, 33.4% and 44.8%, in the respective groups when compared to HCD. With the incorporation of fenugreek, onion or their combination, the activity of LDH were decreased by 34%, 30%, and 36.8%, while hat of alkaline phosphatase was decreased by 32.4%, 33.3%, and 38.7%, respectively, when compared to HCD.

Table 7. Effect of dietary fenugreek and onion combination on the activity of liver antioxidant enzymes in HCD fed mice.

Animal group	Catalase	Glutathione-S-transferase	Glutathione reductase	Glutathione peroxidase	SOD
	mmol/min/mg protein	μmol/min/mg protein			Units/min/mg protein
Basal control	121.4 ± 6.04	0.15 ± 0.02	25.9 ± 2.64	2.04 ± 0.20	11.6 ± 0.4[a]
HCD	132.9 ± 9.07	0.11 ± 0.01	18.3 ± 1.18	1.67 ± 0.12	14.1 ± 0.4
HCD + F	114.5 ± 8.31	0.18 ± 0.03[a]	22.9 ± 0.78	1.90 ± 0.21	12.8 ± 0.9
HCD + O	114.6 ± 7.46	0.13 ± 0.01	25.4 ± 1.64[b]	2.52 ± 0.17[a]	13.1 ± 0.9
HCD + F + O	116.6 ± 5.85	0.16 ± 0.01[a]	24.2 ± 1.93[b]	2.74 ± 0.16[b]	13.2 ± 0.5

Values are mean ± SEM of 6 samples, each sample being pooled from 3 animals
HCD: High cholesterol diet; F: Fenugreek; O: Onion
Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

Table 8. Effect of dietary fenugreek and onion combination on the activity liver function enzymes in high cholesterol diet fed mice.

Animal group	SGPT	SGOT	LDH	Alkaline phosphatase
Basal control	18.2 ± 0.97^{b}	53.4 ± 2.48^{b}	1333.7 ± 64.0^{b}	53.2 ± 3.9^{b}
HCD	50.2 ± 1.39	95.8 ± 4.04	2157.1 ± 51.9	122.8 ± 4.8
HCD + F	22.8 ± 1.71^{b}	55.3 ± 2.95^{b}	1424.7 ± 81.3^{b}	83.0 ± 6.4^{b}
HCD + O	28.7 ± 1.09^{b}	63.8 ± 4.12^{b}	1506.8 ± 61.8^{b}	82.0 ± 9.0^{b}
HCD + F + O	20.0 ± 1.18^{b}	52.9 ± 8.91^{b}	1362.6 ± 30.5^{b}	75.3 ± 2.5^{b}

Values (Units/L) are mean ± SEM of 6 samples, each sample being pooled from 3 animals

HCD: High cholesterol diet; F: Fenugreek; O: Onion

Statistically significant compared to HCD group; a: $p<0.05$, b: $p<0.01$

Effect of dietary fenugreek, onion and their combination on biliary proteins during CGS induction

The SDS-PAGE profile of biliary proteins as given in **Fig.6** indicated the presence of higher amounts of proteins in the HCD treatment compared to all other treatments. It was also evident that low molecular weight proteins, corresponding to 38 kDa, 24 kDa and 20 kDa were present in higher concentrations in HCD group compared to all other groups.

Effect of dietary fenugreek, onion and their combination on the histopathological changes in liver and gallbladder during induction of CGS

The morphological variations in the histology of liver and gallbladder are given in **Fig.7**. Feeding of HCD caused accumulation of fat in the liver and the same was significantly reduced with the addition of fenugreek, onion or their combination to HCD, while there was no fat cell in the basal control group. Gallbladder membrane morphological observation revealed that there is an increased inflammation in the HCD group, while incorporation of fenugreek, onion or their combination into HCD significantly suppressed the increase in inflammation of gallbladder membrane.

Discussion

In the present study, feeding of HCD for ten weeks induced CGS in mice. Inclusion of fenugreek, onion or a combination of these in HCD significantly lowered the incidence of CGS. Addition of fenugreek, onion or their combination to HCD had a significant hypocholesterolemic effect. These spices reduced concentration of serum LDL-cholesterol in particular, which is primarily responsible for the reduction in C: P ratio of serum. There was a significant decrease in liver weight with the incorporation of spices into HCD during CGS induction, which was a result of lowered lipid content in the hepatic tissue. There was also a significant reduction of hepatic cholesterol and triglyceride, and an increase in hepatic phospholipids thus resulting in beneficial lowering of C: P ratio of liver. Many studies have reported hypocholesterolemic influence of various dietary components like, fiber (Jenkins *et al.,* 1993; Anderson *et al.,* 1992), spices: red pepper, turmeric, fenugreek, onion and garlic in several animal models

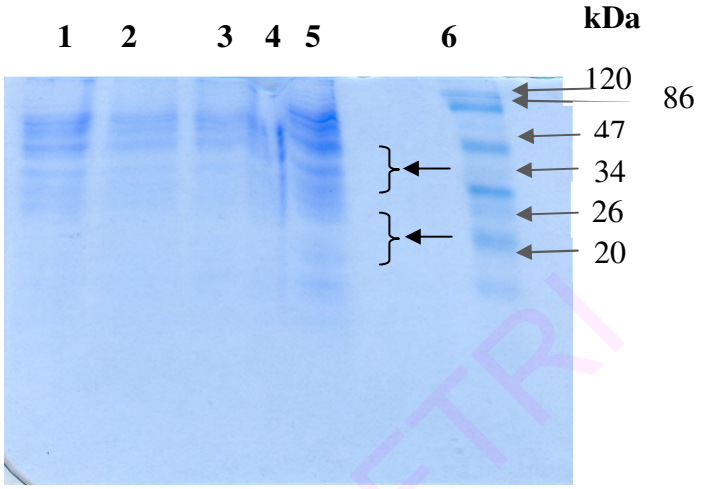


Fig.6. SDS-PAGE profile of biliary proteins of mice maintained under lithogenic diet.

Lanes: 1. Basal control, 2. HCD + Fenugreek, 3. HCD + Onion,
4. HCD + Fenugreek + Onion, 5. HCD, 6. Protein markers.

