EVALUATION OF EFFECT OF SELECTED HERBS ON CHOLESTEROL LEVEL IN GHEE

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SHINGALA KRUPABEN MANASUKHBHAI M. Tech DAIRY CHEMISTRY 2018

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ABSTRACT

Ghee is a heat clarified milk fat and supplies various nutrients. It contains, cholesterol, one of the major unsaponifiable matters. Cholesterol ensures stability of cell membranes, necessary for production of different steroid hormones and vitamin D. However, high cholesterol level causes atherosclerosis, strokes, heart attacks, and peripheral vascular diseases. It is advisable to maintain cholesterol level in body. There are many methods for reducing cholesterol content in foods and that include blending in vegetable oils, extraction using organic solvents, cholesterol degradation by cholesterol oxidases and removal via supercritical carbon dioxide extraction as well as using β-cyclodextrins. However, these processes are costly and they may have side effects because of chemical used for removal of cholesterol. Since the usage of the synthetic drug, statins, leads to various side effects, the plants flavonoids with antiartherosclerotic activity gained much attention. So there is need to develop natural way to reduce cholesterol by using herbs in ghee. Keeping the said idea as a central goal, the study was planned with distribution of the work into 4 different phases: (1) assessment of compatibility of herbs in ghee (2) evaluation of the herbs for their cholesterol lowering ability in ghee (3) optimization of the herbs for application and (4) analysis of ghee for quality parameters and sensory.

Total seven herbs (ajwain leaves, betel leaves, coriander leaves, curry leaves, fenugreek leaves, liquorice, mint) being very common in use were shortlisted for assessment of their compatibility in ghee by sensory evaluation using flavour score for their acceptability. The 9 point hedonic scale was used. On treating ghee with 0.5 per cent (w/w) of these herbs, the flavour score of all the ghee samples were found acceptable. The cholesterol content of the control ghee sample was 209.07 mg/100g of ghee. Upon addition of ajwain leaves, betel leaves, curry leaves and liquorice in ghee, the cholesterol content were 200.33, 187.78, 199.67 and 207.05 mg/100 g respectively. The per cent reduction in cholesterol contents of ghee samples in descending order were observed in betel leaves (10.53 per cent) > ajwain leaves (4.63 per cent) > curry leaves (4.50 per cent)> and licourice (0.96 per cent). However,

cholesterol content was increased in ghee when treated with mint and coriander leaves. The increased in cholesterol content might be attributed to development of turbidity in the ghee samples. Though licourice have ability to lower down the cholesterol content, it gave bit green colour to the ghee sample and it was not carried forward for further study. From the work carried, the three herbs namely ajwain leaves, betel leaves and curry leaves were selected for further study.

For selection of stage for addition of herb in preparation of ghee, the herbs (ajwain leaves, betel leaves and curry leaves) were individually added (@ 0.5per cent) at three different stages during manufacture of ghee. These stages were categorized into (i) Stage 1: Addition at the initial stage of heat clarification of butter (i.e. in melted butter, 50 °C), (ii) Stage 2: Addition at the final stage of heat clarification of butter (i.e. at 105°C temperature) and (iii) Stage 3: In hot ghee at 90 ºC and allow it to cool at 60°C. In stage 1, the highest cholesterol reduction was observed in betel leaves (14.16 per cent). In stage 2, the cholesterol content was also marginally reduced in all the treated ghee samples. The cholesterol contents were significantly reduced in stage 3. The average cholesterol content of control ghee sample was 212.68 mg/100 gm that decreased to 175.93 mg/100 gm in ghee added with ajwain. Thus, highest reduction found in ajwain (17.46 per cent) > betel leaves (16.99 per cent) > curry leaves (11.77 per cent). Among the all stages, the maximum reduction in cholesterol contents was observed in stage 3. Moreover, the flavour score were also acceptable in this stage and hence stage 3 was considered as the best for lowering down the cholesterol content in ghee.

Among the various forms (pieces, paste, coarse and fine particles) of herbs, the coarse particles of ajwain leaves, betel leaves and curry leaves shown 6.98 per cent, 5.75 per cent and 14.7 per cent reduction in cholesterol content when compared to other forms. The selected herbs in the form of coarse particles were added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 per cent in ghee. When ajwain, betel leaves and curry leaves were added at the rate of 0.5 per cent in ghee samples, the maximum reduction of cholesterol content were obtained as 4.25 per cent, 4.54 per cent and 9.55 per cent respectively as compared to other rate of addition in ghee. Among the various heating intervals *viz*. 15 min, 30 min and 45 min at 60 °C, the 30 min of heating interval considered as optimum in maximum reduction of cholesterol content without affecting the flavour score of the ghee. Thus, addition of ajwain leaves, betel leaves and curry leaves in ghee in the form of coarse particle at the rate of 0.50 per cent with heating at 60 °C for 30 min. was found best in reducing the cholesterol content without affecting the flavour score of ghee.

The fresh samples of ghee were fulfilled the requirement of the quality parameters such as moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and baudouin test as prescribed under FSSAI as well as AGMARK. All ghee samples were stored at 40±2°C and evaluated for cholesterol content, peroxide value, free fatty acid and sensory evaluation at the 5 days of interval. The data revealed that changes in these parameter were statistically non-significant (P> 0.05 per cent).

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CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of Effect of Selected Herbs on Cholesterol Level in Ghee "** submitted by **Shingala Krupaben Manasukhbhai (Reg No.:** 04-2993-2016**)** in partial fulfillment of the requirements for the degree of **MASTER OF TECHNOLOGY** in **DAIRY CHEMISTRY** of the Anand Agricultural University is a record of bonafide research work carried out by her under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand **(Dr. B. M. Mehta)**

Date: **Major Advisor**

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Per cent
*	Significant
AGMARK	Agricultural produce(Grading and Marking Rules)
AMF	Anhydrous milk fat
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BIS	Bureau of Indian Standards
BR reading	Butyro-Refractometer reading
C. D.	Critical difference
C. V.	Coefficient of Variation
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
DMF	Dimethylformamide
FFA	Free fatty acid
FID	Flame ionization detector
FSSAI	Food Safety and Standards Authority of India
FTIR	Fourier-transform infrared spectroscopy
GLC	Gas liquid chromatography
gm	Gram(s)
HDL	High-density lipoprotein
HMDS	Hexamethyldisilane
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	High performance liquid chromatography
Kg	Kilogram
L	Liter(s)

LB	Liebermann-Burchard
LDL	Low-density lipoprotein
mg	Milligram
min	Minute(s)
ml	Milliliters
mm	Millimeter
mM	Micro molar
N	Normality
NACE	Non-aqueous capillary electrophoresis
NaOH	Sodium hydroxide
NS	Non-significant
PV	Polenske value
RM	Reichert Meissl
SEm	Standard error of mean
SPE	Solid phase extraction
TMCS	Trimethylchlorsilane
TMS	Trimethylsilyl esters
TPC	Total phenolic content
TS	Total Solids
VLDL	Very Low-density lipoprotein
µ	Micron

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CHAPTER – 1

INTRODUCTION

Ghee is an anhydrous milk fat, occupies a prominent place in the Indian diet. Ghee is manufactured by direct heating of cream or butter churned out of fresh or ripened cream or *dahi* obtained by fermentation of milk with bacteria native to milk or selected starter cultures (Srinivasan, 1976). Chemically ghee (99.5 per cent milk fat) is a complex lipid of mixed glycerides together with a small amount of free fatty acids, phospholipids, sterols and their esters, fat soluble vitamins (A, D, E and K), carotenoids, carbonyl compounds, hydrocarbons, charred casein, moisture and trace elements like copper and iron (Sharma, 1981). Ghee is the elixir of life. The essence of life is unctuous and the vital capacity of the body depends on it. Ayurveda describes the four types of unctuous substances *viz*. oil, ghee, animal fat and bone marrow; these are generally used for human beings and out of these ghee is considered as best. Ghee is considered as far superior owing to its special attribute as it carries the properties of the drug without leaving its own inherent properties. Ghee is prompter of memory, intelligence, power of digestion and metabolism, semen and vital essence. It is refrigerant and emollient and clarifies the voice and complexion. The ghee has very excellent nutritive, medicinal and therapeutic properties (Bhatted and Singh, 2002).

Cholesterol is a steroid alcohol with the molecular formula $C_{27}H_{45}OH$. It is the precursor of bile acids, steroid hormones, and provitamin D_3 and is found in foods of animal origin, such as eggs, meat, fish and dairy products. Substantial amounts of total body cholesterol are obtained from the diet even though a major portion of the cholesterol needed for normal body functions is synthesised endogenously (Sweeney and Weihrauch, 1976). It is essential to our body's cell membrane, to insulate our nerves and for the production of certain hormones. Cholesterol is the major constituent of the unsaponifiable matter of milk fat. The unsaponifiable matter of milk fat generally falls within the range of 0.40 to 0.5 per cent by weight and out of this, 95 per cent is represented by cholesterol (i.e. 0.38 to 0.47 per cent). Cholesterol and its esters are associated primarily with milk fat globules. The total cholesterol content in cow and buffalo ghee is 330 mg/100g fat and 275 mg/100g fat respectively.

Inspite of its numerous health benefits, milk fat has received adverse publicity in terms of its effect on blood cholesterol level over the past few years. Cholesterol is suspected to be associated with coronary heart diseases (CHDs). Cholesterol-lowering and cholesterol-free products are gaining increasing relevance in the modern era. Due to this, people are prone to eliminate or reduce intake of food in the diet containing cholesterol. To eliminate cholesterol, people are also avoiding the consumption of ghee in the diet.

The diet which is completely devoid of ghee is not logical as it play an important role in Indian diet. The supply of other potent and advantageous nutrients (like conjugated linolenic acid, fat soluble vitamins, essential fatty acids etc.) is not readily available if diet is devoid of milk fat/ghee. There are many methods of reducing cholesterol content in foods and that include blending in vegetable oils (Dunkley, 1982), extraction using organic solvents (Larsen and Froning, 1981), cholesterol degradation by cholesterol oxidases (Watanabe *et al.,* 1989) and removal via supercritical carbon dioxide extraction (Ong *et al.,*1990), use of β- Cyclodextrins. However, most of these are relatively non-selective methods and require a great deal of investment. There is a need to eliminate/reduce cholesterol content in food by natural means.

Herbs are any plants used for food, flavouring, medicine, or fragrances for their savoury or aromatic properties. Herbs contain biologically active chemical substances such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds, which have antioxidants, antimicrobial, antifungal, as well as curative properties. Herbs also have medicinal properties like antiglycemic, hypolipidemic and antihypertensive properties. Herbs have long been used as traditional medicine for the treatment of hyperlipidemia in Indian traditional system of medicine and other parts of the world. Recently, the search for appropriate antihyperlipidemic agents have been again focused on plants because of less toxicity, easy availability and easy absorption in the body that may be better treatment than currently used drugs. Thirumalai *et al.* (2014) studied the hypolipidemic activity of betel leaves *in vivo*. They found that the methanolic leaf extract of betel leaves exhibited a significant hypolipidemic effect in high fat diet induced hyperlipidemic rat. However, it appears from the available literature that no attention has been paid so far for use herbs in lowing cholesterol content in ghee. Therefore, there is a need to undertake systematic study to generate data. Keeping in view the above resume, the present study was contemplated with the following objectives:

Objectives

(a) To assess the compatibility of selected herbs in ghee.

(b) To evaluate the compatible herbs for their cholesterol lowering ability in ghee.

(c) To optimize the promising herbs for application in ghee.

(d) To analyse the ghee for quality parameters and sensory attributes.

CHAPTER – 2

REVIEW OF LITERATURE

Ghee is one of the oldest and important traditional milk products of India. *Ghrita* (ghee) was produced in ancient India as far back as 1500 BC (Achaya, 1997). Ancient Sanskrit literature describes ghee as the food fit for Gods and a commodity of enormous value.

In ancient India, ghee was the preferred cooking medium. It was considered pure and was felt to confer purity to foods cooked with it (Achaya, 1997). *Ayurveda* has traditionally considered ghee to be the healthiest source of edible fat, with many beneficial properties. According to *Ayurveda*, ghee promotes longevity and protects the body from various diseases (Tirtha, 1998). It improves memory and strengthens the brain and nerve system, it lubricates the connective tissues, thereby rendering the body more flexible.

Ghee is the elixir of life. Ghee is promoter of memory, intelligence, power of digestion and metabolism, semen, vital essence. It is refrigerant and emollient and clarifies the voice and complexion (Bhatted and Singh, 2002).

2.1 GHEE

Chemically ghee (99.5 per cent milk fat) is a complex lipid of mixed glycerides together with a small amount of free fatty acids, phospholipids, sterols and their esters, fat soluble vitamins (A, D, E and K), carotenoids, carbonyl compounds, hydrocarbons, charred casein, moisture and traces of trace elements like copper and iron (Sharma, 1981). The ghee has maximum 0.5 per cent moisture. Ghee contains relatively higher proportion of saponifiable matter that represents 99.5 per cent while 0.4 to 0.5 per cent is represented by unsaponifiable matter. Among unsaponifiable matter, 95 per cent is represented by cholesterol (i.e. 0.38-0.47 per cent). Cholesterol is principal sterol of milk. It is also known as zoo sterol. Cholesterol present in different three phases: (i) true solution in the fat, (ii) part of milk fat globule membrane and (iii) complex formation with protein in non-fat portion of milk. The total cholesterol content in cow and buffalo ghee is 330 mg/100g fat and 275 mg/100g fat respectively.

2.2 METHOD OF GHEE MANUFACTURE

Ghee manufacture has been practiced traditionally in almost every rural household in India since ancient times. The method followed generally involved conversion of milk into curd, subsequent churning and heating of the fresh butter. However as the technology for commercial manufacture of the products was standardized, other methods evolved which are being delineated below (Rajorhia, 1993; Achaya, 1997; Sserunjogi *et al.,* 1998; Aneja *et al.,* 2007).

2.2.1 Indigenous Method

This is the most commonly used and oldest method to produce ghee in India. Milk is heated and inoculated with buttermilk or curd (i.e. previous day's product) @ 5-10 per cent. Starter is mixed with milk left for 18 - 36 hrs. Ripened milk or curd is churned. Butter floating at top is removed in separate vessel. *Makhan*, fresh or accumulated over a few days, is usually taken in a suitable open mud-pot or metallic vessel, and carefully heated and stirred on a low fire to drive out the moisture. The end point of heating is indicated by the appearance of second effervescence, which is subtler than the first one accompanied by the browning of curd particles. At this stage the typical ghee flavour emanates and this indicates the final stage in the preparation of ghee. After cooling and sedimentation, ghee is filtered through a muslin or filter cloth to separate the ghee residue.

2.2.2 Creamery Butter Method

This is the most commonly used method of ghee making in organized sector. Milk is separated and the resulting cream is ripened overnight using starter followed by churning next day to obtain butter. This is washed to remove buttermilk and then worked to get rid of occluded water without damaging grains. Worked butter is then boiled to 118-120°C to obtain satisfactory quality of ghee. Here, raw material (unsalted creamery butter, otherwise known as white butter) is gradually heated till the entire lot of butter melts resulting in formation of a layer of scum that gathers on the top surface of the liquefied butter. The scum is periodically removed using a ladle. The resulting product is sedimented in pre-stratification tanks. The top layer consists of butterfat, which is transferred to ghee boiler. With continuous heating in the boiler, the moisture content is drastically reduced. The final stage of ghee preparation is indicated by the appearance of second effervescence, which is less pronounced than

the first one along with browning of curd particles. The temperature of heating has to be controlled at this stage. This stage is further confirmed by appearance of characteristic ghee flavour. The final temperature of the liquid mass is increased to 110 - 120°C for clarification before finally cooling and storage in bulk tanks before filling.

The merits for this method are savings in labour, physical exertion and exposure to uncomfortably high temperature and humidity during actual ghee making when compared to the direct cream method. One of the demerits here is cooked flavour in the end product due to higher temperature of heating.

2.2.3 Pre-Stratification Method

When butter is left undisturbed at a temperature of 80–85°C for 15–30 min, it stratifies into three separate layers, *viz.* a top layer of floating denatured particles of curd, a middle layer of fat and a bottom layer of butter milk. This separation of three layers is called pre-stratification. The bottom layer which consists of buttermilk has 60-70 per cent SNF and more than 80 per cent of moisture originally present in butter. The bottom layer is carefully removed without disturbing both top and middle layers. Afterwards, the temperature of two remaining upper layers consisting of denatured curd and fat is raised to the regular clarifying temperature of 110 - 120°C.

2.2.4 Continuous Method

Here the cream separated from milk is directly taken in ghee boiling unit for continuous ghee production. One of the advantages of employing this method is the continuous conversion of excess of by-product of milk separation process into a finished product. One of the limitations is that it uses high amount of heat energy and hence high amount of steam is required. Such process leads to flat flavoured ghee.

2.3 MEDICINAL PROPERTIES OF GHEE

The various milk fat components, such as CLA, sphingomyelin, butyric acid, ether lipids, β-carotene and vitamin A and D, have anticarcinogenic potential (Parodi, 1999; Alkalin and Tokusoglu, 2003; Khanal and Olson, 2004).

Ghee is heavily utilized in *Ayurveda* for numerous medical applications, including the treatment of allergy, skin, and respiratory diseases. Proper digestion, absorption, and delivery to a target organ system are crucial in obtaining the maximum benefit from

any therapeutic formulation; the lipophilic action of ghee facilitates transportation of molecules to a target organ and final delivery inside the cell since the cell membrane also contains lipid (Sharma, 1990).

2.4 GHEE CHOLESTEROL

Sterols are polycyclic alcohols having a secondary -OH group at position 3, and the presence of this group makes the sterols more polar than triglycerides. Cholesterol is the principal mammalian sterol containing 27 carbon atoms with a combination of steroid and alcohol. Cholesterol, both free (90 per cent) and esterified, is one of the constituents of the fat globule membrane in milk fat (Keenan and Dylewski, 1995), particular care is necessary during fat extraction from dairy products to recover the whole sterol fraction.

Cholesterol present exclusively in foods of animal origin including meat, dairy and poultry (Xu *et al.,* 2002). Cholesterol is required by all the mammalian cells for the proper functioning of cellular membranes. It is an important functional, constituent of cellular membrane and brain functions and precursor of important endogenous substances such as sex hormones, and bile acids (Bragagnolo, 2010).

The pathway of cholesterol biosynthesis may be generally divided into four stages (Li and Parish, 1998): (1) The formation of mevalonic acid from three molecules of acetyl CoA, (2) The biosynthesis of squalene from six molecules of mevalonic acid through a series of phosphorylated intermediates, (3) The biosynthesis of lanosterol from squalene via cyclization of 2, 3-epoxysqualene, and (4) The modification of lanosterol to produce cholesterol.

2.5 STRUCTURE AND PROPERTIES OF CHOLESTEROL

Chemically, cholesterol is a fat-like compound, basically, an alcohol which, in its pure form appears as flakes. It is composed of 27-carbon atoms which form three fused cyclohexane (6-carbon) rings, a cyclo-pentane (5 carbons) ring and a side chain of eight carbon atoms. Its molecular weight is 386.66 and the molecular formula is $C_{27}H_{46}O$. The melting point of cholesterol is 148.50℃. Cholesterol contains the polar hydroxyl group at C-3, which gives cholesterol a slightly hydrophilic nature, can be esterified to a fatty acid, producing cholesterol ester. Cholesterol is insoluble in water, sparingly soluble in cold alcohol or petroleum ether, and completely soluble in hot alcohol and in most other organic solvents.

2.6 SYNTHESIS OF CHOLESTEROL

All the 27 carbon atoms of cholesterol are derived from acetyl CoA through the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway. First of all Formation of mevalonic acid from three molecules of acetyl CoA than Biosynthesis of squalene from six molecules of mevalonic acid through a series of phosphorylated intermediates is carried out. After that biosynthesis of lanosterol from squalene via cyclization of 2, 3-epoxysqualene, and Modification of lanosterol to produce cholesterol (Li and Parish, 1998) is done.

2.7 SIGNIFICANCE OF CHOLESTEROL

Cholesterol is more abundant in tissues having densely packed membranes, like the liver, spinal cord, and brain. There are mainly two types of cholesterol: High-density lipoprotein (HDL) cholesterol and Low-density lipoprotein (LDL) cholesterol. If cholesterol is high, it builds upon the walls of the arteries leading to a heart attack or a stroke Cholesterol is a major determinant of membrane fluidity due to its hydrophobic and hydrophilic regions. Cholesterol is a structural component of the cell membranes. Approximately 80 per cent of cholesterol is used by the liver to form the bile acids. This surrounds nerves and (c) the brain, though its function in these areas is not completely understood. Cholic acid is the most abundant bile acid in human bile. Eventually, bile acids get coupled to glycine and taurine to form salts of these substances which are water soluble and act as powerful emulsifiers, important in the digestion of fat. Cholesterol is a structural component of the cell membranes particularly skin and the myelin sheath. Cholesterol is a chemical ancestor of the hormones like progesterone, cortisone and estrogen containing 21, 19, and 18 carbon atoms, respectively. These hormones are found in the ovaries, testis and adrenal gland. Cholesterol is a precursor for the synthesis of vitamin D (Makwana 2007).

2.8 CHOLESTEROL IN MILK AND MILK PRODUCTS

Cholesterol is rich in animal food products like milk and milk products. Cholesterol accounts for 0.25-0.40 per cent of the total lipids in milk (Jenness and Patton 1959). In milk, it is present in the fat globule membrane (FGM), in the fat core and in association with milk protein particularly in skimmed milk (Schlimme and Kiel 1989). Any process disrupting the membrane structure will result in the transfer of cholesterol along with ruptured membrane material to the aqueous phase. Tylkin *et al.*

(1975) reported 9 times higher cholesterol per gram fat in buttermilk than that in butter. Vyshemirskii *et al.* (1977) reported that 80-90 per cent cholesterol initially present in cream passed into butter and 10-20 per cent to buttermilk. Bindal and Jain (1973) estimated free and esterified cholesterol in desi ghee and reported their contents as 0.288 and 0.038 per cent, 0.214 per cent and 0.056 per cent in cow and buffalo ghee, respectively. The cholesterol in cheese was found to contain 69.3 mg/100 g of cheese (Fuke and Matsuoka 1974). The cholesterol content in unsalted butter and 10 per cent cream were reported to contain 244 and 31.4 mg / 100g of product, respectively (Aristova and Bekhova 1976). Arul *et al.* (1988) studied the distribution of cholesterol in various milk fat fractions viz., solid fraction (melting point 390℃), semisolid fraction (melting point 210℃) and liquid fraction (melting point. 120℃) and reported that 80 per cent of the total cholesterol content was present in the liquid fraction of the milk fat. Bindal and Jain (1973) estimated free and esterified cholesterol in desi ghee and reported their contents as 0.288 and 0.038 per cent, 0.214 and 0.056 per cent in cow and buffalo ghee, respectively. The cheese was found to contain 52.3 to 76.6 mg/100 g of cheese and 198 to 298 mg / 100 g of cheese fat (Fuke and Matsuoka 1974). Unsalted butter and 10 per cent cream were reported to contain 244 and 31.4 mg/100g of product, respectively (Aristova and Bekhova 1976). Singh and Gupta (1982) have estimated cholesterol content in different species and reported that goat ghee contains higher cholesterol (236 mg/100 g) than cow (230 mg/100 g) and buffalo (196 mg/100 g) ghee. Pantulu *et al*. (1975) reported cholesterol content for buffalo milk fat in the range of 183 to 383 mg/100 g of fat. Prasad and Pandita (1987) reported the cholesterol content of ghee prepared from the milk of Haryana, Sahiwal and Sahiwal X Friesian cows and from Murrah buffaloes as 303, 310, 328 and 240 mg/100 g fat, respectively. These workers also observed that seasons also affected the cholesterol content of ghee with the highest content in winter (301 mg/100g) and lowest in summer (291 mg/100g fat).

2.9 HEALTH IMPLICATION OF CHOLESTEROL

Cholesterol is bound to one of the various lipoproteins, and transported into the circulatory system. The main types of lipoproteins are low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and they carry the cholesterol from and to the liver (Patton and Jensen, 1976). Abnormally high cholesterol levels (hypercholesterolemia) and abnormal proportions of LDL and HDL are associated

with cardiovascular disease through the development of atheroma in arteries (atherosclerosis) (Richard and Ostlund, 2007). This disease process leads to myocardial infarction (heart attack), stroke, and peripheral vascular disease. As high LDL contributes to this process, it is termed as "bad cholesterol," while high levels of HDL ("good cholesterol") offer a degree of protection. The balance can be redressed with exercise, healthy diet, and medications (Isomaa *et al.*, 2001).

2.10 METHODS FOR REMOVAL OF CHOLESTEROL

There are various methods and /or processes available in the literature for removal of cholesterol content in butter oil and foods in general and they generally categories into physical, chemical, biological and complexation processes. However, these processes are costly.

2.10.1 Physical Processes

a) Vacuum steam distillation

In this process, steam is bubbled through liquid milk fat under vacuum. The Omega Source Corporation (Burnsville, MN) has acquired this proprietary technology and is currently producing 109.1 kg/day of decholesterolized anhydrous milk fat (Boudreau and Arul, 1993). The process reduces free cholesterol by 93 per cent.

Firma Hoche (Speikem, Germany) is also utilizing vacuum stem distillation technology on a commercial scale. The milk fat was reported to be almost neutral in smell and taste with 75 per cent less cholesterol content (Boudreau and Arul, 1993).

b) Short path molecular distillation

Short-path molecular distillation is based on the transfer of molecules from the hot surface of an evaporating liquid to the cooled surface of a condenser through a short path. Lanzani *et al*. (1994) investigated a new short-path molecular distillation system for the reduction of cholesterol in butter fat and lard. This method however does not remove the high boiling cholesterol ester.

c) Supercritical fluid extraction (Supercritical Carbon Dioxide Extraction)

Bradley (1990) have shown that it was technically feasible to produce a milk fat product (80 per cent plastic cream) for a reasonable cost, with at least 90 per cent of the cholesterol extracted using Supercritical carbon dioxide. The finished milk fat retained its color and flavors. In the same study, he described a batch process that

removed 90 per cent of the cholesterol from butter by this technology and subsequently yielded a soft spread product (Bradley, 1990).

Some studies have shown that supercritical carbon dioxide (SC-CO_2) can be used to fractionate AMF with evidence that cholesterol can be concentrated into selected fractions. Kaufmann *et al*. (1982) obtained two fractions of milk fat by SC-CO_2 extraction at a pressure of 200 bar and temperature of 800 ℃. In this, the liquid fractions were enriched in total cholesterol. However, Huber *et al*. (1996) observed that direct supercritical extraction of cholesterol over AMF is not feasible because of the low selectivity of cholesterol and poor solubility of AMF. Moreover, under these conditions, important milk flavours also get separated with the cholesterol. Therefore they proposed another process for cholesterol removal from AMF, dissolved in SC-CO_2 under high solubility conditions for AMF (40 MPa at 700℃) to achieve rapid extraction. In this process, the dissolved AMF in SC-CO_2 is then passed isobarically and isothermally through a high-pressure column, filled with a suitable adsorbent (e.g. silica gel) to eliminate cholesterol. Finally, the supercritical mixture is fractionated by either descending or ascending temperature profile in separators connected in series.

d) Reaction with Cyclic Anhydride

Gu *et al*. (1994) developed a method for cholesterol removal from milk fat based on the reaction between the hydroxyl group of cholesterol and a cyclic anhydride such as succinic anhydride. The conversion of cholesterol into an acid derivative makes it possible to remove these from fats by extraction with aqueous alkali. Addition of acetic acid increases the rate of reaction and prevents the distillation of cyclic anhydride from reaction mixture. The process should be carried out in CO_2 atmosphere to prevent oxidation.

2.10.2 Chemical Process

Gu *et al*. (1994) described chemical method based on the reaction between cyclic anhydride and the hydroxyl group of cholesterol. The reaction forms monoesters with acryl chains having a terminal acid group. This process could effectively and economically remove up to 40 per cent of the cholesterol from animal fats. Wrezel *et al.* (1992) were issued a patent "Method for Removing Cholesterol from Edible Oils" based on a procedure that was similar to that of Gu *et al*. (1994).

2.10.3 Biological Processes

a) Cholesterol reductase

Cholesterol reductase is an enzyme that converts cholesterol to coprostanol which is poorly absorbed by humans. Dehal *et al*. (1991) investigated several cholesterol reductases produced by bacteria or by extraction from leaves of several green plants. They found that cholesterol reductase could convert cholesterol to coprostanol in milk, cream, ground beef and pork. They concluded that these reductases from bacterial or plant sources could be used to decrease the cholesterol content of foods.

b) Cholesterol Oxidases

Several workers (Christodoulou *et al.*, 1994) have reported that cholesterol in foods could be nearly eliminated by cholesterol-degrading enzymes produced by some bacterial strains. These enzymes have been used by Xiansheng *et al*. (1990).