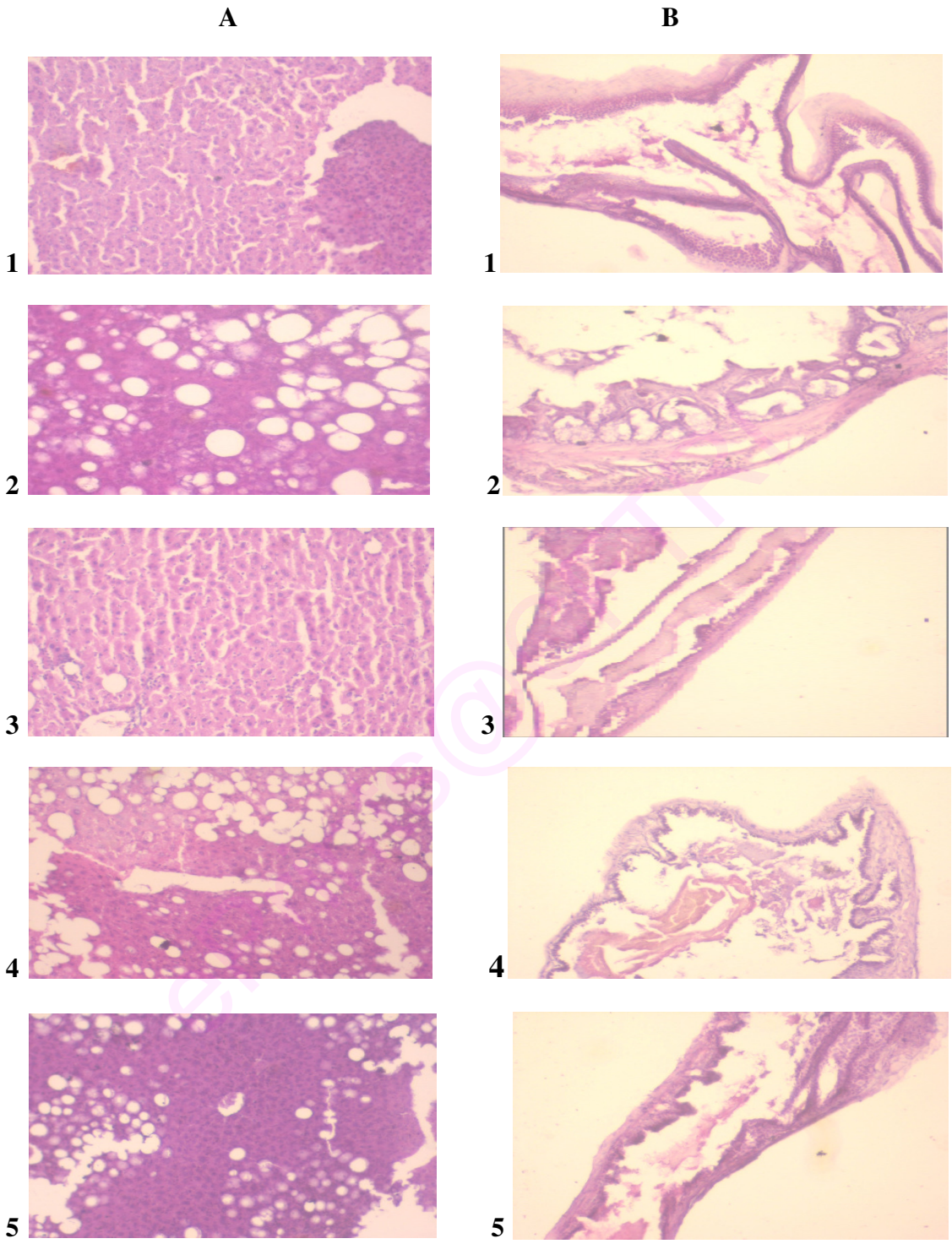


Fig.7. Effect of dietary fenugreek and onion combination on (A) liver histology and (B) gallbladder histology in mice maintained on lithogenic diet.

1. Basal control, 2. HCD, 3. HCD + Fenugreek,
4. HCD + Onion; 5. HCD + Fenugreek + Onion

(Srinivasan *et al.,* 2004), fenugreek seeds particularly in mice (Previous chapters II and III), onion in mice (Vidyashankar *et al.,* 2009).

Diets supplemented with cholesterol have shown to produce lithogenic bile and gallstones in experimental animals including prairie dogs, squirrel monkeys and hamsters (DenBesten *et al.,* 1974; Pearlman *et al.,* 1979), rats and mice (Hussain & Chanrasekhara, 1992; 1994; Vidyashankar *et al.,* 2009; 2010). Continuous feeding of HCD increased the cholesterol content, and hence elevated the ratio of C: P, the ratio of C: BA, and CSI in bile, which eventully resulted in cholesterol crystallization. Supplementation of fenugreek, onion or their combination resisted these changes thus ameliorating cholesterol crystallization in gallbladder. Feeding *Allium* spices, especially onion significantly reduced serum cholesterol, especially LDL-cholesterol. With attendent reduction in liver cholesterol, along with a significant improvement in the phospholipid content (Vidyashankar *et al.,* 2009). Our present results indicated that there was an improvement in the HDL_3 levels with the inclusion of fenugreek / onion / their combination in the diet. Halldestam *et al.,* (2009) reported that there was a positive relation between total cholesterol, LDL-cholesterol and CGS. Acalocschi (2001) has reported that plasma total HDL or HDL_3 cholesterol was inversely related associated to gallstone and suggested that HDL has a protective role against CGS.

Many authors have reported similar results; Sowmya and Rajyalakshmi (1999) have reported that feeding diet with fenugreek resulted in reduced level of total cholesterol with simultaneous reduction in LDL-cholesterol. Among various mechanisms of hypocholesterolemic effect of fenugreek, was due to the increased excretion of bile acids and neutral steroids through feces. Dietary fenugreek stimulates the conversion of cholesterol to bile acids (Bhat *et al.,* 1985), and inhibits intestinal uptake of cholesterol and bile acids (Oakenfull & Sidhu, 1990; Sauvaire *et al.,* 1991). Crude saponin fraction of fenugreek has been found to reduce serum cholesterol in rats (Sharma, 1986; Sharma, 1986a). Feeding of the diet containing fenugreek significantly decreased plasma cholesterol, without affecting triglyceride levels. It was believed that large mixed micelles were formed containing bile salts and saponins and these large molecules were less

efficiently absorbed due to the formation of the physical barrier (Sidhu & Oakenfull, 1986; Trowell, 1975).

Feeding of the diet enriched with cholesterol markedly elevated the lithogenic index of bile. Decrease in the lithogenic index with incorporation of fenugreek to HCD reflects a favorable effect; reduced precipitation of cholesterol in bile. Similar results were reported for curcumin and capsaicin (Hussain & chandrasekhara, 1992; 1994), onion and garlic (Vidyashankar *et al.,* 2009; 2010) and in fenugreek (Previous chapters-II and -III) which evidenced significant reduction in biliary cholesterol, C: P ratio and CSI of the bile and improvement in bile flow.

Administration of spice principles stimulated bile formation in liver and also increased bile acid secretion into bile. Antilithogenic effect of fiber was mainly attributed to its influence on the intestinal transit and reduction of secondary bile acids (Marcus & Heaton, 1986; 1986a; Cuevas *et al.,* 2004). Fiber also acts as physical barrier for absorption (Sidhu & Oakenfull, 1986). Supplementation of fiber with lithogenic diet inhibits cholesterol stone formation by reducing biliary cholesterol saturation (Schwesinger *et al.,* 1999).

Addition of fenugreek, onion or their combination to HCD had a significant countering effect on the liver function enzymes in serum during the induction of CGS, suggesting their hepatoprotective influence. Besides, there was also a significant antioxidant effect both in serum and liver with the addition of fenugreek, onion or their combination. These spices improved the concentration of antioxidant molecules and enhanced the activity of antioxidant enzymes. Ravikumar and Anuradha (1999) have reported that supplementation of fenugreek in diet lowers lipid peroxidation.

The activity of hepatic HMG-CoA reductase, CYP7A1 and CYP27A1 increased with the addition of fenugreek, onion or combination of both compared to HCD alone. Increased activity of cholesterol metabolizing enzymes: CYP7A1 and CYP27A1 have a role in cholesterol excretion and hence hypocholesterolemic influence. Increased activity of HMG-CoA reductase by inclusion of these specific spices in the diet was an adaptive response, wherein the suppressed enzyme activity during HCD feeding (feedback since no need for *de novo* synthesis) was countered due to accelerated excretion rate. Feeding of

HCD continuously shattered the feed back mechanism and affects the equilibrium by depositing more and more cholesterol in the solution than its carrying capacity (Carey, 1989); the turn around effect was observed with the incorporation of fenugreek, onion and combination of both to HCD. The overall impairment of bile acids absorption results in the up-regulation of CYP7A1 with a consequent rise in bile acid synthesis (Matheson & Story, 1994).

Biliary protein profile showed high amounts of proteins in the bile of HCD group, few additional proteins of low molecular weight which were significantly reduced with the incorporation of fenugreek, onion or their combination with HCD. Hahm *et al.,* (1992) has reported the presence of more amounts of proteins in the bile of gallstone patients and also the presence of 14.2 kDa glycoprotein in the gallstone subjects.