2.10.4 Complexation Process

a) Saponins

Saponins from plants can also be used to selectively bind and precipitate cholesterol. Riccomini *et al.* (1991) reported an 80 per cent cholesterol reduction in cream by simply mixing cream and saponins for 1 hour at 65°C, filtering the mixture through Celite and washing with water. The same treatment on anhydrous butter fat produced a 90 per cent reduction in cholesterol. No adverse flavors could be detected in the final products. Micich *et al*. (1992) investigated polymer-supported saponins to improve the removal of cholesterol from butter oil. This new support was reusable and reduced the residual saponin in the butter oil. One g polymer-supported saponins can bind 3 mg pure cholesterol in hexane. It was also shown that cholesterol removal from butter oil (dissolved in hexane) was 10-40 per cent less efficient than with pure cholesterol.

b) β-cyclodextrin

Several studies have been reported on the removal of cholesterol from animal fats with β-cyclodextrin (Davidson, 1990; Ockenfull *et al*., 1991). Yen and Tsai (1995) studied cholesterol removal from a lard-water mixture with β-cyclodextrin, founded that about 90 per cent of cholesterol could be removed from lard.

β-Cyclodextrin, a cyclic Oligosaccharide of seven glucose units joined head to tail by β -1,4 linkage, is produced by the action of enzyme cyclodextrin glycosyl transferase (CGT) on hydrolyzed starch.

One drawback of this method is that it removes fat soluble vitamins also by binding them into hydrophobic core.

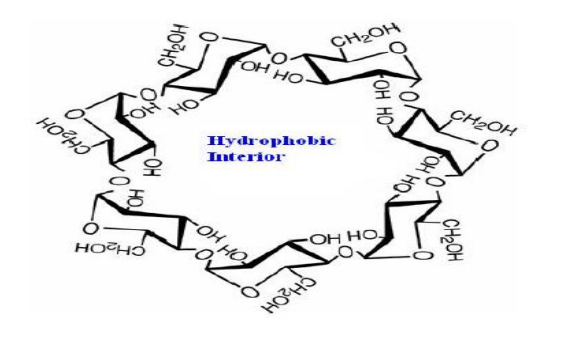

Figure 2.1 : Structure of β-Cyclodextrin

Seth and Singh (1994) observed that from buffalo milk samples on treatment with beta-cyclodextrin at 1, 2 and 4 per cent , the cholesterol level was reduced from 36 mg to 25 mg, 19.5 mg and 8 mg per 100 ml, respectively, corresponding to 30.5, 45.8 and 77.6 per cent reduction, respectively. Higher than 4 per cent β-cyclodextrin, did not show any beneficial effects.

Kwak *et al*., (2001) prepared Mozzarella cheese from milk treated with 1 per cent β-cyclodextrin followed by homogenization at pressure 70.0 kg/cm2 and temperature 700C for removal of cholesterol. He reported that meltability, stretchability and oiling-off in cholesterol-reduced cheese were significantly lower than those in control. Hardness, gumminess and chewiness were significantly reduced while cohesiveness and elasticity increased. Appearance and flavour of the cheese were superior, but texture inferior to the control. Kwak *et al*. (2003) reported that β-cyclodextrin treated milk with low pressure homogenization results to an effective cholesterol reduction and rapid cheese ripening with no capture of flavour compounds by β-cyclodextrin.

From the above review, it is evident that because of the non-toxic, edible, non-hygroscopic, chemically stable and easily separable properties of β-cyclodextrin, it has been used as a commercial tool for the successful and easier removal of cholesterol from milk & milk products in recent years. Therefore, a systematic study

was planned to explore the possibility of preparation of low-cholesterol ghee using β-cyclodextrin.

2.10.5 Adsorption Methods

Cholesterol can be removed by its adsorption on certain material. Adsorbents, which have been used to remove cholesterol, include activated charcoal, saponins or β-cyclodextrin.

2.10.6 Using Activated Charcoal

Bindal *et al*., (1994) could remove half of the cholesterol present in milk fat through treatment of liquid fat with activated charcoal. Another activated charcoal method claimed 95 per cent of cholesterol removal from AMF but many other compounds including yellow pigments were also removed simultaneously (Sharma *et al*., 1999).

2.10.7 Using Saponins

The methods using activated charcoal or saponins are relatively nonselective and remove flavour and nutritional components also when cholesterol is removed (Lee *et al*., 1999; Sharma *et al*., 1999).

2.11 HERBS AND THEIR IMPORTANCE

Herbs contain high amounts of phenolic compounds which possess great antioxidant properties. The natural antioxidant properties of herbs have made their use in the formulation of functional foods specifically targeted for the people suffering from CVD (Najgebauer *et al*., 2009).

The term herb is used loosely to refer not only to herbaceous plants but also to bark, roots, leaves, seeds, flowers and fruits of trees, shrubs and woody vines and extracts that are valued for their savory, aromatic or medicinal qualities. Herbal remedies are also a part of this renewed public interest in seeking health solutions through alternative approach to conventional medication. Herbal medicine is based on the concept that plants contain certain natural substances that can promote health and alleviate illness. Research interest has been focused on various herbs that possess hypolipidemic, anti-platelet, antitumor or immune – stimulating properties that may be useful adjuncts in reducing the risk of CVD and cancer. In different herbs, a wide variety of active phytochemicals including the flavonoids, terpenoids, lignans, sulfides, polyphenols, carotenoids, coumarins, saponins, plants sterols, curcumins, S-

methyl cysteine sulfoxide, Inulin, Mono and , oligosaccharides, Triterpine, Pectin, citrus , Gums , Fixed oil, volatile oil, Polyphenolic compounds, Flavonoids, Saponins, Metamucil and phthalides have been identified. In recent past these is rapid upsurge in usage and demand of herbal medicines and food preparation (Sawale *et al.,* 2012). Thirumalai *et al*.(2014) studied the hypolipidemic activity of betel leaves *in vivo*. They found that the methanolic leaf extract of betel leaves exhibited a significant hypolipidemic effect in high fat diet induced hyperlipidemic rat.

Anthocyanins are the water-soluble pigments responsible for the red, pink, mauve, purple, blue, and violet hues of many types of flowers and fruit. Hawthorn and juniper berries and rose hips are examples of herbs that contain anthocyanins. Because anthocyanins are effective in inhibiting LDL-cholesterol oxidation and platelet aggregation, these herbs may provide some protection against heart disease. Anthocyanins may also be useful for the treatment of vascular disorders and capillary fragility (Winston, 1999). Herbs that are rich in flavonoids, vitamin C, or the carotenoids may enhance immune function. The flavonoid rich herbs may also possess mild anti-inflammatory action (Winston, 1999). Several commonly used herbs have been identified by the National Cancer Institute as possessing cancer-preventive properties. These herbs include members of the *Allium* sp. (garlic, onions, and chives); members of the *Labiatae* (mint) family (basil, mints, oregano, rosemary, sage, and thyme); members of the *Zingiberaceae* family (turmeric and ginger); licorice root; green tea; flax; members of the *Umbelliferae* (carrot) family (anise, caraway, celery, chervil, cilantro, coriander, cumin, dill, fennel, and parsley); and tarragon (Winston, 1999).

Ghee carries the therapeutic properties of herbs to all the body's tissues. It is an excellent anupana (vehicle) for transporting herbs to the deeper tissue layers of the body (Lad, 1998).

2.11.1 Ajwain Leaves

Coleus aromaticus is a commonly available medicinal herb in India. The plant chosen for study *Coleus aromaticus*, belonging to the Family: Lamiaceae is grown as a household herb in Tamilnadu (vernacular Tamil name: ommam or ommavalli). This herb is native to East Indies and is widely cultivated in Africa and almost all tropical countries. It is also popularly known as "Indian Oregano" (Thilagavathi and Hariram,

2016). The search for antioxidants from natural sources has received much attention to replace the synthetic ones have shown antioxidant activities of the ethanol extract of *Coleus aromaticus*. Phenolic and polyphenol constituents namely carvacrol, flavonoids, osmarinic acid, caffeic acid and chlorogenic acid are reported to be responsible for antioxidant activity of *Coleus aromaticus* (Gulcin, 2006; Rasineni *et al.,* 2008). Three components showing strong DPPH radical scavenging activity were shown to be rosmarinic acid, chlorogenic acid, caffeic acid. Rosmarinic acid was major constituent for free radical scavenging potential. The broad spectrum of the *Coleus aromaticus* plants is therapeutic potential of antioxidant, antimicrobial, antitumor, and natural antibiotic was cured (Thilagavathi and Hariram, 2016).

2.11.2 Betel Leaves

Piper betle L., commonly called betel, belongs to the Piperaceae family. It originated from South and South East Asia. People in India, China, Taiwan, and South East Asia have used it as a traditional medicine due to its medicinal properties. Due to strong pungent aromatic flavour betel leaves are used as masticatory by the Asian people. Its common names are betel (in English), paan (in Indian), phlu (in Thai) and sirih (in Bahasa Indonesian). Grown abundantly in many parts of India, betel is an evergreen herb that needs warm and moist growth conditions for its growth (Kumari and Rao, 2015). The betel plant is an evergreen and perennial creeper, with glossy heart-shaped leaves and white catkin (Uddin *et al.,* 2015).

Betel contains saponins, flavonoids and polyphenols, essential oils hydroxyl chavicol, eugenol, ascorbic acid and beta carotene. The aqueous leaves extract of *Piper betle* L. has been reported to have antifungal activity, antibacterial activity and antioxidant activity. Betel leaves (*Piper betel*) are also known to contain significant amount of antioxidants like hydroxyl chavicol, eugenol, ascorbic acid and beta carotene (Risdian *et al.,* 2011).

Thirumalai *et al*. (2014) studied the hypolipidemic activity of betel leaves *in vivo*. They found that the methanolic leaf extract of betel leaves exhibited a significant hypolipidemic effect in high fat diet induced hyperlipidemic rat.

2.11.3 Coriander Leaves

Coriander (*Coriandrum sativum*) is an umbelliferous annual plant of the parsley family, native to the eastern Mediterranean region and southern Europe and is found

in many other parts of the world. It is valued for the dry ripe fruits, called coriander seeds and also the fresh green leaves called cilantro. The major compounds in the plants are tocopherols, carotenoids and chlorophylls and sugars, ascorbic acid, phenolics, flavonols, and anthocyanins (Dias *et al.*, 2011).

The ethanolic, methanolic, chloroform, ethyl acetate, and water extracts of coriander were found to have high total phenolic content with constituents like pyrogallol, caffeic acid, glycitin, etc. It has been reported by Angelo and Jorge (2008) that extracts from coriander serve as an excellent antioxidant which is stable even at high temperature and hence be a substitute for synthetic antioxidants.

2.11.4 Curry Leaves

Murraya koenigii commonly known as curry plant belongs to the family Rutaceae. The plant is a medicinal plant and native to India, Sri Lanka and other South Asian countries. It is usually found in tropical and sub-tropical region and cultivated in China, Australia, Nigeria etc. The plant is highly valued for its leaves which are used for flavouring and spicing of food. The curry leaf is believed to have several medicinal properties such as anti-diabetic, anti- oxidant, antimicrobial, anti-inflammatory, anticarcinogenic and hepatoprotective properties. Curry leaves have the richest source of carbazole alkaloids such as koenigine and mahanimbine extracted from the leaves which have been found to demonstrate anti-cancer and anti-oxidant properties **(**Igara *et al.*, 2016).

Lalwani *et al.* (2014) reported the total phenolic content 24.44±0.12 mg GAE per g of ethanolic extract curry leaves. Ishtiaque *et al.* (2015) determined the total phenolic content and antioxidant activity of methanolic extract of *Murraya koenigii* (curry leaves). Result from their study indicated that curry leaves contained 170 mg GAE per 100g total phenolic content and 20-64 per cent DPPH activity.

2.11.5 Fenugreek Leaves

Fenugreek (*Trigonella foenum graecum*) is an annual plant belongs to the family (leguminosae). Native to India and Southern Europe and the Mediterranean region, the plant is cultivated in central and southeastern Europe, western Asia, India, and northern Africa.

The leaves contain seven saponins, known as graecumins. These compounds are glycosides of diosgenin (Wani and Kumar, 2016). Alkaloids such as trigocoumarin,

nicotinic acid, trimethyl coumarin and trigonelliene are present in leaves (Snehlata and Payal, 2012). Yadav and Sehgal (1997) found that fresh leaves of fenugreek contain ascorbic acid of about 220.97 mg per 100g of leaves and β-carotene is present about 19 mg/ 100g.

The hypoglycemic and hypolipidemic effects of fenugreek *(T. foenumgraecum L.*) are well documented in animal studies and these observations have been translated to humans. For example, in a clinical trial study with 24 Type 2 diabetic patients supplemented with 10 g/day powdered fenugreek seeds mixed with yoghurt or soaked in hot water for 8 weeks, it was found that supplementation with fenugreek (seeds soaked in hot water) resulted in significant decreases in fasting blood glucose, TGs and VLDL cholesterol by 25, 30 and 30.6 per cent, respectively. (Tappia *et al*., 2013)

In Ayurveda, both fenugreek leaves and seeds are used to prepare extracts or powders for medicinal use (Basch *et al.,* 2003). Premanath *et al.* (2011) revealed that the level of total phenols in the ethanolic extract of fenugreek leaves was 4.9±0.25 mg per g and 59.7±0.46 per cent DPPH activity.

2.11.6 Liquorice/ Licorice

Licorice is one of the oldest and most popular herbal medicines in the world are reduced in the pharmacopoeias of many countries. The *Glycyrrhiza* genus contains about 30 species, and is widely distributed all over the world.

Flavonoid rich fractions includes liquirtin, isoliquertin liquiritigenin and rhamnoliquirilin and five new flavonoids- glucoliquiritin apioside, prenyllicoflavone A, shinflavanone, shinpterocarpin and 1-methoxyphaseolin isolated (Rastogi and Mehrotra, 1994; Roshan *et al.,* 2012).

Four new isoprenoid - substituted phenolic constituents – semi licoisoflavone B, 1-methoxyficifolinol, isoangustone A and licoriphenone isolated from licorice (Rastogi and Mehrotra, 1994). The presence of many volatile components such as pentanol, hexanol, linalool oxide A and B, tetramethyl pyrazine, terpinen-4-ol, α-terpineol, geraniol and others in the roots is reported. Presence of propionic acid, benzoic acid, ethyl linoleate, methyl ethyl ketine, 2, 3-butanediol, furfuraldehyde, furfuryl formate, 1-methyl-2 formylpyrrole, trimethylpyrazie, maltol and any other compounds is also isolated from the essential oil (Roshan *et al.,* 2012).