Histopathological observation of liver and gallbladder showed that feeding of HCD increased the fat accumulation in liver and inflammation of the gallbladder membrane, these effects of fat accumulation in liver and inflammation were reduced with the addition of fenugreek, onion and combination of both to HCD. Similar observations have been made by Rege and Prystowsky (1998), who have reported that feeding lithogenic diet developed cholesterol crystals and gallstones at 2 and 6 weeks. Mucus layer was progressively increased during this period and the inflammation was an early and necessary event in the formation of gallstones in bile. Histopathological observation revealed that feeding of lithogenic diet to gallstone susceptible mice (C57L) progressively increased the gallbladder wall thickness. In contrast AKR mice which were resistant to gallstones displayed no or mild increase in gallbladder thickness. Both strains accumulated sub-epithelial inflammatory cells and edema, which was highly significant in C57L mice. These findings suggest that during formation of CGS, gallbladder undergoes progressive changes that ultimately result in increased edema and decreased motility. He also reported similar results in prairie dogs, where the inflammation was an essential event of CGS cholelithogenesis. Thus in our study, fenugreek, onion and their combination showed a beneficial effect by reducing the accumulation of fat in the liver and reducing the inflammation of gallbladder membrane.

In conclusion feeding of HCD containing 0.5% cholesterol and 0.125% bile salts for 10 weeks induced CGS in mice. Incorporation of fenugreek (12%), onion (2%) and their combination to HCD significantly reduced the incidence of CGS. The antilithogenic influence was associated with a significant reduction in serum LDL cholesterol, C: P ratio. Incorporation of spices either singly or in combination beneficially moderated the CSI in bile which was a result of reduction in cholesterol and also an increase in bile acids concentration in bile. These spices individually or in combination effectively reduced the accumulation of fat in the liver and also reduced the inflammation caused by feeding of HCD. Apart from this antilithogenic influence, addition of fenugreek, onion or their combination had a beneficial antioxidant and hepatoprotective effect under lithogenic condition. The results of this investigation suggested the antilithogenic influence was maximum with fenugreek alone; and the presence of onion along with it did not bring about any further increase in this health effect. Generally, there was no additive effect either with respect to hepatoprotective influence or the antioxidant molecules and the activity of antioxidant enzymes.

Summary

An animal study was carried out to evaluate the antilithogenic effect of a combination of dietary of fenugreek seeds and onion under lithogenic diet fed conditions in mice. Lithogenic condition was induced by feeding a HCD containing 0.5% of cholesterol and 0.125% bile salts. Addition of fenugreek (12%), onion (2%) and their combination to HCD showed significant reduction in the incidence of CGS, which was attendant with a reduction in the cholesterol content by 38-42%, 50-72% and 61.5-80.5% in serum, liver and bile, respectively. The triglyceride content of serum and liver was reduced by 15-20% and 10-54% respectively. C: P ratio was reduced to 0.62-0.75, 0.65-1.5 and 0.6-1.2 from 1.32, 2.81 and 2.00 in serum, liver and bile, respectively. There was a significant reduction in the CSI of bile from 4.14 to 1.38 in the case of spice combination, while it was 2.33 in case of onion alone. There was also an improvement in the phospholipid content bile acid output of bile. The activity of hepatic HMG-CoA reductase was increased by 2-3 times, CYP7A1 activity was increased by 2-17%, and while CYP27A1 activity was increased by 2-40% with the incorporation of fenugreek, onion and their combination to HCD. Hepatic

lipid peroxides and ROS were reduced by 19-22% and 39-45% with the addition of fenugreek, onion and their combination to HCD. Microscopic examinations of liver and gallbladder sections showed that HCD feeding increased accumulation of fat in liver and inflammation of gallbladder membrane, while these effects were reduced by the feeding of fenugreek, onion and their combination along with HCD. The results of this investigation suggested the antilithogenic influence was maximum with fenugreek alone; and the presence of onion along with it did not bring about any further increase in this health effect. Generally, there was no additive effect either with respect to hepatoprotective influence or the antioxidant molecules and the activity of antioxidant enzymes.

oo O oo

GENERAL SUMMARY

- Fenugreek (*Trigonella foenum-graecum*) seed is one among the common spices which are food adjuncts being used to enhance the organoleptic properties of food. In recent decades, several health beneficial physiological attributes of fenugreek seeds have been experimentally evidenced in animal studies as well as in human trials. These include antidiabetic effect, hypocholesterolemic influence, antioxidant potency, digestive stimulant action, *etc.*. Among these beneficial physiological effects, the antidiabetic and hypocholesterolemic property of fenugreek, both of which were mainly attributable to the intrinsic dietary fibre constituent, have promising nutraceutical value.

- Fenugreek seeds were documented to have excellent cholesterol lowering property. In this investigation, the possible antilithogenic property of dietary fenugreek seeds in experimental animal model both with respect to formation of cholesterol gallstones and regression of cholesterol gallstones was studied.

- Dietary hypocholesterolemic adjunts may have beneficial role in the prevention and treatment of cholesterol gallstones (CGS). In this investigation, fenugreek seed was evaluated for this potential on the experimental induction of CGS in laboratory mice.

- CGS was induced in groups of mice by maintaining on a lithogenic diet (0.5% cholesterol) for 10 weeks. Fenugreek seed powder was included at 5, 10 and 15% of this lithogenic diet.

- Dietary fenugreek significantly lowered the incidence of CGS in these mice; the incidence was 63, 40 and 10% in 5, 10 and 15% fenugreek group respectively, as compared to 100% in lithogenic control.

- The antilithogenic influence of fenugreek was attributable to its hypocholesterolemic effect. Serum cholesterol level was decreased by 26–31% by dietary fenugreek, while hepatic cholesterol was lowered by 47–64% in these HCD fed animals.

- Biliary cholesterol was 8.73–11.2 mM as a result of dietary fenugreek, as compared to 33.6 mM in HCD feeding without fenugreek. CSI in bile was reduced to 0.77–0.99 in fenugreek treatments as compared to 2.57 in high-cholesterol group. Thus, fenugreek seeds offer the health beneficial antilithogenic potential by virtue of its beneficial influences on cholesterol metabolism.

- An animal study was carried out to evaluate the beneficial influence of dietary fenugreek seeds in terms of regression of pre-established CGS. CGS was induced by feeding a HCD for a period of 10 weeks. After the CGS induction, groups of these animals were maintained for further 10 weeks on HCD/ basal control diet/ 6% fenugreek powder / 12% fenugreek powder diets. Incidence of CGS and its severity were evaluated at the end of this feeding regimen.

- The incidence of CGS was significantly lowered as a result of dietary fenugreek seeds, the extent of regression being 61 and 64% in the lower and higher dose groups when compared to 10% regression in basal control group.

- The antilithogenic influence of dietary fenugreek was accompanied by significant reductions in serum cholesterol concentration which was more than 35%. Hepatic cholesterol concentration was also profoundly lowered by dietary fenugreek, the decrease being 53-63% compared to basal control diet.

- Biliary cholesterol concentration was significantly lower as a result of dietary fenugreek during post-CGS induction period resulting in decreased C: P ratio (0.44 and 0.40 as compared to 0.79 in the basal control group). Biliary C: BA ratio was lowered upon feeding fenugreek (by 67 and 73%) much more than in the basal control group. The CSI in the bile was also beneficially lowered by fenugreek treatment during post-CGS induction period (which was 0.90 and 0.42 as compared to 1.86 in the basal control diet group).

- The present study has evidenced the potency of hypolipidemic fenugreek seeds in regressing the pre-established CGS and this beneficial antilithogenic influence was attributable to its primary influence on cholesterol levels.

- This finding was significant in the context of evolving a dietary strategy to address CGS, which could help in the prevention of incidence, regression of existing CGS and preventing possible recurrence.

- Dietary fenugreek seed was also evaluated for hepatoprotective and antioxidant influence in mice fed HCD to result in lithogenic condition. After feeding HCD for 10 weeks, groups of animals were maintained for further 10 weeks on HCD / basal diet / basal diet containing 6 or 12% fenugreek.