The potential of licorice extract as an antiatherosclerotic agent has been further examined in hypercholesterolemic patients (Fuhrman *et al.,* 2002). In this study, supplementation of licorice root extract (0.1 g/day) was given to patients for one month. It was found that licorice supplementation resulted in a reduction in LDL susceptibility to oxidation and increased the resistance of plasma LDL to oxidation. In addition, it was revealed that plasma cholesterol levels were reduced by 5 per cent, plasma LDL‑C levels were decreased by 9 per cent and plasma triacylglycerol levels by 14 per cent. Of note, after 1 month of placebo administration, these parameters reversed toward baseline levels. Interestingly, licorice extract supplementation also reduced systolic blood pressure by 10 per cent, which was not reduced by placebo consumption. It was concluded that dietary supplementation of licorice-root extract by hypercholesterolemic patients may act as a moderate lipid-lowering nutrient and a potent antioxidant agent and, thus, may be beneficial in CVD (Fuhrman *et al.,* 2002) through attenuation of production of the atherogenic LDL oxidation. It shows a variety of pharmacological activities, including antiulceric, antioxidative, antiallergic and antiviral (El-sherif *et al.,* 2011).

2.11.7 Mint

Mentha citrata is a member of family *Lamiaceae* and is a popular plant in Egypt and Arabic countries and is frequently consumed in form of hot beverage. A tea made from the fresh or dried leaves of *Mentha citrata* has traditionally been used for stomach aches, nausea, parasites and other digestive disorders and for fevers and headaches.

In the oil of *M. citrata*, 27 compounds were identified, representing about 96.72 per cent of the whole oil. The oxygenated monoterpenes was the dominant class, accounting for 74.02 per cent of the total oil followed by the monoterpenes (14.57 per cent), sesquiterpene hydrocarbons (5.58 per cent) and caryophylene oxide (2.55 per cent) which was the only identified oxygenated sesquiterepene hydrocarbon. The major components identified in the oil were, linalyl acetate (35.01 per cent), linalool (20.99 per cent) and limonene (5.57 per cent) (Al- okbi *et al.,* 2015).

Linalool was the major constitutes (59.76 per cent) in the oil of *M. citrata* grown in India followed by linalyl acetate (18.4 per cent), nerol (2.0 per cent), trans-p-menth-1-en-2-ol (1.8 per cent), α- terpineol (1.5 per cent) and limonene (1.1 per cent) (Al-juhaimi and Ghafoor, 2011).

2.12 ANTIHYPERLIPIDEMIC ACTIVITY OF CERTAIN MEDICINAL PLANTS AND THEIR PARTS

Hyperlipidemia is the condition of abnormally elevated levels of any or all lipids that include cholesterol, cholesterol esters, phospholipids and triglycerides in the blood. Numbers of plant species are reported to possess anti-hyperlipidemic activity. Some of them are mentioned in Table 2.1

Table 2.1 List of medicinal plants having Hypolipidemic activity

Plant name	Part
Abelmoschus esculentus	Whole plant
Achyranthus aspera	Roots
Aegle marmelos	Leaf
Allium cepa	Fresh bulbs
Allium sativum	Fresh fruits
Alstonia scholaris	Leaves
Amaranthus viridis	Leaves
Andrographis serpyllifolia	Roots
Anethum graveolens	Essential oil
Asparagus racemosus	Roots
Bauhinia variegata	Roots & Stems
Carica	*papaya* Seed
Cassia fistula	Legume
Catharanthus roseus	Leaves
Curcuma longa	Rhizome
Cymbopogon citrates	Leaves
Eclipta prostate	Leaves
Garcinia combogia	Peel of matured fruits
Glyccyrrhiza glabra	Roots
Hibiscus rosa sinesis	Root
Morus alba	Leaves
Nelumbo nucifera	Leaves
Ocimum basilicum	Whole plant
Pongamia pinnata	Pods,flowers
Ruta graveolens	Leaves,Whole plant
Terminalia chebula	Pericarp
Vernonia anthelmentica	Seeds
Withania somnifera	Roots and Leaf
Zingiber officinale	Rhizome
Source: Geetha (2013). *Studies on Anti-hyperlipidemic and Anti-atherosclerotic Activities of Selected Indian Medicinal Plants*.(Doctoral thesis, Andhra University, Visakhapatnam, India).	

2.13 METHODS FOR ESTIMATION OF CHOLESTEROL

Increasing awareness about health consciousness among consumers, formulation of mandatory guide lines which include the declaring of nutritional facts on the label of food packet have stressed much emphasis on estimation of cholesterol in food products. A number of methods are available in the literature and many of these are being tried in clinical and food laboratories. Most of these methods for cholesterol estimation were initially developed for blood plasma and then applied to foods. Various analytical approaches employed for cholesterol estimation are listed bellow and discussed in subsequent sections.

2.13.1 Gravimetric Methods

Windous (1910) presented a reaction between alcoholic solution of cholesterol and alcoholic solution of digitonin (C56H92O29) which results in precipitation of 1:1 molecular complex of cholesterol and digitonin as cholesterol digitonoids.

$$\mathbf{C_{56}H_{92}O_{29} + C_{27}H_{46}O \longrightarrow C_{56}H_{92}O_{29} \cdot C_{27}H_{46}O}$$

This reaction became a platform for several gravimetric methods of estimating cholesterol in fats and oils. Bloor (1916) observed that saponification was not necessary in the above method, as cholesterol in both forms gives colour. He suggested a method for extraction of cholesterol but the results obtained by his method gave much higher result than the Windous method (1910).

2.13.2 Colorimetric Methods

(a) Liebermann-burchard (LB) reaction method

Bindal and Jain (1973) applied modification suggested by Reinhold and Shiels (1936) for estimating cholesterol in blood serum/plasma, for the determination of total cholesterol in ghee and found it to be very successful.

Ghee (0.2 to 0.25) g of ghee dissolved in chloroform (3 ml), 4 ml L.B. reagent (acetic anhydride: H_2SO_4:: 20:1, freshly prepared at 0^oC) was added and the mixture incubated at 25^oC for 12 min. The optical density was recorded at 650 nm in the colorimeter, taking care that the measurements were completed within 15 min after the addition of LB Reagent. The amount of cholesterol was calculated by plotting a standard curve, prepared under same conditions simultaneously. They compared their results with that of gravimetric method of Windaus (1910) and found that the method

gave comparable result with gravimetric method. Cholesterol content in cow and buffalo ghee was found to be 310 and 270 mg per 100 g of ghee, respectively.

(b) *o*-Phthaldehyde method

Bachman *et al*. (1976) used *o*-phthaldehyde method for determination of cholesterol in dairy products. Accurately weighed dairy products were saponified and unsaponifiable matter was extracted using 10 ml of hexane. An aliquot (4ml) of hexane extract was evaporated and 4 ml of *o*-phthaldehyde reagent (50 mg/100 ml in glacial acetic acid) was added and mixed properly. After 10 min, 2 ml of concentrated H_2SO_4 was added and absorbance was taken after 10 min at 550 nm. These results were compared with GLC method for cholesterol estimation and found good agreement between the two methods. Using this method, they reported 13.2, 46.1, 104.1, 95.3, 97.4, 78.4 and 24.2 mg of cholesterol per 100 g of sample in milk, vanilla ice-cream, cheddar, swiss, processed cheese and Creamed Cottage Cheese, respectively. Recovery of the method was found to be 99.79 per cent.

2.13.3 Chromatographic Methods

(a) Gas liquid chromatographic methods

Cholesterol determination by gas liquid chromatography (GLC) is usually more accurate than colorimetric procedures (Vanzetti, 1964). Numerous studies have been done on GLC method of cholesterol determination, but in all cases a trimethylsilyl (TMS) ether derivatization of sterols is involved which is time consuming**.** In the past, most studies focused on a quick, accurate and simple method to analyze cholesterol. For example, gas chromatography (GC) with capillary columns, in many cases, a method for routine analysis of sterols, especially for cholesterol (Itoh *et al.*, 1973).

Punwar (1975) developed a widely used approach for determining cholesterol in foods. The method involved lipid extraction, saponification and silyl derivatization followed by estimation through gas chromatography. However, the lipid extraction and saponification steps in this method were cumbersome, the derivatizing reagents were unstable and cholesterol was thermally decomposed in the GC column (Kovacs *et al*., 1979).

To overcome these problems, Kovacs *et al*. (1979) proposed a method in which derivatization step of cholesterol was removed. They used a gas chromatograph equipped with flame ionization detector (FID) and glass column packed with Gas

Chrom Q and coated with 3 per cent OV-17. The column was operated at 230℃ using helium as carrier gas at a flow rate of 40 ml/min. Injector and detector temperature were 235°C & 240℃ respectively. For quantification purpose, 5α-cholestane was used as the internal standard and all sterols were identified by comparing their retention times with authentic standards. Prior to GLC analysis, sterols were saponified and extracted into hexane.

Tsui (1989) developed a method for rapid determination of total cholesterol in homogenized milk. The milk sample was saponified in ethanolic KOH in the presence of an internal standard, 5α-cholestane. Cholesterol and cholestane were then isolated by solid phase extraction on a non-polar absorbent and eluted with organic solvent. The evaporated extract was derivatized and analyzed by capillary gas chromatography. Average recovery of cholesterol added to milk prior to saponification was 95 per cent and the average relative standard deviation for repeated analyses was 2 per cent. The limit of detection for this method was 2 mg per 100 g.

Fletouris *et al*. (1998) developed a rapid method to determine cholesterol in milk and milk products by direct saponification and capillary gas chromatography. 0.2 g of samples was saponified with 0.5 *M* methanolic KOH solution by heating for 15 min at 80°C with intermittent vortexing for 10 sec. after every 5 min. To extract unsaponifiable fractions, 1 ml water and 5 ml hexane were added and the contents were vortexed vigorously for 1 min and then centrifuged for 1 min at 2000 x *g*. An aliquot of the upper phase was transferred into the auto sampler GC. The conditions maintained in the GC were, oven temperature at 285°C, injection port temperature at 300°C, and flame ionization detector temperature at 300°C. The flow rates were 2 ml/min for helium, 30 ml/min for hydrogen, and 300 ml/min for air. The injection volume was 1 ml with a split ratio of 20:1. Because of the rapid sample preparation and gas chromatographic procedures, a single sample could be analyzed in 30 min. Overall recovery was 98.6 per cent, and the linearity was excellent for the fortification range examined. .

Standard method of AOAC (2005) for estimating cholesterol in foods involves saponification, extraction, derivatization, and GC analysis. In the method, 2-3 g of sample was saponified with 40 ml of 95 per cent ethanol and 8 ml of 50 per cent KOH solution by heating on water bath for 70 ± 10 min. Unsaponifiable matter was extracted into 100 ml toluene and it was washed with 110 ml of 1 M KOH, 40 ml 0.5

M KOH, and 40 ml water (washing with water was carried out for 3 times), respectively. Then the toluene layer was passed through Na2SO4 and the solvent layer was allowed to stand for 15 min. The sterols present in the solvent layer were derivatized to trimethylsilyl esters (TMS) using dimethylformamide(DMF), hexamethyldisilane (HMDS), and trimethylchlorsilane (TMCS). For quantification purpose, 5α-Cholestane was used as internal standard. In GC analysis, 1 µl of test portions was injected into capillary column and hydrogen flame ionized detector was employed to detect the sterol peaks. The operating conditions maintained were; injector temperature 250°C, detector temperature 300°C, and column temperature 190°C.

(b) High performance liquid chromatographic (HPLC) methods

Oh *et al*. (2001) developed a sensitive high-performance liquid chromatographic method to determine the cholesterol content in milk and milk products. They compared various extraction process and various mobile phases for separating cholesterol by HPLC in order to develop a simple and accurate method. They observed that solid phase extraction (SPE) had a high recovery with shorter extraction time and the process was highly reproducible in comparison to liquid-liquid extraction. Further, acetonitrile: methanol: 2-propanol was found to be superior to the other mobile phase systems for separating cholesterol. HPLC was performed using a multisolvent delivery pump equipped with a 20 µl injection loop and a C18 column. Maintaining the flow rate of mobile phase at 1.6 ml/min, elution of cholesterol was monitored at 205 nm using UV detector method, again, accurately predicted the cholesterol content in a variety of dairy products.

2.13.4 Methods Based On Infra-Red Spectroscopy

Paradkar and Irudayaraj (2002a, b) developed two methods based on principle of FT-IR and FT-NIR spectroscopy for determination of cholesterol in dairy products. In FT-IR method, 1.5-2.5 g of sample was mixed with 9 ml of ethanol and 1 ml of potassium hydroxide solution (50 g per 100 ml) and vortex mixed for 20 sec. The capped tube was then placed in a water bath maintained at 60°C for 1 hr in order to have complete saponification and stirred continuously at 200 rpm. After cooling to room temperature, 5 ml of deionized water and 10 ml of hexane were added and vortexed for approximately 2 min. The sample was then centrifuged for 3 min at 2000

rpm and the hexane layer was transferred into a clean tube. Another 10 ml of hexane was added to the aqueous phase. The extraction and centrifugation steps were repeated. The combined hexane extract was then used for cholesterol determination. The hexane extract was evaporated to dryness and the sample was re-dissolved in 5 mL of chloroform and used for FTIR analysis. They concluded that FTIR (mid-infrared) spectroscopy was successfully used for the rapid estimation of cholesterol in commercial dairy products such as milk, milk powder, yoghurt, cheese, grated cheese and butter. The Partial Least Square (PLS) calibration model in the region between 2800 cm-1 and 3200 cm-1 was found to be the most accurate ($R2$ = 0.99). The method developed was fast and less expensive than the conventional method and could be accomplished in less than 5 min.

The FT-NIR spectrum of pure cholesterol was also characterized and the most significant region found to be between 3550 and 3650 cm-1. Cholesterol was extracted as in FTIR spectroscopy and then subjected to FT-NIR. This method, again, accurately predicted the cholesterol content in a variety of dairy products.

2.13.5 Electrophoretic Method

Xu *et al*. (2002) described a simple non-aqueous capillary electrophoresis (NACE) method for the rapid quantification of cholesterol in egg yolk and milk. The samples were subjected to saponification and then quantified by NACE, in which 100 mM sodium acetate-acetic acid in methanol was employed as the running buffer. The correlation coefficient between the cholesterol concentration and the corresponding peak area was 0.999. The detection limit of cholesterol was 5 g/ml (twice the signal-to-noise ratio). They recommended this method as a routine method for the rapid and sensitive determination of cholesterol in foods.

2.13.6 Enzymatic Method

Generally, the enzymatic measurement is more specific to cholesterol than the colorimetric method, and also expensive analytical instruments are not required. The cholesterol content in the ghee samples were estimated using method developed by Fletouris *et al.* (1998).