- Activities of serum aspartate aminotrasferase, alanine aminotransferase, lactate dehydrogenase and alkaline phosphatase were increased with prolonged feeding of HCD. Activities of these enzymes were lower in animals fed basal control/ fenugreek containing diets after initial exposure to HCD, and were prominent in fenugreek groups.

- Hepatic lipid peroxides were decreased and antioxidant molecules increased in fenugreek fed groups. Activities of hepatic antioxidant enzymes – glutathione reductase, glutathione-S-transferase and glutathione peroxidase were higher in fenugreek treatment.

- These results suggested hepatoprotective and antioxidant potential of fenugreek seeds under conditions of lithogenicity.

- Formation of CGS in gallbladder was controlled by pro-crystallizing and anti-crystallizing factors present in bile. In the context of dietary fenugreek seed being observed to possess antilithogenic potential in experimental mice, the effect of dietary fenugreek on compositional changes in the bile, particularly effect on glycoproteins,

low molecular weight (LMW) and high molecular weight (HMW) proteins, cholesterol nucleation time, and cholesterol crystal growth was evaluated.

- Incorporation of fenugreek into HCD decreased the cholesterol content (70.5%), total protein (58.3%), glycoprotein (27.5%), lipid peroxides (13.6%) and CSI (from 1.98 to 0.75), and increased the bile flow rate (19.5%), There was an improvement in the phospholipid content (33%) and total bile acid content (49%) in HCD + fenugreek group as compared to HCD.

- Dietary fenugreek prolonged the cholesterol nucleation time and reduced the vesicular form of cholesterol (65%) accompanied with an increase in the smaller vesicular form (94%).

- Electrophoretic separation of LMW proteins showed the presence of high concentration of 28 kDa protein which might be responsible for the prolongation of cholesterol nucleation time in the fenugreek fed groups.

- These findings indicate that the beneficial anti-lithogenic effect of fenugreek which was primarily by reducing the cholesterol content in the bile, but it also affected through a modulation of the nucleating and anti-nucleating proteins which in turn affect cholesterol crystallization.

- An animal study was carried out to evaluate the antilithogenic effect of a combination of dietary of fenugreek seeds and onion under lithogenic diet fed conditions in mice.

- Addition of fenugreek (12%), onion (2%) and their combination to HCD showed significant reduction in the incidence of CGS, which was attendant with a reduction in the cholesterol content by 38-42%, 50-72% and 61.5-80.5% in serum, liver and bile, respectively. The triglyceride content of serum and liver was reduced by 15-20% and 10-54%.

- Cholesterol: phospholipid ratio was reduced to 0.62-0.75, 0.65-1.5 and 0.6-1.2 from 1.32, 2.81 and 2.00 in serum, liver and bile, respectively. There was a significant

reduction in the CSI of bile from 4.14 to 1.38 in the case of spice combination, while it was 2.33 in case of onion alone. There was also an improvement in the phospholipid and bile acid output of bile.

- The activity of hepatic HMG-CoA reductase was increased by 2-3 times, while CYP7A1 activity was increased by 2-17%, while CYP27A1 activity was increased by 2-40% with the incorporation of fenugreek, onion and their combination to HCD.

- Hepatic lipid peroxides and reactive oxygen species were reduced by 19-22% and 39-45% with the addition of fenugreek, onion and their combination to HCD.

- Microscopic examinations of liver and gallbladder sections showed that HCD feeding increased accumulation of fat in liver and inflammation of gallbladder membrane, while these effects were reduced by the feeding of fenugreek, onion and their combination along with HCD.

- There was a significant decrease in the activities of liver function enzymes in the serum of animal groups with the additions of fenugreek, onion or their combination to HCD

- The results of this investigation suggested the antilithogenic influence was maximum with fenugreek alone; and the presence of onion along with it did not bring about any further increase in this health effect. Generally, there was no additive effect either with respect to hepatoprotective influence or the antioxidant molecules and the activity of antioxidant enzymes.

oo O oo

Bibliography

Acalovschi M. (2001) Cholesterol gallstones: from epidemiology to prevention. P*ostgrad Med. J.* **77**: 221-229

Aebi H. (1984) Catalase *in vitro. Methods Enzymol*. **105**: 121-126.

Ahmadiani A, Javan M, Semnanian S, Barat E & Kamalinejad M. (2001) Anti-inflammatory and antipyretic effects of *Trigonella foenum-graecum* leaves extract in the rat. *J. Ethnopharmacol.* **75**: 283-286

Akiyoshi T, Uchida K, Takase H, Nomura Y & Takeuchi N. (1986) Cholesterol gallstones in alloxan-diabetic mice. *J. Lipid Res*. **27**: 915-924.

Ambasta SP. (2000) *Useful Plants of India*. National Institute of Science Communication, New Delhi.

Anderson JW, & Chen WL. (1979) Plant fibre: Carbohydrate and lipid metabolism. *Am. J. Clin. Nutr.* **32**: 346–363.

Anderson JW, Riddell-Mason S, Gustafson NJ, Smith SF & Mackey M. (1992) Cholesterol-lowering effects of psyllium-enriched cereal as an adjunct to prudent diet in the treatment of mild to moderate hypercholesterolemia. *Am. J. Clin. Nutr*. **56**: 93–98.

Anuradha CV & Ravikumar P. (2001) Restoration of tissue antioxidants by fenugreek seeds (*Trigonella Foenum-graecum*) in alloxan-diabetic rats. *Indian J. Physiol. Pharmacol*. **45**: 408-420.

Apstein MD & Carey MC. (1996) Pathogenesis of cholesterol gallstones a parsimonious hypothesis. *Eur. J. Clin. Invest*. **26**: 343-352.

Babu PS & Srinivasan K. (1998) Amelioration of renal lesions associated with diabetes by dietary curcumin in experimental rats. *Mol. Cell. Biochem.* **181**: 87–96

Babu PS & Srinivasan K. (1999) Renal lesions in streptozotocin induced diabetic rats maintained on onion or capsaicin diet. *J. Nutr. Biochem.* **10**: 477–483

Banerjee I, Saha S, Dutta J. (1992) Comparison of the effects of dietary fish oils with different n-3 polyunsaturated fatty acid compositions on plasma and liver lipids in rats. Lipids. **27**: 425-8

Basch E, Ulbricht C, Kuo G, Szapary P & Smith M. (2003) Therapeutic application of fenugreek. *Altern. Med. Rev*. **8**: 20-27.

Belguith-Hadriche O, Bouaziz M, Jamoussi K, El-Feki A, Sayadi S & Makni-Ayedi F. (2010) Lipid-lowering and antioxidant effects of an ethyl acetate extract of fenugreek seeds in high-cholesterol-fed rats. *J. Agric. Food Chem*. **58**: 2116–2122.

Bellows CF, Berger DH & Crass RA. (2005) Management of gallstones. *Am. Fam. Physician.* **72**: 637-642

Bergman F & van der Linden W. (1971) Reaction of the Mongolian gerbil to a cholesterol-cholic acid-containing gallstone inducing diet. *Acta Path. Microbiol. Scand*. **79**: 476–486.

Beutler E, Duron O & Kelly B.M. (1963) Improved method for the determination of blood glutathione, *J. Lab. Clin. Med*. **61**: 882-888.

Bhat BG, Sambaiah K & Chandrasekhara N. (1985) The effect of feeding fenugreek and ginger on bile composition in the albino rats. *Nutr. Rep. Int*. **32**: 1145-1151.

Biddinger SB, Haas JT, Yu BB, Bezy O, Jing E, Zhang W, Unterman TG, Carey MC & Kahn CR. (2008) Hepatic insulin resistance directly promotes formation of cholesterol gallstones. *Nat. Med.* **14**: 778-782

Bligh EG & Dyer WJ. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. **37**: 911-917.