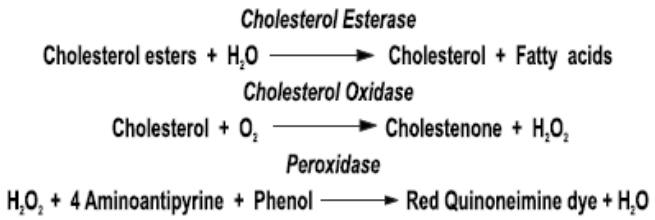

Figure 2.2 Principle of cholesterol estimation by enzymatic kit

The method comprises of saponify the ghee samples followed by extraction of unsaponifiable matter and then the colour developed by using enzymatic diagnostic kit (Makwana, 2007).

CHAPTER – 3

MATERIALS AND METHODS

The material used and the methodology employed in the present study are dealt in this chapter.

3.1 MATERIALS

Particulars regarding different chemicals, glasswares and instruments used in this study are delineated here.

3.1.1 Chemicals

All the chemicals used in the present study were of analytical (AR) grade supplied by authorized dealers. For cholesterol estimation cholesterol test kit (Arkray Healthcare Pvt. Ltd., Surat, India) was used. The kit contained cholesterol monoreagent 1(comprised of cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine) and cholesterol standard reagent 2(comprised of cholesterol standard 200mg/dL).

3.1.2 Glasswares

All glasswares used in the study were of standard quality supplied by authorized dealers.

3.1.3 Equipment

Equipment used includes hot air oven (SAIF Surgical & Scientific Equipment, Anand), hot plate (Macro Scientific Works Pvt. Ltd., Delhi), digital incubator (NOVA Instruments Pvt. Ltd., Ahmedabad), analytical balance (Reptech, Ahmedabad and Denver Instrument, Germany), water bath (SAIF Surgical & Scientific Equipment, Anand), butyrorefractometer (ATAGO, Japan), centrifuge (Labline, Ahmedabad), vortex mixer (Labline, Ahmedabad) and spectrophotometer (SYSTRONICS, Ahmedabad).

3.1.4 Herbs

Seven herbs including ajwain leaves (*Coleus aromaticus*), betel leaves (*Piper betle*), coriander leaves (*Coriandrum sativum*), curry leaves (*Murraya koenigii*), fenugreek leaves (*Trigonella foenum graecum*), liquorice (*Glycyrrhiza glabra*), mint (*Mentha*

citrata), were brought from Directorate of Medicinal and Aromatic Plants Research, Anand Agricultural University, Anand as well as The Directorate of Medicinal and Aromatic Plants Research, Boriyavi, Gujarat, India. Fenugreek leaves and coriander leaves were brought from local market. The herbs were manually cleaned to remove dirt and damage parts. The herbs were vacuum tray dried at 40 ±5 °C (Make: Perfect Engineering and Allied Works Pvt Ltd., Baroda, INDIA) at the vaccum of 650 mm of Hg for 4-5h, installed at Anubhav Dairy, S.M.C. College of Dairy Science, Anand Agricultural University, Anand. After drying, samples were manually crushed into coarse size particles and transferred in to zip lock plastic cover, then put in air tight plastic bottle and stored at room temperature.

3.2 PREPARATION OF GHEE

White butter was procured from a commercial dairy plant (Amul Dairy), Anand, Gujarat. Ghee was prepared by creamery butter method (De, 2004) in our laboratory. The white butter was taken in a stainless steel vessel (pan) and clarified (120 ^{o}C for no hold) into ghee with continuous stirring. The clarified fat was allowed to cool at room temperature until it attained a temperature of 80 ^{o}C and filtered through six folds of muslin cloth in dry glass beaker (2000 ml).

3.3 COMPATIBILITY ASSESSMENT OF HERBS FOR USE IN GHEE

Ghee was prepared as per the procedure discussed in section 3.2 and prepared ghee was divided into eight portions of 100g each. To portion 1 nothing was added which was served as control. To the remaining seven portions, above listed herbs (section 3.1.4) i.e. ajwain leaves, betel leaves, coriander leaves, curry leaves, fenugreek leaves, liquorice and mint were added at the rate of 0.5 per cent (w/w) respectively. The mixtures of ghee and herbs were thoroughly mixed using glass rod and allowed to stand at 80°C for 30 min. in a hot air oven. The treated ghee samples were filtered through 6 layers of muslin cloth. The resultant ghee samples were subjected to sensory evaluation using flavour score for acceptability. The 9 point hedonic scale was used. The samples were evaluated for their flavour (9-like extremely, 5-neither like nor dislike and 1-dislike extremely). Three replications were conducted for evaluating compatibility of selected herbs as additive in ghee. The samples of ghee considered as unacceptable when flavour score became < 5 in sensory evaluation.

3.4 EVALUATION OF COMPATIBLE HERBS FOR CHOLESTEROL LOWERING CAPACITY IN GHEE

The compatible herbs which were selected from section 3.2 were analyzed for cholesterol lowering capacity. The selected herbs were added at the rate of 0.5 per cent (w/w) in ghee. The mixtures of ghee and herbs were thoroughly mixed using glass rod and allowed to stand at 80ºC for 30 min. in a hot air oven. The treated ghee samples were filtered through 6 layers of muslin cloth. The resultant ghee samples were subjected to cholesterol content. The herbs giving more cholesterol lowering capacity were selected for further study. Three replications were conducted for evaluating the compatibility of herbs in ghee. In this phase ajwain leaves, betel leaves and curry leaves were selected as per the results obtained.

3.5 OPTIMIZATION OF THE HERBS FOR APPLICATION IN GHEE

The herbs used in ghee were optimized using various criteria such as stages, forms, rate of addition as well as duration of heating.

3.5.1 Selection of Stage to Add Herbs in Preparation of Ghee

For selection of stage for addition of herbs in preparation of ghee, the selected herbs (ajwain leaves, betel leaves and curry leaves) were added at three different stages during manufacture of ghee: (stage 1) Addition at the initial stage of heat clarification of butter (in melted butter), (stage 2) Addition at the final stage of heat clarification of butter (105ºC temperature) and (stage 3) In hot ghee at 90 ºC and allow it to cool at 60ºC.

For evaluating three different stages in preparation of ghee for treatment with the selected herbs (@ 0.5 per cent) (and without herb as control), the sample of butter (50 g) was taken into 250 ml glass beakers. The beakers containing butter were arranged in round shaped sand bath in such a way that each beaker remained at equal distance from the center of the sand bath. The sand bath was heated by gas fired burner with flame at the center.

In stage 1, when butter was melted completely, ajwain leaves, betel leaves and curry leaves were added in 3 beakers. For stage 2, ajwain leaves, betel leaves and curry leaves were added separately when butter temperature reached to 105°C (nearer to heat clarification). In both the stages, heating was continued till temperature reached

to 120°C. In third stage herbs were added in hot ghee at 90 ºC and allow it to cool at 60ºC. One sample in each stage was not treated with any herb to serve as a control.

In the entire process (from beginning to end) for preparation of ghee each samples were mixed gently with stainless steel spatula turn by turn. The content of each beaker was then filtered through 6 folded muslin cloths; ghee was collected in 150 ml glass beakers and analyzed for cholesterol content. Simultaneously, the samples were also monitored for flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted. The stages for addition of herbs giving better cholesterol reduction in ghee were selected for further study. The higher reduction in cholesterol content was observed in stage 3 (i.e. herb added in hot ghee at 90 ºC).

3.5.2 Selection of Form to Add Herbs in Preparation of Ghee

For selection of form for addition of herbs in preparation of ghee, the selected herbs (ajwain leaves, betel leaves and curry leaves) were added (@ 0.5 per cent) in four different forms in hot ghee at 90 ºC.

(i) As fresh herbs

- (a) Pieces
- (b) paste

(ii) Dried herbs

- (a) Coarse particles
- (b) Fine particles

For evaluating four different forms in ghee (Pieces, Paste, Coarse particles, Fine particles) for treatment with the selected herbs, were added in hot ghee at 90 ºC in four different forms and allow it to cool at 60ºC. The one sample was not treated with any herb to serve as a control. The samples were kept for 20 minutes at 60 ºC in hot air oven. The content of each beaker was then filtered through 6 folded muslin cloth, ghee was collected in 150 ml glass beakers and analyzed for cholesterol content. Simultaneously, the samples were also subjected for flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted. The form of addition of herbs giving better cholesterol reduction and compatibility in ghee was selected for further study. The form of coarse particles gave better result.

3.5.3 Optimization of Rate for Addition of Herb in Ghee

The selected herbs (ajwain leaves, betel leaves and curry leaves) in the form of coarse particles were added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 per cent in ghee. The herb treated ghee was prepared as per the procedure described in section 3.5.1 (i.e. in hot ghee at 90 ºC). The sample of ghee without addition of herb was also prepared to serve as control. The ghee samples were analyzed for cholesterol content. Simultaneously, the samples were also subjected for flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted. The 0.5 per cent rate of addition of the coarse particles gave better result in this phase.

3.5.4 Optimization of Duration of Exposure

The herbs were added in hot ghee at 90 ºC and allow it to cool at 60ºC. The one sample was not treated with any herb to serve as a control. The samples were kept for 15, 30 and 45 minutes at 60 ºC in hot air oven. The sample of ghee without addition of herb was also prepared to serve as control. All fresh ghee samples were analyzed for cholesterol content as well as flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted.

3.6 SENSORY EVALUATION

All the samples of ghee made in laboratory were evaluated for their sensory characteristics on a 9 point hedonic scale. The samples were evaluated for their flavour score. The 9 point hedonic scale score card for sensory analysis is given in following table.

Table 3.1 Flavour score of ghee using 9 point hedonic scale

Hedonic rating	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Sensory score card for ghee on 9 point hedonic scale

Sensory attribute	1	2	3	4	5
Flavour					

Comment if any:

Date: Name of panelist:

3.7 ESTIMATION OF CHOLESTEROL CONTENT USING ENZYMATIC DIAGNOSTIC KIT

The cholesterol content in the ghee samples were estimated using method developed by Fletouris *et al.* (1998). The method comprises of saponify the ghee samples followed by extraction of unsaponifiable matter and then the colour developed by using enzymatic diagnostic kit (Makwana, 2007).

(a) Saponification of ghee

The saponification of ghee was carried out using following flow diagram (Figure 3.1).

Ghee (0.1-0.15 g) in test tube with teflon lined screw cap

↓

Add 5 ml of (5 per cent) methanolic KOH and mix thoroughly

↓

Incubate the capped tubes in water bath for (90 °C/ 20 min) with

Intermittent shaking after every 5 min

↓

Cool the contents under tap water

Figure 3.1 Protocol for saponification

After saponification, add 1 ml of distilled water followed by the addition of 5 ml of hexane. Mix the contents thoroughly using vortex mixer for 1 min followed by centrifugation at 2000 rpm for 2 min. Transfer 0.2 ml of this extract in a dry test tube and evaporate at 75-85°C. Add 10 µl ethanol to dissolve dried unaponifiable matter.

After that add 1 ml cholesterol reagent 1. The standard was prepared using 1 ml cholesterol reagent 1 and 10 µl cholesterol reagent 2 another test tube. All the test tubes were then incubated at 37°C for 10 minutes. The colour intensity was measured at 505 nm using spectrophotometer and cholesterol content was calculated from following formula.

$$\text{Cholesterol (mg/100g)} = \frac{0.2 \times \text{OD of sample} \times \text{ml of hexane (5 ml)} \times 100}{\text{OD of std.} \times \text{ml of hexane aliquot (0.2 ml)} \times \text{wt of sample (g)}}$$

(0.02 is the concentration (mg) of cholesterol in 10 µl of standard solution)

Table 3.2 Protocol for cholesterol estimation using enzymatic diagnostic Kit reagent

Reagents	Blank	Standard	Test
Hexane Aliquot	-	-	0.2 ml and evaporate
Reagent 1: Cholesterol reagent	-	10 µl	-
Reagent 2: Working cholesterol standard Solution	1000 µl	1000 µl	1000 µl

3.8 ANALYSIS OF GHEE FOR QUALITY STANDARDS

The fresh samples of ghee prepared were analyzed for quality standards such as moisture content, free fatty acid content, Reichert Meissl value, Polenske value, Butyro-Refractometer reading at 40ºC and Baudouin test using the procedures described in IS: SP: 18 (part XI) (1981). Moreover, these samples of ghee were also stored at 40±2 ºC. The samples were analyzed for changes in cholesterol content, free fatty acids, peroxide value as well as flavour score at 5 days of interval. The details of the procedures for these parameters are discussed below.

3.8.1 Determination of Moisture

Ten grams of ghee sample was weighted in a clean dry aluminum dish. The dish with ghee sample was placed in hot air oven maintained at 105 ± 1ºC for approximately for 1 h. The dish was removed from the oven and cooled to room temperature in a desiccator. The dish was then weighed. The steps of heating, cooling and weighing were repeated after half an hour each time until the difference between the two successive weighing's did not exceed 1 mg.

The moisture content of ghee was calculated as follows:

Moisture content, per cent by weight = 100 (W1-W2)/W1-W

Where,

W1= Weight in g of the dish with ghee before drying

W2= Weight in g of the dish with ghee after drying, and

W= Weight in g of empty dish

3.8.2 Determination of Free Fatty Acids (FFA)

In a clean and dry 150 ml conical flask, 10 g of ghee sample was taken. In a second flask about 50 ml of ethyl alcohol (95 per cent v/v) was taken and brought to boil, while hot it was neutralized with 0.1 N sodium hydroxide to the end point of phenolphthalein (1 per cent solution in ethyl alcohol). The neutralized alcohol was poured on ghee in a flask, the contents were mixed thoroughly and brought to boil and still hot, the contents were titrated with 0.1 N sodium hydroxide. The end point was noted when the addition of a single drop produced a slight but definite pink colour persisting for at least 15 s.