Bouchier AD. (1990) New Drugs for Gallstones. *Br. Med. J.* **300**: 5927-5931.

Burkitt DP & Trowell HC. (1975) Refined carbohydrate foods and disease. London: Academic press.

Burnstein MJ, Ilson RG, Petrunka CN, Taylor RD & Strasberg SM. (1983) Evidence for a potent nucleating factor in the gallbladder bile of patients with cholesterol gallstones. *Gastroenterology* **85**: 801–807.

Busch N & Matern S. (1991) Current concepts in cholesterol gallstone pathogenesis. *Eur. J. Clin. Invest*. **21**: 453-460.

Busch N, Matiuck N, Sahlin S, Pillay SP & Holzbach RT. (1991) Inhibition and promotion of cholesterol crystallization by protein factors from normal human gallbladder bile. *J. Lipid Res*. **32**: 695-702.

Carey MC. (1978) Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res*. **9**: 945-955.

Carey MC. (1989) Formation of cholesterol gallstones: the new paradigms. In: Paumgartner G, Stiehl A, Gerok W, Eds. *Trends in Bile Acid Research.* Lancaster: Kluwer Academic Publishers, pp.259–281.

Carlberg I & Mannervik B. (1985) Glutathione reductase, *Methods Enzymol*. **113**: 484-490.

Cayen MN & Dvornik D. (1979) Effect of diosgenin on lipid metabolism in rats. *J. lipid Res.* **20**: 162-174.

Chadha YR. (1985) *The Wealth of India*. Council of Scientific and Industrial Research, New Delhi.

Chatterjee A & Prakashi SC. (1995) *Treatise on Indian Medicinal Plants* - Vol.2, Council of Scientific and Industrial Research, New Delhi.

Chaturvedi V & Pant MC. (1988) Effect of feeding fenugreek leaves on excretion of total lipids and steroids in rabbits. *Curr. Sci.* **57**: 81-82.

Chen HC. (2001) Molecular mechanisms of sterol absorption, *J. Nutr.* **131**: 2603–2605.

Chijiiwa K, Koga A, Yamasaki T, Shimada K, Noshiro H & Nakayama F. (1991) Fibronectin: a possible factor promoting cholesterol monohydrate crystallization in bile. *Biochim. Biophys. Acta.* **1086**: 44-48.

Chopra RN, Nayar SL & Chopra IC. (1986) *Glossary of Indian Medicinal Plants.* Council of Scientific and Industrial Research, New Delhi.

Choudhary D, Chandra D, Choudhary S & Kale RK. (2001) Modulation of glyoxalase, glutathione-S-transferase and antioxidant enzymes in the liver, spleen & erythrocytes of mice by dietary administration of fenugreek seeds. *Food Chem. Toxicol*. **39**: 989-997.

Cuevas A, Miquel JF, Reyes MS, Zanlungo S & Nervi F. (2004) Diet as a risk factor for cholesterol gallstone disease. *J. Am. Coll. Nutr.* **23**: 187-196.

DenBesten L, Safaie-Shirazi S, Connor WE & Bell S. (1974) Early changes in bile composition and gallstone formation induced by a high cholesterol diet in Praire dogs. *Gastroenterology* **66**: 1036–1045.

Dixit P, Ghaskadbi S, Mohan H & Devasagayam TP. (2005) Antioxidant properties of germinated fenugreek seeds. *Phytother. Res.* **19**: 977-983.

Dowling RH. (2000) Review: pathogenesis of gallstones. *Alim. Pharmacol. Ther.* **14**: 39-47.

Dreher ML. (1987) *Handbook of Dietary Fibre. An Applied Approach*. New York, Dekker, p.199.

Driver.AS, Kodavanti.PR & Mundy.WR. (2000) Age-related changes in reactive oxygen species production in rat brain homogenates. *Neurotoxicol Teratol*. **22**: 175-181.

Dubrac S, Parquet M, Blouquit Y, Gripois D, Blouquit MF, Souidi M & Lutton C. (2001) Insulin injections enhance cholesterol gallstone incidence by changing the biliary cholesterol saturation index and Apo A-I concentration in hamsters fed a lithogenic diet. *J. Hepatol.* **35**: 550-557

Ebihara K & Kiriyama S. (1985) Prevention of CGS by a water soluble fibre - Konjac Mannan in mice. *Nutr. Rep. Int*. **32**: 223-229.

Eder MI, Jungst D, Meyer G, Paumgartner C & Von Ritter C. (1995) Lipid peroxidation products are increased in lithogenic human gallbladder bile. *Gastroenterology* **114**: 548–552.

Fan Y, Wu SD & Fu BB. (2007) Effect of intestinal transit on the formation of cholesterol gallstones in hamsters Hepatobiliary *Pancreat. Dis. Int.* **6**: 513-515

Fillips R & Foy N. (1990) *Herbs.* Pan Books Ltd., London.

Finzi L, Barbu V, Burgel PR, Mergey M, Kirkwood KS, Wick EC, Scoazec JY, Peschaud F, Paye F, Nadel JA & Housset C. (2006) MUC5AC a gel-forming mucin accumulating in gallstone disease is overproduced via an epidermal growth factor receptor pathway in the human gallbladder. *Am. J. Pathol.* **169**: 2031-2041.

Fletcher MJ. (1968) A colorimetric method for estimating serum triglycerides. *Clin. Chim. Acta.* **22**: 303-307.

Flohe L & Gunzler WA. (1984) Assays of glutathione peroxidase, *Methods Enzymol*. **105**: 114-121.

Flohe L & Otting F. (1984) Superoxide dismutase assays, *Methods Enzymol*. **105**: 93-104.

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

Forman DT, Garvin JE, Forestner JE & Taylor CB. (1968) Increased excretion of fecal bile acids by an oral hydrophilic colloid. *Proc. Soc. Exp. Biol. Med*. **127**: 1060–1063.

Gaby AR. (2009) Nutritional Approaches to prevention and treatment of gallstones. *Altern. Med. Rev.* **14**: 258-267

Genet S, Kale RK & Baquer NZ. (2002) Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (*Trigonella foenum-graecum*). *Mol. Cell. Biochem*. **236**: 7-12.

Grunhage F & Lammert F. (2006) Pathogenesis of gallstones: a genetic perspective. *Best Pract. Res. Clin. Gastroenterol.* **20**: 997-1015

Gurr MI. (1983) The role of lipids in the regulation of the immune system. *Prog. Lipid Res.* **22**: 257-287

Hahm JS, Sung IK, Yang SC, Rhee JC, Lee MH, Kee CS & Park KN. (1992) Biliary proteins in patients with and without gallstones. *Kor. J. Intern. Med*. **7**: 18-24.

Halldestam I, Kullman E & Borch K. (2009) Incidence of and potential risk factors for gallstone disease in a general population sample. *Br. J. Sur.* **96**: 1315-1322

Halliwell B & Gutteridge JMC. (2006) Free Radicals in Biology and Medicine, Ed 4. Clarendon Press, Oxford

Halliwell B. (2001) Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging* **18**: 685–716.

Halpern Z, Dudley MA, Lynn MP, Nader JM, Breuer AC & Holzbach RT. (1986) Vesicle aggregation in model systems of supersaturated bile: relation to crystal nucleation and lipid composition of the vesicular phase. *J. Lipid Res.* **27**: 295-306.

Hirano T, Nohtomi K, Koba S, Muroi A & Ito Y. (2008) A simple and precise method for measuring HDL-cholesterol subfractions by a single precipitation followed by homogenous HDL-cholesterol assay. *J. Lipid Res*. **49**: 1130-1136.

Holan KR, Holzbach RT, Hermann RE, Cooperman AM & Claffey WJ. (1979) Nucleation time; a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* **77**: 611-617.

Holzbach RT. (1995) Cholesterol nucleation in bile. *Ital. J. Gastroenterol.* **27**: 101-105

Hopkins PN & Williams RR. (1981) A survey of 246 suggested coronary risk factors. *Atherosclerosis* **40**: 1-52.

Hulcher FH & Oleson WH. (1973) Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl coenzyme-A reductase by measurement of coenzyme A. *J. Lipid Res*. **14**: 625-631.

Hussain MS & Chandrasekhara N. (1992) Effect of curcumin on cholesterol gallstone induction in mice. *Indian J. Med. Res.* **96**: 288–291.

Hussain MS & Chandrasekhara N. (1993) Influence of curcumin and capsaicin on cholesterol gallstone induction in hamsters and mice. *Nutr. Res.* **13**: 349–357.

Hussain MS & Chandrasekhara N. (1994) Effect of curcumin and capsaicin on the regression of pre-established CGS in mice. *Nutr. Res.* **14**: 1561–1574

Hussaini SH, Pereira SP, Murphy GM & Dowling RH. (1995) Deoxycholic acid influences cholesterol solubilization and microcrystal nucleation time in gallbladder bile. *Hepatology* **22**: 1735-1744.

Igimi H & Carey MC. (1981) CGS dissolution in bile: dissolution kinetics of crystalline (anhydrate and monohydrate) cholesterol with chenodeoxy cholate, ursodeoxy cholate, and their glycine and taurine conjugates. *J. Lipid Res*. **22**: 254-270.

Ikeda I, Tanaka K, Sugano M, Vahouny VK & Gallo LL. (1988) Inhibition of cholesterol absorption in rats by plant sterols. *J. Lipid Res.* **29**: 1573-1582

Janssen YM, van Houten B, Borm PJ & Mossman BT. (1993) Cell and tissue responses to oxidative damage. *Lab. Invest*. **69**: 261-274.

Jenkins DJ, Wolever TM, Rao AV, Hegele RA, Mitchell SJ, Ransom TP, Boctor DL, Spadafora PJ, Jenkins AL & Mehling C. (1993) Effect on blood lipids of very high intakes of fiber in diets low in saturated fat & cholesterol. *N. Engl. J. Med*. **329**: 21–26.

Johnston DE & Kaplan MM. (1993) Pathogenesis and treatment of gallstones. *New Engl. J. Med.* **328**: 412-421.

Joshi R & Rajani M. (2007) Lipid profile of rats fed on domestically processed fenugreek. *J. Food Sci. Technol*. **44**: 542-544.

Judd PA. (1985) Dietary fibre and gallstones. In: Leeds, A.R. (Ed.), Dietary fibre perspective: Reviews and bibliography. London, John Libbey, pp.40-46.

Juvonen T. (1994) Pathogenesis of gallstones. Scand. *J. Gastroenterol*. **29**: 577-582.

Kansal VK. (1995) Advances in milk fat and its role in human health. *Indian. Dairyman* **47**: 20-29

Kibe A, Dudley MA, Halpern Z, Lynn MP, Breuer AC & Holzbach RT. (1985) Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J. Lipid Res*. **26**: 1102-1111.

Kibe A, Holzbach RT, LaRusso NF & Mao SJ. (1984) Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science* **225**: 514-516.

Kritchevsky D, Tepper SA & Story JA. (1979) Influence of sapogenins on cholesterol metabolism in rats. *Proc. Soc. Exp. Biol. Med.* 160: 126-129.

Kritchevsky D. 1982, Dietary fiber and disease. *Bull N Y Acad Med*. **58**:230-41

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Lee TJ & Smith BF. (1989) Bovine gallbladder mucin promotes cholesterol crystal nucleation from cholesterol-transporting vesicles in supersaturated model bile. *J. Lipid Res*. **30**: 491–498.

Li X, Hylemon P, Pandak MW & Ren S (2006) Enzyme activity assay for cholesterol 27-hydroxylase in mitochondria. *J. Lipid. Res*. **47**: 1507–1512.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**: 265–275.

Mantle M & Allen A. (1978) A colorimetric assay for glycoproteins based on the periodic acid-schiffs stain. *Biochem. Soc. Transactions* **6**: 607-609.

Marcus SN & Wheaton KW. (1986) Intestinal transit rate, deoxycholic acid and the cholesterol saturation of bile-three interrelated factors. *Gut.* **27**: 550–558.

Marcus SN & Wheaton KW. (1986a) Effects of a new, concentrated wheat fiber preparation on intestinal transit, deoxycholic acid metabolism and the composition of bile. *Gut.* **27**: 893-900.

Marschall HU & Einarsson C. 2007 Gallstone disease. J Intern Med. **261**:529–542

Marzolo MP, Rigotti A & Nervi F. (1990) Secretion of biliary lipids from the hepatocyte. *Hepatology* **12** (Suppl.): 134-142.

Matheson HB, Colon IS & Story JA. (1995) Cholesterol-7-α-hydroxylase activity is increased by dietary modification with psyllium hydrocolloid, pectin, cholesterol and cholestyramine in rats. *J. Nutr.* **125**: 454–458.

Maurer KJ, Carey MC & Fox JG, (2009) Roles of infection, inflammation and the immune system in cholesterol gallstone formation. *Gastroenterol.* **136**: 425-440

Mendez-Sanchez N, Zamora-Valdes D, Chavez-Tapia.NC & Uribe M. (2007) Role of diet in cholesterol gallstone formation. *Clin. Chim. Acta.* **376**: 1-8.

Miettinen TA. (1987) Dietary fibre and lipids. *Am. J. Clin. Nutr.* **45**: 1237-1242.

Miquel JF, Rigotti A, Rojas E, Brandan E & Nervi F. (1992) Isolation and purification of human biliary vesicles with potent cholesterol nucleation promoting activity. *Clin. Sci.* (Lond) **82**: 175-180.

Mott GE, Jackson EM & McMohan CA. (1992) Effect of dietary cholesterol, type of fat and sex on bile lipid composition of adult baboons. *Am. J. Clin. Nutr.* **56**: 511-515.

Narender, T, Puri A, Shweta, Khaliq T, Saxena R, Bhatia G & Chandra R. (2006) 4-Hydroxyisoleucine an unusual amino acid as antidyslipidemic and antihyperglycemic agent, *Bioorg.Med. Chem. Lett.* **16**: 293-296.

Oakenfull DG & Sidhu GS. (1990) Could saponins be a useful treatment for hyper-cholesterolemia? *Eur. J. Clin. Nutr*. **44**: 79–88.

Oakenfull DG. (1981) Dietary fiber, saponins and plasma cholesterol. *Food Tech*. (Australia) **33**: 432-435.

Ohkawa H, Ohishi N & Yagi K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem*. **95**: 351-358.

Omaye ST, Turnbull JD & Sauberlich HE. (1973) Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol*. **62**: 3-11.

Osuga T & Portman DW. (1971) Experimental formation of gallstones in the squirrel monkey. *Proc. Soc. Exp. Biol. Med.* **136**: 722–726.

Ozben T. (1989) Biliary lipids, composition and gallstone formation in rabbits fed on soya protein, cholesterol, casein and modified casein. *Biochem. J*. **263**: 293-296.

Pattinson.NR, Willis KE & Frampton CM. (1991) Comparative analysis of cholesterol transport in bile from patients with and without CGS. *J. Lipid Res*. **32**: 205-214.

Pearlman BH, Bonorris GG & Phillips MJ. (1979) Cholesterol gallstone formation and prevention by chenodeoxycholic and ursodeoxycholic acids: A new hamster model. *Gastroenterol*. **77**: 634– 641.

Petit PR, Sauvaire YD, Hillaire-Buys DM, Leconte OM, Baissac YG, Ponsin GR & Ribes GR. (1995) Steroid saponins from fenugreek seeds: extraction, purification, and pharmacological investigation on feeding behavior and plasma cholesterol. *Steroids* **60**: 674-680.