The free fatty acids content was calculated as follows

Free fatty acids (per cent oleic acid) = (2.82 x T)/W

Where,

T= Volume in ml of 0.1 N Sodium hydroxide required for titration, and

W= Weight in g of ghee sample taken

3.8.3 Determination of Reichert-Meissl (RM) and Polenske Value

Accurately 5.0 g of sample was weighed into a Polenske flask and then saponified with 20.0 g of glycerol and 2.0 ml of 50 per cent (w/w) sodium hydroxide solution on a direct flame. Then 93 ml of freshly boiled distilled water were added followed by 50 ml of dilute sulphuric acid. The flask was immediately connected with the distillation apparatus and 110 ml of the distillate was collected within 21 minutes. The flask was replaced with 25 ml cylinder and the flame was removed. The distillate was cooled in a water bath maintained at 15°C for 10 min. Then it was filtered through a dry 9 cm Whatman No.4 filter paper and 100 ml of the filtered distillate were titrated against

0.1 N sodium hydroxide solution using phenolphthalein as an indicator. Similarly, a blank test was also done by using all reagents in similar fashion except fat sample. From this, the RM value was calculated as follows:

Reichert-Meissl Value = 1.10 (T1-T2)

Where,

T1 = Volume (in ml) of 0.1 N NaOH solution used for sample titration

T2 = Volume (in ml) of 0.1 N NaOH solution used for blank titration

For Polenske value, the condenser, 25 ml cylinder, 110 ml flask and the filter paper were washed with three successive washings of 15 ml portions of cold water followed by neutralized alcohol. The washings with neutralized alcohol were collected and then titrated against 0.1 N sodium hydroxide solution using phenolphthalein as an indicator. Similarly, a blank was also done. From this, the polenske value was calculated as follows:

Polenske Value = T3-T4

Where;

T3 = Volume (in ml) of 0.1 N NaOH solution used for sample titration

T4 = Volume (in ml) of 0.1 N NaOH solution used for blank titration

3.8.4 Determination of Butyro-Refractometer (BR) Reading at 40°C

Before determining the BR reading of a sample, the temperature of the samples was brought to 40 °C. The digital refractometer was calibrated with the standard provided by the company before taking the reading of the different samples. A drop of the molten fat sample was placed on the prism of the refractometer and digital readings were recorded.

3.8.5 Baudouin Test

To 5 ml of melted fat in a test tube, 5ml of concentrated hydrochloric acid (HCl) was added followed by 0.4ml of furfural solution. After stoppering the test tube it was vigorously shaken for 2 min. the mixture was allowed to separate in to different layers. Development of pink colour in the lower acid layer indicated presence of sesame oil as described in IS: SP: 18 (part XI) (1981).

3.8.6 Determination of Peroxide Value of Ghee

The peroxide value of ghee was determined by the method (iodometric method) as described by the Indian Standards Institution (IS: 3508-1966).

One gram of ghee sample was taken in a 150 x 25 mm test tube and 1 g of potassium iodide and 20 ml of the solvent mixture (prepared by mixing two volumes of glacial acetic acid, AR and one volume of chloroform, AR) was added. The contents were heated to boil within 30 sec in a boiling water bath and allowed to boil for not more than 30 sec. The test tubes were transferred to a 250 ml conical flask containing 20 ml of freshly prepared 5 per cent potassium iodide solution. The test tube was rinsed well with about 25 ml of distilled water and all washings were transferred to the above flask. The contents were titrated against 0.002 N Sodium thiosulphate solutions using 2 ml of starch indicator, near to end point. A blank was also performed.

The peroxide value of ghee was calculated as milliequivalents of peroxide oxygen per kg of ghee.

Peroxide value (milliequivalents of peroxide oxygen/kg of ghee) = 2T/W

Where,

T= Volume in milliliters of 0.002 N Sodium thiosulphate, and

W= Weight in g of sample

3.9 STATISTICAL ANALYSIS

The results were analyzed using Analysis of Variance (ANOVA). All tests were conducted at the 5 per cent significance level. The data obtained for each of the attributes under study were subjected to statistical analysis in completely randomized design (Steel and Torrie, 1980). The following statistical model was used for analysis.

$$\mathbf{Y_{ij} = \mu + T_i + R_j + C_{ij}}$$

Where,

Y_{ij} = response due to the ith treatment in j^{th} replication

μ = general mean

T_i = effect due to i^{th} treatment

R_i = effect due to j^{th} replication

C_{ij} = uncontrolled variation due to i^{th} treatment in j^{th} replication.

$$\mathbf{Y_{ijk} = \mu + A_j + B_k + (AB)_{jk} + E_{ijk}}$$

Where,

Y_{ijk}= Response from the jth and k^{th} receiving i^{th} replication

μ = General mean

A_j = Effect of A at j^{th} level (Treatment)

B_k = Effect of B at k^{th} level (Storage period)

$(AB)_{jk}$ = Interaction effect of A at j^{th} level and B at k^{th} level

E_{ijk} = Uncontrolled variation due to i^{th} replication in the jth and k^{th} level

CHAPTER - 4

RESULTS AND DISCUSSION

It is common knowledge that cholesterol ensures stability of cell membranes in the wide interval of temperatures. Cholesterol is necessary for production of different steroid hormones and vitamin D by adrenal glands and, according to the most recent figures, cholesterol takes significant part in the activity of cerebrum synapse and immune system, including cancer prevention. The high cholesterol level causes atherosclerosis. Atherosclerosis is generally associated with cardiovascular diseases such as strokes, heart attacks, and peripheral vascular diseases. It is advisable to maintain cholesterol level for proper functioning of the body. There are various methods and /or processes available in the literature for removal of cholesterol content in butter oil and foods in general. They generally categorized into physical, chemical, biological and complexation processes like vacuum steam distillation, short path molecular distillation, supercritical fluid extraction, use of various enzymes (like cholesterol reductase, cholesterol oxidases, etc), saponin and β-cyclodextrin. However, these processes are costly and have side effects due to use of chemicals. β-cyclodextrin not only remove cholesterol from ghee by binding it in its hydrophobic core but it also remove fat soluble vitamins. It causes loss to nutritive value of ghee. Other way to lower down the cholesterol level in body is using cholesterol lowering drugs. Since the usage of the synthetic drug, statins, leads to various side effects. The plants flavonoids with antiartherosclerotic activity gained much attention and were proven to reduce the risk of atherosclerosis *in vitro* and *in vivo* based on different animal models. The flavonoids compounds also exhibit lipid lowering effects and anti-inflammatory and antiatherogenic properties. Flavonoids are scientifically substantiated to have capability to regulate penetrability of blood vessel walls, to improve their elasticity, and to prevent sclerotic lesion with cholesterol. So there is need to develop natural way to reduce cholesterol by using herbs in the product itself. Till date, there is no report available to evaluate herbs for its cholesterol lowering capacity in ghee. The present study was undertaken to evaluate the effect of selected herbs on cholesterol level in ghee.

The first phase of the study dealt with the compatibility of herbs added in ghee. This was followed by the screening of herbs for cholesterol lowering property. The selected herbs, then, studied for optimization of processing parameters (viz. stage of addition, form of addition, the rate of addition and duration of heating exposure) and evaluating its effect on cholesterol level. Final ghee samples were subjected to its common physicochemical properties. The results obtained during these studies are presented and discussed in this chapter.

4.1 COMPATIBILITY ASSESSMENT OF HERBS USE IN GHEE

In the selection of herbs for cholesterol-lowering capacity, the first and foremost point for consideration was the compatibility of the herbs to be used for the study. Since, herbs were to be incorporated during preparation of ghee, some preliminary work required to assess their compatibility in ghee. The compatibility assessment of the herbs used in ghee was carried out by sensory evaluation using flavour score. Flavour of any edible substance is the most important sensory attribute, which significantly affecting acceptance or rejection of the product. It is said that in sensory evaluation of product its overall acceptability score and flavour score go hand-in-hand. The total seven commonly used herbs namely ajwain leaves, betel leaves, mint, coriander leaves, fenugreek leaves, liquorice and curry leaves were selected for the assessment of their compatibility in ghee. They were added at the rate of 0.5 per cent (w/w) in ghee. The resultant ghee samples were subjected to sensory evaluation using flavour score for their acceptability. The 9 point hedonic scale (9-like extremely, 5-neither like nor dislike and 1-dislike extremely) was used. The samples of ghee treated with different herbs were given to judges of the sensory panel to evaluate the samples of ghee for acceptability of their flavour in terms. The samples of ghee considered as unacceptable when flavour score became < 5 in sensory evaluation. The sensory evaluation based on flavour score is shown in Table 4.1

Table 4.1 Flavour score of ghee samples added with different herbs

Sr. No	Samples of ghee	Range	Average	Remark
1	Control	8.31-8.69	8.50±0.05	Acceptable
2	Ajwain leaves	7.56-8.31	7.92±0.10	Acceptable
3	Betel leaves	6.88-7.88	7.50±0.25	Acceptable
4	Mint	6.50-7.13	6.75±0.06	Acceptable
5	Coriander leaves	7.25-7.50	7.38±0.03	Acceptable
6	Fenugreek leaves	5.56-6.50	5.92±0.09	Acceptable
7	Liquorice	7.25-7.81	7.52±0.14	Acceptable
8	Curry leaves	7.63-7.50	7.56±0.03	Acceptable

Source of variation	Treatment (herbs)
SEm	0.20
CD (0.05)	0.60
CV %	4.72
Test	*

The result indicated that the flavour score of all ghee samples varied from 5.92 to 8.50. The flavour score of the ghee samples added with fenugreek leaves was lowest having the range from 5.56 to 6.50. The flavour score of control ghee sample varies from 8.31-8.69 representing highest score. The average flavour score of ghee samples added with betel leaves, liquorice and curry leaves were much closed. The flavour score of ghee samples obtained by sensory evaluation were >5.0 in all treated ghee samples. All the herbs were considered as compatible in flavour score and all seven herbs were selected for further study.

Patel and Rajorhia (1979) noticed that when betel (Piper betel) and curry (Murraya koeniji) leaves were added to butter during clarification into ghee, the samples of ghee were rated excellent at beginning of the experiment. The authors opined that the judges preferred ghee samples treated with betel and curry leaves. Kapadiya (2016) studied the compatibility of various herbs like ajwain leaves, betel leaves, mint, coriander leaves, fenugreek leaves, liquorice and curry leaves in ghee and found that

all the samples of ghee treated with herbs were acceptable for their flavour score. Therefore, the acceptability of the betel leaves and curry leaves treated ghee samples for flavour score were very well in corroboration with results reported by these authors.

4.2 EVALUATION OF THE HERBS FOR THEIR CHOLESTEROL LOWERING ABILITY IN GHEE

In the present study, the seven compatible herbs (ajwain leaves, betel leaves, mint, coriander leaves, fenugreek leaves, liquorice and curry leaves) were incorporated in ghee for evaluating cholesterol lowering ability in ghee. The coarse forms of the each herbs were added separately at the rate of 0.5 per cent (w/w) to the ghee samples. The ghee samples were prepared as mentioned in Section 3.4. Ghee sample without addition of any herbs was used as a control sample. The resultant samples were subjected to cholesterol estimation and the result is presented in the Table 4.2.

Table 4.2 Total cholesterol content of ghee samples added with herbs

Sr. No	Samples of ghee	Cholesterol content (mg/100gm)		% Reduction in cholesterol
		Range	Average	
1	Control	188.07-233.14	209.07±7.43	-
2	Ajwain leaves	199.00-207.00	200.33±2.14	4.63
3	Betel leaves	163.00-193.00	187.78±4.43	10.53
4	Mint	206.88-241.00	221.29±6.59	-
5	Coriander leaves	191.00-233.00	218.33±3.98	-
6	Fenugreek leaves	199.33-235.00	214.78±6.64	-
7	Liquorice	191.00-236.16	207.05±10.79	0.96
8	Curry leaves	182.00-218.00	199.67±5.39	4.50

Source of variation	Treatment (herbs)
SEm	11.57
CD (0.05)	NS
CV %	9.65

Data presented as means ± SD (n=3). NS: Non-significant

Upon careful examination of the data given in Table 4.2, it was found that ajwain leaves, betel, fenugreek, curry leaves and licourice were able to lower down the cholesterol content of the ghee. The per cent reduction in cholesterol contents observed in ghee samples were betel leaves (10.53 per cent) > ajwain leaves (4.63 per cent) > curry leaves (4.50 per cent) > and licorice (0.96 per cent).

However, cholesterol content was increased in ghee when treated with mint, fenugreek and coriander leaves. The increase cholesterol content might be attributed to the development of turbidity in the ghee samples even after filtration as well as doing the analysis of these three ghee samples. Though licorice has the ability to lower down the cholesterol content of the ghee samples it gave bit green colour to the ghee sample and it was not carried forward for further study.

There are no reports available on the use of herbs in oils/fat to lower down the cholesterol content. However, in vivo studies were carried out using various herbs for their hypolipidemic activity. Ghasi *et al*. (2000) reported that moringa leave extract at the concentration of 200 mg/ml for 30 days gave hypocholesterolaemic action in the rat. The potential of licorice extract as an antiatherosclerotic agent was studied in hypercholesterolemic patients (Fuhrman *et al*., 2002). In this study, supplementation of licorice root extract (0.1 g/day) was given to patients for one month. It was found that licorice supplementation resulted in the reduction of plasma cholesterol levels by 5 per cent. Kassaian *et al.* (2009) observed 30 per cent reduction in VLDL-cholesterol when fenugreek was soaked in hot water and gave to the patients. Jain *et al*. (2010) studied the hypolipidemic activity of Moringa oleifera in rats. They observed 6-38 per cent reduction in cholesterol level when the methanolic extract of moringa fed to rats at the rate of 150-600 mg/kg. Thirumalai *et al.* (2014) studied the hypolipidemic activity of betel leaves in vivo. They found that the methanolic leaf extract of betel leaves significantly lower down the total cholesterol content in the rat when high-fat diet was fed.

A number of epidemiological studies provide evidence that use of fruit and vegetables in our diet is associated with reduced risk of cardiovascular disease. This cardioprotective effect is thought to be due, at least in part, to minor components of fruit and vegetables called flavonoids (Cook and Samman, 1996). Borradaile *et al.* (1999) have shown that flavonoids and limonoids from citrus, as well as unextracted citrus juices, have cholesterol-lowering properties. James *et al.* (2007) and (Guthrie,

2007) suggested that both citrus flavonoids and palm tocotrienols reduce cholesterol levels (20-30 per cent) in laboratory animals. Sadovoy *et al.* (2013) studied the mechanism of interaction of red grape flavonoids with cholesterol. They have established that flavonoids like rutin, catechine, quercetin, resveratrol, epicatechin, and epicatechin-gallate form a stable complex in combination with cholesterol.

From the above listed seven herbs, the three herbs namely betel leaves, ajwain leaves and curry leaves have the ability to reduce the cholesterol content as well as compatible in ghee and these three herbs were selected for further study.