Petrack B & Latario BJ. (1993) Synthesis of 27-hydroxycholesterol in rat liver mitochondria: HPLC assay and marked activation by exogenous cholesterol. *J. Lipid Res.* **34**: 643-649.

Platel K & Srinivasan K. (1996) Influence of dietary spices or their active principles on digestive enzymes of small intestinal mucosa in rats. *Int. J. Food Sci. Nutr.* **47**: 55–59

Platel K & Srinivasan K. (2000) Stimulatory influence of select spices on bile secretion in rats. *Nutr. Res.* **20**: 1493–1403

Platel K & Srinivasan K. (2000) Studies on the influence of dietary spices on food transit time in experimental rats. *Nutr. Res.* **21**: 1309–1314.

Platel K & Srinivasan K. (2000a) Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. *Nahrung* **44**: 42–46.

Platel K & Srinivasan K. (2001) A study of the digestive stimulant action of select spices in experimental rats. *J. Food Sci. Technol.* **38**: 358–361.

Platel K & Srinivasan K. (2004) Digestive stimulant action of spices: A myth or reality? Indian *J. Med. Res.* **119**: 167-179

Portincasa P, Moschetta A & Palasciano G. (2006) Cholesterol gallstone disease. *The Lancet* **368**: 230-239

Portincasa P, Moschetta A, van Erpecum KJ, Calamita G, Margari A, vanBerge-Henegouwen GP & Palasciano G. (2003) Pathways of cholesterol crystallization in model bile and native bile. *Diges. Liver Dis.* **35**: 118-126.

Premsingh RS, MacPherson BR & Scott GW. (1987) Mucus hypersecretion in the gallbladder epithelium of ground squirrels fed a lithogenic diet for the induction of cholesterol gallstones. *Hepatology* **7**: 1267-1271.

Rao PU, Sesikeran B, Rao PS, Naidu AN, Rao VV & Ramachandran EP. (1996) Short term nutritional and safety evaluation of fenugreek. *Nutr. Res*. **16**: 1495-1505.

Ravikumar P & Anuradha CV. (1999) Effect of fenugreek seeds on blood lipid peroxidation and antioxidants in diabetic rats. *Phytother. Res*. **13**: 197–201.

Rege RV & Prystowsky JB. (1998) Inflammation and a thickened mucus layer in mice with cholesterol gallstones. *J. Surg. Res*.**74**: 81-85.

Roslyn IT, Binns GS & Mughes BF. (1993) Open cholecystectomy. A contemporary analysis of 42,474 patients. *Ann. Surg*. **218**: 129-137.

Russell DW. (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Ann. Rev. Biochem.***72**: 137-174

Sambaiah K & Srinivasan K. (1991) Secretion and composition of bile in rats fed diets containing spices. *J. Food Sci. Technol.* **28**: 35–38

Sauvaire Y, Ribes G, Baccon JC & Loubatieres MM. (1991) Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. *Lipids* **26**: 191–197.

Schwesinger WW, Kurtin WE, Page CP, Stewart RM & Johnson R. (1999) Soluble dietary fiber protects against CGS formation. *Am. J. Surg*. **177**: 307–310

Scobey MW, Johnson FL, Parks JS & Rudel LL. (1991) Dietary fish oil effects on biliary lipid secretion and CGS formation in the African green monkeys. *Hepatology* **14**: 679-684.

Searcy RL & Bergquist LM. (1960) A new colour reaction for the quantification of serum cholesterol. *Clin. Chim. Acta.* **5**: 192-199.

Secknus R & Holzbach RT. (1997) Role of biliary proteins in pathogenesis of CGS. *Z Gastroenterol*. **35**: 41-46.

Sedlak J & Lindsay RH. (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem*. **25**: 192-205.

Shaffer E. (2006) Epidemiology of gallbladder stone disease *Best Pract. Res. Clin. Gastroenterol.* **20**: 981-996

Sharma RD & Raghuram TC. (1990) Hypoglycaemic effect of fenugreek seeds in non-insulin dependent diabetic subjects. *Nutr. Res.***10**: 731-739.

Sharma RD, Raghuram TC & Rao NS. (1990a) Effect of fenugreek seeds on blood glucose and serum lipids in type I diabetes. *Eur. J. Clin. Nutr*. **44**: 301–306.

Sharma RD, Sarkar A, Hazra DK, Mishra B, Singh JB, Sharma SK, Maheshwari BB & Maheshwari PK. (1996) Use of fenugreek seed powder in the management if NIDDM. *Nutr. Res*. **16**: 1331-1339.

Sharma RD. (1984) Hypocholesterolemic activity of fenugreek (*T. foenum graecum*) An experimental study in rats. *Nutr. Rep. Int.* **30**: 221–231

Sharma RD. (1986) An evaluation of hypocholesterolemic factor in fenugreek seeds (*T. foenum graecum*). *Nutr. Rep. Int.* **33**: 669–677.

Sharma RD. (1986a) Effect of fenugreek seeds and leaves on blood glucose and serum insulin responses in heman subjects. *Nutr. Res*. **6**: 1353-1364.

Siddiqui MR, Taha A, Moorthy K, Hussain ME, Basir SF & Baquer NZ. (2005) Amelioration of altered antioxidant status and membrane linked functions by vanadium and *Trigonella* in alloxan diabetic rat brains. *J. Biosci*. **30**: 483-490.

Sidhu GS & Oakenfull DG. (1986) A mechanism for the hypocholesterolaemic activity of saponins. *Br. J. Nutr*., **55**: 643-649.

Simon JE, Chadwick AF & Craker LE. (1984) Herbs: an indexed bibiliography 1971-1980, the scientific literature on selected herbs, and aromatic and medicinal plants of the temperate zone, archon books, pp770., Hamden CT.

Singhal PC, Gupta RK & Joshi LD. (1982) Hypocholesterolemic effect of *T. foenum graecum. Nutr. Rep. Int.* **33**: 669–677

Smith LC, Pownall HJ & Gotto AM Jr. (1978) The Plasma Lipoproteins: Structure and Metabolism. *Ann. Rev. Biochem.* **47**: 751-777

Soares DG, Andreazza AC & Salvador M. (2003) Sequestering ability of butylated hydroxy toluene, propyl gallate, resveratrol and vitamins C and E against ABTS, DPPH and hydroxyl free radicals in chemical and biological systems. *J. Agric. Food Chem*. **51**: 1077-1080.

Sowmya P & Rajalakshmi P. (1999) Hypocholesterolemic effect of germinated fenugreek seeds in human subjects. *Plant Food Hum. Nutr.* **53**: 359–365.

Srinivasan K & Sambaiah K. (1991) The effect of spices on cholesterol 7a-hydroxylase activity and on serum hepatic cholesterol levels in the rats. *Int. J. Vit. Nutr. Res.* **61**: 364–369

Srinivasan K, Sambaiah K & Chandrasekhara N. (2004) Spices as beneficial hypolipidemic food adjuncts: A Review. *Food Rev. Int.* **20**: 187-220

Srinivasan K. (2005) Role of spices beyond food flavouring: nutraceuticals with multiple health effects. *Food Rev. Int.* **21**: 167–188

Srinivasan K. (2006) Fenugreek (*Trigonella foenum-graecum*): A Review of health beneficial physiological effects. *Food Rev. Int.* **22**: 203-224

Srinivasan K. (2010) Antioxidant potential of spices and their active constituents. *Crit. Rev. Food Sci. Nutr.* **50**: In press.

Steiner M, Khan AH, Holbert D & Lin RI. (1996) A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged garlic extract and placebo administration on blood lipids. *Am. J. Clin. Nutr.* **64**: 866-870

Stewart JCM. (1980) Colorimetric estimation of phospholipids with ammonium ferrothio-cyanate. *Anal. Biochem*. **104**: 10-14.

Strasberg SM. (1998) The pathogenesis of cholesterol gallstones: A review. *J. Gastrointest. Surg*. **2**: 109-125.

Swobodnik W, Wenk H, Janowitz P, Hagert N, Kratzer W, Berghold J, Zhang Y, Bittner R, Schusdziarra V & Ott R. (1991) Total biliary protein, mucus glycoproteins, cyclic-AMP, and apolipoproteins in the gallbladder bile of patients with cholesterol stones and stone-free controls. *Scand. J. Gastroenterol*. **26**: 771-778.

Tazuma S, Mizuno S, Sasaki H, Sagawa H, Hatsushika S, Tao S, Yamashita G, Aihara N, Kajiyama G & Yamamoto M. (1991) A study on the mechanisms whereby apo-lipoprotein A-1(apo A-1) inhibits cholesterol crystal nucleation in human gallbladder bile. *Nippon Shokakibyo Gakkai Zasshi* **88**: 1083-1088.

Tepperman J, Caldwell FT & Tepperman HM. (1964) Induction of gallstones in mice by feeding a cholesterol-cholic acid containing diet. *Am. J. Physiol.* **206**: 628–634.

Teramen K, Tazuma S, Ohya T & Kajiyama G. (1995) Comparative effects on biliary concanavalin A-bound glycoproteins and calcium ion on cholesterol crystal nucleation and growth in model bile. *J. Gastroenterol*. **30**: 500-507.

Thimmayamma BVS, Rao P & Radhaiah G. (1983) Use of spices and condiments in the dietaries of urban and rural families. *Indian J. Nutr. Dietet*. **20**: 153-162.

Thirunavukkarasu V, Anuradha CV & Viswanathan P. (2003) Protective effect of fenugreek (*Trigonella foenum-graecum*) seeds in experimental ethanol toxicity. *Phytother. Res.* **17**: 737-743.

Thompson JC, Gerald M, Fried W, Ogden D, Fagan CJ, Inoue K, Wiener I & Watson LC. (1982) Correlation between Release of Cholecystokinin and Contraction of the Gallbladder in Patients with Gallstones *Ann. Surg.* **195**: 670–675

Thornton JR, Emmett PM & Heaton KW. (1983) Diet and gallstone effect on refined and unrefined carbohydrate diets on bile cholesterol saturation and bile acid metabolism. *Gut.***24**: 2-6.

Trowell HC. (1975) Letter: Dietary fibre hypothesis. *Br. Med. J*. **13**: 649.

Turley SD & Dietschy JM. (1970) Reevaluation of 3α-hydroxysteroid dehydrogenase assay for total bile acids. *J. Lipid Res*. **19**: 924-928.

Udayasekhara Rao, P, Sesikeran B, Rao PS, Naidu AN, Rao VV & Ramachandran EP. (1996) Human study Short term nutritional and safety evaluation of fenugreek. *Nutr. Res.***16**: 1495-1505.

Valette G, Sauvaire Y, Baccou J & Ribes G. (1984) Hypocholesterolaemic effect of Fenugreek seeds in dogs. *Atherosclerosis* **50**: 105-111

Van Erpecum KJ, van Berge-Henegouwen GP, Stoelwinder B, Schmidt YM & Willekens FL. (1990) Bile concentration is a key factor for nucleation of cholesterol crystals and CSI in gallbladder bile of gallstone patients. *Hepatology* **11**: 1-6

Van Erpecum KJ, Wang DQ, Moschetta A, Ferri D, Svelto M, Portincasa P, Hendrickx JJ, Schipper M & Calamita G. (2006) Gallbladder histopathology during murine gallstone formation: relation to motility and concentrating function. *J. Lipid Res.* **47**: 32-41

Van Erpecum KJ. (2004) Cholesterol-gallstone formation: More than a biliary lipid defect? *J. Lab. Clin. Med.***44**: 121-123

Vidyashankar S, Sambaiah K & Srinivasan K. (2009) Dietary garlic and onion reduce the incidence of atherogenic diet-induced CGS in experimental mice *Br. J. Nut.***101**: 1621-1629

Vidyashankar S, Sambaiah K & Srinivasan K. (2010) Regression of pre-established CGS by dietary garlic and onion in experimental mice. *Metabolism* **59**: In Press.

Von Erpecum KJ & Von Berge-Henegouwen GP. (1989) Pathogenic factors in cholesterol gallstone disease. *Scand. J. Gastroenterol*. **24**(Suppl): 83-90.

Warholm M, Guthenberg C, Bahr CV & Mannervik B. (1985) Glutathione transferase from human liver. *Methods Enzymol.* **113**: 499-504.

Warnick GR & Albers JJ. (1978) A comprehensive evaluation of the heparin-manganese chloride precipitation procedure for estimating HDL-cholesterol. *J. Lipid Res*. **19**: 65-76.

Wayne SW, Kurtin WE, Page CP, Stewart RM & Johnson R. (1999) Soluble dietary fiber protects against cholesterol gallstone formation. *Am. J. Surg*. **177**: 307–310.

Wilhelmi M, Jüngst C, Mock M, Meyer G, Zündt B, Del Pozo R, Jüngst D. (2004) Effect of gallbladder mucin on the crystallization of cholesterol in bile. *Eur. J. Gastroenterol. Hepatol.* **16**: 1301-1307.

Wu J, Yang H, Zhou J, Tian X & Zhou M. (2007) Cholesterol phospholipid cholic acid and mucoprotein in the crystallization of gallbladder stone. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi*. **24**: 389-393.

Xu QW, Scott RB, Tan DT & Shaffer EA. (1996) Slow intestinal transit: a motor disorder contributing to CGS formation in the ground squirrel. *Hepatology* **23**: 1664-1672.

Yamasaki T, Chijiiwa K & Endo M. (1993) Isolation of mucin from human hepatic bile and its induced effects on precipitation of cholesterol and calcium carbonate in vitro. *Diges. Dis. Sci.* **38**: 909-915.

Zak A, Zeman M, Hrubant K, Vecka M & Tvrzicka E. (2007) Effect of hypolipidemic treatment on the composition of bile and the risk or cholesterol gallstone disease. *Cas Lek Cesk* **146**: 24-34.

List of Publications:

1. **Reddy RLR,** and Srinivasan K. 2009, Fenugreek seeds reduce atherogenic diet-induced cholesterol gallstone formation in experimental mice. ***Can J Physiol Pharmacol***. **87**:933-43
2. **Reddy RLR** and Srinivasan K. 2009, Dietary fenugreek seed regresses pre-established cholesterol gallstones in mice. ***Can J Physiol Pharmacol***. **87**:684-93
3. **Reddy RLR** and Srinivasan K. 2010, Hepatoprotective and antioxidant effect of Fenugreek (*Trigonella foenum-graecum*) seeds in mice under lithogenic condition (In press ***J. Food Biochemistry***)
4. Shubha.M.C, **Reddy R.L.R.** and K.Srinivasan 2010, Influence of dietary Curcumin and Capsaicin on induction of CGS in mice (In press).

Manuscript communicated/to be communicated:

1. **Reddy RLR** and Srinivasan K. Effect of dietary fenugreek seeds on biliary proteins and their influence on cholesterol nucleation in model bile.
2. **Reddy RLR** Suresha BS and Srinivasan K. Influence dietary fenugreek and onion on biochemical changes in serum, liver and bile and histopathological changes in liver and bile mice fed with high cholesterol diet

Manuscript in preparation

1. **Reddy RLR** and Srinivasan K. Dietary fenugreek, onion under lithogenic condition modulates hepatic mRNA expression of cholesterol 7α- hydroxylase, sterol-27-hydroxylase and LDL-receptor in lithogenic fed mice