4.3 OPTIMIZATION OF THE HERBS FOR APPLICATION IN GHEE

To optimize the application of herbs in ghee, following three criteria were worked out:

(i) Stage of addition of herbs in ghee

(ii) Form of addition of herbs in ghee

(iii) Rate of addition of herbs in ghee

(iv) Duration of exposure of herbs in ghee

4.3.1 Stage of Addition of Herbs in Ghee

Dairy industry would abruptly stop without heating. Heating is very essential processing step in manufacturing of milk and milk products. It is well known that many properties of food can be significantly lost as a consequence of food processing such as sterilization, pasteurization and dehydration as well as during storage and handling and cooking (Nicoliy *et al*., 1999). Food processing involves changes in structural integrity of the plant material and this produces both negative and positive effects on their various properties. Thermal processes lead to an either decrease or increase phenolic compounds (flavonoid) which depends on their magnitude and duration. Gerard and Roberts (2004) observed 50 per cent increases in flavonoid content when apple juice was heated from 40°C to 70°C. A roasting of 130°C, 33 min increased the phenol content of cashew nuts (Chandrasekara and Shahidi, 2011); same results were noticed for peanuts (Yu *et al*., 2005). In these cases, an increase of temperature improved the extraction of phenolic compounds from foods; others results showed losses of phenolic compounds in different quantities. A loss of about 22 per cent in total flavonoids has been observed in boiled products at a temperature

of 50°C during 90s (Vina and Chaves, 2008). A few studies identified phenolic compounds in foods and followed their degradation during heat treatment. They noticed that individual phenolic compounds were also subjected to heat degradation. The identification and quantification of these compounds were performed with high performance liquid chromatography. Rutin in buckwheat groats was reported to be more stable to heat then vitexin, isovitexin, homoorientin and orientin during roasting at 160°C for 30 min (Zielinski *et al.*, 2009). Thermal pasteurization treatments (90°C for 60s) for strawberry juices have no effect on quercetin and kaempferol contents (Odriozola-Serrano *et al*., 2008), whereas it reduced naringin, rutin, quercetin and naringenin content for grapefruit juices (Igual *et al*., 2011).

However, the above results may not be comparable, because on the one hand, the food matrix is different from one assay to another and on the other hand, the food matrix can act as a barrier to heat effect or induce the degradation. It is not easy then to dissociate the thermal processing effect from the food matrix effects. Thus, some authors studied the effects of thermal processes on model solutions of phenolic compounds (especially on flavonoids). The data indicated that flavonoids in aqueous solutions show different sensitivity to heat treatment depending on their structures (Ioannou and Ghoul, 2012).

Lal and Narayanan (1980) reported that the direct addition of antioxidants to ghee was slightly less effective in suppressing the development of peroxides in comparison to that when added to butter before clarification into ghee. Ghee is obtained by clarification of milk fat at high temperature. Basically, the high heat applied to cream or butter for removal of moisture. Both are usually clarified at 110 to 120°C temperature. However, in southern India, clarification is carried out at higher temperature (120 to 140°C) (Ganguli and Jain, 1973). Exposure of the herbs to such a high temperature may adversely affect the stability of major and/ or minor flavonoid components present in the herbs. Even possibility also exists about interaction of phenolic compounds present in herbs with ghee residue, leading to change in cholesterol lowering property. There is possibility of improvement in extraction of these phenolic compounds due to their better leaching from the herbs to ghee at higher temperature.

Therefore, it is necessary to find out appropriate stage for addition of herb in process method adopted for preparation of ghee. From examination of manufacturing process

for ghee it can be envisaged that there are three possible stages to add herbs (@ 0.5per cent) into ghee as listed below.

(1) Stage 1: Initial stage of heat clarification (in melted butter).

(2) Stage 2: Final stage of heat clarification after reaching temperature near to 105°C.

(3) Stage 3: In hot ghee at 90°C and allow it to cool at 60°C.

An adoption of third possibility (stage 3) for treatment of ghee with herb (i.e. in hot ghee after the heat clarification and separation from ghee residue) avoids possibilities of loss of flavonoids due destruction by heat or interaction with ghee residue. Moreover, adopting the practice of adding herb in prepared ghee sample is technically the most appropriate in the study particularly when large number of samples to be treated alike, to avoid sample to sample variation in intensity of heat clarification.

The 3 different herbs namely ajwain leaves, betel leaves and curry leaves were added to ghee and analyzed for flavour score by sensory evaluation using 9-point hedonic scale as well as for cholesterol content of the ghee samples. Total four replications were conducted.

4.3.1.1 Effect of stages of addition of herbs on cholesterol content

The results of cholesterol content of ghee obtained from various stages of addition of herbs are shown in Table 4.3.

The cholesterol content of control ghee samples varies from 210.80 to 238.4 mg/100 gm with an average of 219.50 mg/100 gm of ghee in stage 1. The cholesterol content of ghee samples treated with ajwain leaves, betel and curry leaves were 221.42, 188.88 and 194.48 mg/100 gm of ghee samples respectively. The highest cholesterol reduction was observed in betel leaves (14.16 per cent) whereas ghee treated with ajwain leaves slightly increased the cholesterol content during study of stage 1.

In stage 2, the cholesterol content was marginally reduced in all the treated ghee samples. The per cent reduction in cholesterol content of ghee obtained in stage 2 was very closed and highest reduction was found only 5.97 per cent in ghee when treated with curry leaves i.e. reduced from 218.90 mg/100 gm (control) to 205.15 mg/100 gm (curry leave).

Table 4.3 Changes in cholesterol content of ghee upon treatment with different herbs in various stages

Herbs used to treat the ghee	Changes (%) in cholesterol content (mg/100gm) of ghee upon treatment with different herbs in various Stages						
	Stage-1		Stage-2		Stage-3		Mean (Herbs)
	Content	Changes	Content	Changes	Content	Changes	
Ajwain leaves	221.42±11.2	+0.91	207.47±10.54	-5.05	175.93±6.38	-17.46	201.61
Betel leaves	188.88±0.36	-14.16	208.75±9.16	-4.27	176.45±5.51	-16.99	191.36
Curry leaves	194.48±6.36	-11.42	205.15±8.26	-5.97	191.81±0.76	-11.77	197.15
Control	219.50±6.48	-	218.90±4.92	-	212.68±2.23	-	217.03
Mean (Stage)	206.07		210.07		189.22		

Source of variation	Herbs	Stage of addition	Stage of addition x herbs
SEm	3.98	3.45	6.90
CD (0.05)	11.47	9.94	19.88
CV (%)	6.22		

- : indicates decreased in cholesterol content in (%)
+ : indicates increased in cholesterol content in (%)

When data observed in the treated ghee samples obtained from Stage-3, the cholesterol contents was significantly reduced. The average cholesterol content was 212.68 mg/100 gm that decreased to 175.93 mg/100 gm in ghee added with ajwain leaves. Thus, highest reduction in cholesterol content of ghee found in descending order of ajwain leaves (17.46 per cent) > betel leaves (16.99 per cent) > curry leaves (11.77 per cent). Among the all stages, the maximum reduction in cholesterol contents was observed in Stage 3 and hence stage 3 was selected for further study.

4.3.1.2 Effect of stage of addition of herbs on flavour score

The result of flavour score of ghee obtained from various stages of addition of herbs are shown in Table 4.4.

The flavour score of the treated samples of ghee were acceptable in all the stages. It was observed that the flavour score of ghee added with curry leaves was lowest in all stages as compared to that of other treated herbs samples. Similarly, the highest scores were observed in ghee samples treated with ajwain leaves as well as curry leaves in all the stages. The highest reduction of cholesterol content and high flavour scores was observed in Stage 3 and hence, Stage 3 was carried forward for further study.

4.3.2 Addition of various Forms of Herbs in Ghee

The work was carried out to select the form of herbs added in ghee for evaluating its capacity to lower down the cholesterol content in ghee. From the result obtained in previous Section 4.3.1, it was decided to add herbs in hot ghee at 90^0 C for selection of the form in this Section.

The selected herbs (ajwain leaves, betel leaves, and curry leaves) were added in the form of pieces, paste, coarse and fine particles at the rate of 0.5 per cent of each in ghee samples. The surface area varies depending upon the forms of herbs. The various forms of herbs might be affected in extracting out the flavonoid components in ghee and ultimately affect the cholesterol-lowering capacity. Looking to these concepts, various forms of herbs were selected in this study. The sample of ghee without the addition of herb was also prepared to serve as the control. The ghee samples were analyzed for cholesterol content. Simultaneously, the samples were also monitored for changes in flavor score by sensory evaluation using the 9-point hedonic scale. Total four replications were conducted.

Table 4.4 Changes in flavour score of ghee upon treatment with different herbs in various stages

Herbs used to treat the ghee	Changes (%) in flavour score of ghee upon treatment with different herbs in various Stages			
	Stage-1	**Stage-2**	**Stage-3**	**Mean (Herbs)**
Ajwain leaves	8.25±0.02	8.06±0.04	8.33±0.04	8.21
Betel leaves	8.21±0.04	7.99±0.13	8.15±0.03	8.12
Curry leaves	7.60±0.05	7.60±0.21	7.90±0.04	7.7
Control	8.98±0.02	8.98±0.20	9.00±0.00	8.99
Mean(Forms)	8.26	8.16	8.35	

Source of variation	Herbs	Stage of addition	Stage of addition x herbs
SEm	0.050	0.043	0.086
CD (0.05)	0.143	0.124	NS
CV (%)	2.09		

4.3.2.1 Effect of various forms of herbs on cholesterol content of ghee

The results of the cholesterol content of ghee samples are presented in Table 4.5.

The data on cholesterol content of ghee samples indicated that the form of the addition of herbs was significant (P <0.05) but interaction effect of herbs with forms was non-significant. Upon careful examination from the data given in Table 4.5, it was found that the per cent reduction in cholesterol contents of ghee when treated with ajwain leaves in the form of coarse particles was highest (6.98 per cent), followed by pieces and paste. However, when ajwain leaves added in the form of fines particle, cholesterol content was increased slightly. Similarly, highest reduction in cholesterol content was also observed in both betel leaves (5.75 per cent) and curry leaves (14.7 per cent) when added in the form of coarse particles as compared to other three forms.

From Table 4.5, it is cleared that among all forms, the maximum reduction in cholesterol content was observed in coarse particles and hence coarse particles were selected for further phase.

4.3.2.2 Effect of form of addition of herbs on flavour score of ghee

The results for changes in flavour score of ghee samples are presented in Table 4.6

The data on flavour score of ghee samples indicated that the form of addition of herbs was statistically significant (P<0.05) but interaction effect of herbs and forms was non-significant. From the data, it is cleared that when coarse particles added in ghee gave highest flavour score in all treated ghee samples as compared to other forms of herbs.

Thus, from Table 4.5 and 4.6, it is cleared that among the all forms of herbs, the maximum reduction in cholesterol content as well as highest flavour score was observed in ghee added with coarse particles and hence coarse particles was selected for further phase.

Table 4.5 Changes in cholesterol content of ghee upon treatment with different herbs in various forms

Herbs used to treat the ghee	Changes (%) in cholesterol content (mg/100gm) of ghee upon treatment with different herbs in various forms									
	Paste		**Pieces**		**Coarse**		**Fines**		**Control**	**Mean (Herbs)**
	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	
Ajwain leaves	212.37±2.27	-1.40	208.41± 2.99	3.26	200.96± 2.89	-6.98	221.05±12.58	-2.79	215.93±5.16	211.75
Betel leaves	206.87± 2.82	-1.44	212.72± 6.22	1.43	197.82±0.89	-5.75	201.87± 3.03	-3.83	209.80± 1.27	205.82
Curry leaves	203.89± 3.40	-3.80	210.71± 4.01	0.48	180.86± 6.49	-14.7	208.70± 1.29	-1.43	211.01± 1.16	203.03
Mean (Forms)	207.71		210.61		193.21		210.54		212.24	

Source of variation	Treatment (herbs)	Form	Treatment (herbs)*Form
SEm	2.12	2.74	4.74
CD (0.05)	6.07	7.84	NS
CV (%)	4.59		

- : indicates decreased in cholesterol content in (%)
+ : indicates increased in cholesterol content in (%)

Table 4.6 Changes in flavour score of ghee upon treatment with different herbs in various forms

Herbs used to treat the ghee	Changes (%) in flavour score of ghee upon treatment with different herbs in various forms					
	Paste	**Pieces**	**Coarse**	**Fines**	**Control**	**Mean (Herbs)**
Ajwain leaves	7.80 ±0.07	7.83±0.04	8.91±0.00	8.39±0.03	9.00±0.00	8.39
Betel leaves	7.44±0.16	7.71±0.21	8.75±0.14	8.27±0.02	9.00±0.00	8.23
Curry leaves	7.60±0.06	7.96±0.02	8.87±0.02	8.21±0.02	9.00±0.0	8.33
Mean Forms)	7.61	7.83	8.84	8.29	9.00	

Source of variation	Treatment (herbs)	Form	Treatment (herbs)*Form
SEm	0.037	0.048	0.082
CD (0.05)	0.105	0.136	NS
CV (%)	1.98		

4.3.3 Optimization of Rate for Addition of Selected Herbs in Ghee

Ghee has the supreme status as an indigenous product in India because of its pleasing aroma and taste (Ganguli and Jain, 1973). The typical aroma and taste associated with herbs in turn adversely affect the organoleptic property of the products like ghee, because people are not accustomed with it. Therefore, it was essential to optimize rate of addition of the selected herbs used in treatment of ghee.

Until now the various herbs were added at the rate of 0.5 per cent of the expected yield of the ghee in the form of coarse particles in different phases like compatibility assessment of different herbs (Section 4.1), evaluation of herbs for their cholesterol lowering potential (Section 4.2) and selection of stage of addition of herbs (Section 4.3.1) in ghee. Now to optimize rate of selected herbs (ajwain leaves, betel leaves, curry leaves) for treatment of ghee, a concentration range of 0.1 to 0.6 per cent was chosen for the study.

The selected herbs (ajwain leaves, betel leaves and curry leaves) in the form of coarse particles were added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 per cent in ghee. Ghee was prepared as per the procedure described in section 3.3.3 (i.e. in hot ghee at 90 ºC). The sample of ghee without addition of herb was also prepared to serve as control. The ghee samples were analyzed for cholesterol content. Simultaneously, the samples were also subjected for flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted.

4.3.3.1 Changes in cholesterol content by different rate of addition of herbs

The results of cholesterol content of ghee samples are presented in Table 4.7

From the data, it was revealed that when ajwain leaves was added at different rates in the ghee samples, the cholesterol contents were changed. The average cholesterol content of the control ghee samples was 214.22 mg/100 gm which was changed to 223.06, 217.63, 231.49, 206.03 ,205.91 mg/100 and 212.22gm when ajwain leaves added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 % respectively. The cholesterol content was increased (1-7 per cent) initially with increased in rate of addition from 0.1 to 0.3 per cent. However, cholesterol content was reduced to maximum of 4.25 per cent at 0.5 per cent rate of addition of ajwain leaves. Simultaneously, the flavour score (Table 4.8), though in the acceptable limit, were decreased with increased in level of addition. Based on this, the rate of addition of 0.5 per cent of ajwain leaves was selected for further study

Table 4.7 Changes in cholesterol content of ghee upon treatment with different herbs in various rate

Herb used	Changes (%) in cholesterol content (mg/100gm) of ghee upon treatment with different herbs in various Rate													
	0.1%		0.2%		0.3%		0.4%		0.5%		0.6%		Control	Mean (Herbs)
	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	
Ajwain leaves	223.06 ±3.85	+4.20	217.63 ±3.71	+1.40	231.49± 10.75	+7.94	206.03 ±3.04	-3.74	205.91 ±3.69	-4.25	212.22 ±3.83	-0.94	214.24 ±2.07	215.80
Betel leaves	211.13 ±0.65	-1.41	213.36 ±0.85	-0.47	213.78± 1.52	-0.15	206.03 ±2.03	-3.77	204.3± 4.52	-4.54	210.41 ±4.90	-1.87	214.3± 2.07	210.47
Curry leaves	211.16 ±1.47	-0.47	214.35 ±1.02	+1.90	217.74± 2.46	+3.33	211.93 ±3.78	+1.01	189.96 ±3.63	-9.55	198.94 ±1.43	-5.38	210.23 ±1.10	207.76
Mean (Rate)	215.12		215.12		221.00		207.99		200.05		207.19		212.92	

Source of variation	Treatment (herbs)	Rate	Treatment (herbs)*Rate
SEm	1.39	2.11	3.66
CD	5.99	3.92	10.37
CV%	3.47		

- \- : indicates decreased in cholesterol content in (%)
- \+ : indicates increased in cholesterol content in (%)

When betel leaves was added at different rates in the ghee samples, the cholesterol contents was reduced. The average cholesterol content of the control ghee samples was 214.30 mg/100 gm which was reduced to 211.13, 213.36, 213.78, 206.03, 204.30 and 210.41 mg/100 gm when betel leaves was at the rate of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 respectively. The maximum reduction was 4.54 per cent found at 0.5 per cent level. Simultaneously, the flavour score (Table 4.8), though in acceptable limit, were decreased with increased in level of addition. Based on this, the rate of addition of 0.5 per cent of betel leaves was selected for further study.

From the data, it was revealed that when curry leaves were added at different rates in the ghee samples, the cholesterol contents were changed. The average cholesterol content of the control ghee samples was 210.23 mg/100 gm which was changed to 211.16, 214.35, 217.74, 211.93, 189.96 and 198.94 mg/100 gm when curry leaves added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 respectively. The cholesterol content was increased (0.47-3.33 per cent) initially with increased in rate of addition from 0.1 to 0.4 per cent. However, cholesterol content was reduced to maximum of 9.55 per cent at 0.5 per cent rate of addition of curry leaves. Simultaneously, the flavour score (Table 4.8), though in acceptable limit, were decreased with increased in level of addition. Based on this, the rate of addition of 0.5 per cent of curry leaves was selected for further study.

Thus, when ajwain leaves, betel leaves and curry leaves were added at the rate of 0.5 per cent in ghee samples, the maximum reduction of cholesterol content were obtained as 4.25 per cent, 4.54 per cent and 9.55 per cent respectively. Among three herbs used, the maximum reduction in cholesterol content was found in ghee added with curry leaves.

4.3.3.2 Effect of rate of addition of herbs on flavour score of ghee

The results of flavour score of ghee samples are presented in Table 4.8

The data indicated that the rate of addition of herbs was statistically significant ($P<0.05$) but interaction effect of herbs with rate was non-significant. The flavour score of ghee samples added with ajwain leaves, betel leaves and curry leaves at the rate of 0.5per cent were 7.63, 8.25 and 8.13 respectively. However, at 0.6 per cent rate of addition of the herbs, the flavour scores were decreased.

Table 4.8 Changes in flavour score of ghee upon treatment with different herbs in various rate

Herb used	Changes (%) in Flavour score of ghee upon treatment with different herbs in various Rate							
	0.1%	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**	**Control**	**Mean (Herbs)**
Ajwain leaves	8.50±0.20	8.38±0.07	8.19±0.12	8.00±0.20	7.63±0.13	7.38±0.24	8.75±0.25	8.30
Betel leaves	8.50±0.06	8.50±0.06	8.38±0.13	8.31±0.12	8.25±0.14	7.13±0.13	8.88±0.13	8.30
Curry leaves	8.56±0.06	8.44±0.16	8.25±0.14	8.25±0.14	8.13±0.13	7.25±0.14	8.75±0.14	8.28
Mean(Rate)	8.41	8.29	8.45	8.41	8.29	7.37	8.83	

Source of variation	Herb	Rate	Rate* Herb
SEm	0.053	0.081	0.140
CD	0.15	0.229	NS
CV%	4.19		

From the Table 4.7 and 4.8., it is cleared the 0.5 per cent rate of addition of these herbs were able to lower down the cholesterol content without affecting the sensory evaluation in terms of flavour score and hence 0.5 per cent rate of addition of these three herbs were carried forward for further study.

4.3.4 Effect of Duration of Heating Exposure on Ghee

Exposure of the herbs to high temperature may affect the stability of major and/ or minor flavonoid components present in the herbs. Exposure of the herbs to high temperature may also affect the sensory quality of ghee. Even possibility also exists about interaction of phenolic compounds present in herbs with ghee residue, leading to change in cholesterol lowering property. There is possibility of improvement in extraction of these phenolic compounds due to their better leaching from the herbs to ghee at higher temperature for long duration. Ghee (clarified butter) is produced from butter by removing water at a clarification temperature between 105 and 118℃. Therefore, it is necessary to find out appropriate duration of exposure of herb in process adopted for preparation of ghee.

The selected herbs (ajwain leaves, betel leaves and curry leaves) were added according to results obtained from the selected stage in the form of coarse particles at the rate of 0.5 per cent. The heating intervals of treated with these selected herbs were 15 min, 30 min and 45 min at 60 °C. The herb treated ghee samples were prepared as per the procedure described in Section 3.3. The sample of ghee without addition of herb was also prepared to serve as control. The ghee samples were analyzed for cholesterol content. Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. Total three replications were conducted.

4.3.4.1 Changes in cholesterol content during heating at various duration

The results on changes of cholesterol content of ghee samples are presented in Table 4.9

Upon care examination from the data given in Table 4.9, it was found that ghee samples treated with ajwain leaves at different duration of exposure affected the cholesterol content. The cholesterol content of the control ghee samples was 211.31 mg/100g. When duration of exposure of heating kept for 30 min, the per cent reduction in cholesterol content was 6.11. However, reduction of cholesterol content

Table 4.9 Changes in cholesterol content of ghee upon treatment with different herbs at various duration of exposure

Herbs used to treat the ghee	Changes (%) in cholesterol content (mg/100gm) of ghee upon treatment with different herbs in various Stages							
	15 min		30 min		45 min		Control	Mean (Herbs)
	Content	Changes	Content	Changes	Content	Changes	Content	
Ajwain leaves	210.12±1.38	-0.58	198.42±3.32	-6.11	207.56±2.99	-1.78	211.31±0.21	206.85
Betel leaves	210.86±3.94	-1.97	186.30±1.67	-6.72	209.16±1.47	-2.22	211.31±0.21	205.55
Curry leaves	207.16±0.63	-0.22	197.13±0.42	-11.84	206.63±2.19	-1.02	211.31±0.21	204.40
Mean (Stage)	209.38		193.94		207.78			

Source of variation	Treatment (herbs)	Duration of exposure	Duration of exposure*Herbs
SEm	1.041	1.203	2.48
CD (0.05)	NS	3.47	6.02
CV (%)	2.03		

- : indicates decreased in cholesterol content in (%)
+ : indicates increased in cholesterol content in (%)

was found only 1.78 per cent when ghee samples were exposed to 45 min. indicating the loss of polyphenol compounds (Vina and Chaves, 2008). Simultaneously, the flavour score (Table 4.10), though in acceptable limit, were decreased with increased in duration of exposure of heating. Based on this, the duration of exposure of heating was 30 min. considered as optimum in maximum reduction of cholesterol content in ghee added with ajwain leaves.

Similarly, ghee samples treated with betel leaves at different duration of exposure affected the cholesterol content. The cholesterol content of the control ghee samples was 211.31 mg/100g. When duration of exposure of heating kept for 30 min, the per cent reduction in cholesterol content was 6.72. However, reduction of cholesterol content was found only 2.22 per cent when ghee samples were exposed to 45 min. indicating the loss of polyphenol compounds (Vina and Chaves, 2008). Simultaneously, the flavour score (Table 4.10), though in acceptable limit, were decreased with increased in duration of exposure of heating. Based on this, the duration of exposure of heating was 30 min. considered as best in maximum reduction of cholesterol content in ghee added with betel leaves.

When ghee samples treated with curry leaves at different duration of exposure affected the cholesterol content. The cholesterol content of the control ghee samples was 211.31 mg/100g. When duration of exposure of heating kept for 30 min, the per cent reduction in cholesterol content was 11.84. However, reduction of cholesterol content was found only 1.02 per cent when ghee samples were exposed to 45 min. indicating the loss of polyphenol compounds (Vina and Chaves, 2008). Simultaneously, the flavour score (Table 4.10), though in acceptable limit, were decreased with increased in duration of exposure of heating. Based on this, the duration of exposure of heating was 30 min. considered as best in maximum reduction of cholesterol content in ghee added with curry leaves.

Thus, when ajwain leaves, betel leaves and curry leaves added ghee were exposed to heating at 45 min., the maximum reduction of cholesterol content were 6.11 per cent, 6.72 per cent and 11.84 per cent respectively. Among three herbs used, the maximum reduction in cholesterol content was found in ghee added with curry leaves.

4.3.4.2 Changes in flavour score of ghee during heating at various duration

The results obtained for changes in flavour score of ghee samples are presented in Table 4.10

The statistical analysis of the data indicated that duration of exposure of heating significant ($P<0.05$) but interaction effect of herbs with heating interval were non-significant. The flavour score of ghee samples added with ajwain leaves, betel leaves and curry leaves at heating interval of 30 min were 8.92, 8.33 and 8.92 respectively. However, at 45 min of heating interval, the flavour scores were decreased.

From the Table 4.9 and 4.10., it is cleared that when heating interval was kept to 30 min., these added herbs were able to lower down the cholesterol content without affecting the sensory evaluation in terms of flavour score and hence 30 min. of heating exposure of these three herbs were considered as best.

4.4 PHYSICO-CHEMICAL PROPERTIES OF HERB ADDED GHEE

Ghee, as in the case of other fats and oils, is characterized by certain physico-chemical properties, which have been found to be the basis for the fixation of certain physico-chemical constants for defining the chemical quality of the product. Ghee samples prepared under the optimized conditions as described above were subjected to analysis for some of the physico-chemical properties and the results obtained are presented in the Table 4.11 This indicated that these values have not shown much variation between control ghee and herb added ghee. Moreover, these physico-chemical parameters of herb added ghee were within the limits of the standards prescribed for milk fat/ghee (FSSAI, 2011; AGMARK, 1991).

Table 4.10 Changes in flavour score of ghee upon treatment with different herbs at various duration of exposure

Herbs used to treat the ghee	Changes (%) in flavour score of ghee upon treatment with different herbs in various Stages				
	15 min	**30 min**	**45 min**	**Control**	**Mean (Herbs)**
Ajwain leaves	8.41±0.03	8.92±0.01	7.75±0.03	9.00±0.00	8.53
Betel leaves	8.33±0.02	8.33±0.02	7.50±0.24	9.00±0.00	8.43
Curry leaves	8.00±0.02	8.92±0.02	7.50±0.06	9.00±0.00	8.36
Mean (Forms)	8.20	8.85	7.71	9.00	

Source of variation	Treatment (herbs)	Duration of exposure	Duration of exposure*Herbs
SEm	0.038	0.044	0.076
CD (0.05)	0.11	0.127	NS
CV (%)	1.81		

Table 4.11 Physico-Chemical Properties of Herb Added Ghee

Quality parameter of ghee						
Samples of ghee	**Moisture (%)**	**BR reading at 40°C**	**RM value**	**Polenske value**	**FFA (% oleic acid)**	**Peroxide value (meq of O2 per kg fat)**
Ajwain leaves	0.23	45.56	28.16	1.77	0.23	0
Betel leaves	0.18	45.56	28.30	1.79	0.22	0
Curry leaves	0.19	45.57	29.15	1.82	0.23	0
Control	0.21	45.58	28.95	1.80	0.25	0
Source of variation						
SEm	0.08	0.009	0.46	0.05	0.007	-
CD(0.05)	NS	NS	NS	NS	NS	NS
CV %	6.47	0.04	3.12	5.52	5.99	-

4.4.1 Moisture Content

It is observed that the moisture content in different ghee samples varied on an average from 0.18 to 0.23 per cent. The moisture content of all ghee samples was statistically at par, there was no significant difference in moisture content of different ghee samples.

As per FSSAI standards moisture content of ghee should not be more than 0.5 per cent (FSSAI, 2011). As per AGMARK standards moisture content of ghee should not be more than 0.3 per cent (AGMARK, 1991). It is almost impossible in practice, to remove all traces of moisture from ghee. If high moisture is present in ghee as a result of improper clarification, the keeping quality of ghee would be adversely affected. The presence of moisture accelerated the hydrolysis of fats, and thereby released free fatty acids, which were prone to quicker autoxidation than intact glycerides (Lalitha and Dastur, 1953). The results revealed that the moisture content in all the ghee samples was very well below the maximum limits prescribed by both FSSAI (2011) and AGMARK (1991) for ghee.

4.4.2 BR Reading

This property which concerns the degree of bending of light waves passing through a liquid or transparent solid is a characteristics for the particular liquid or solid. For the oils and fat, it increases with the unsaturation and decrease with the rise in

temperature. In case of the milk fat, the constant may be determined readily with an Abbe refractometer at 40ºC. The instrument is calibrated in Butyro-refractometer (BR) reading instead of absolute refractive indices. The refractive index of milk fat generally, ranges between 1.4538 and 1.4578.

BR reading of different ghee samples at 40°C varied on an average from 45.56 to 45.58. The difference in BR reading of different samples of ghee was statistically non-significant (P> 0.05). The BR reading of ghee at 40°C is reported to vary from 39.2-43.1 (Achaya, 1949). As per the FSSAI standards, BR reading of ghee should be 40.0 to 43.5 for areas other than cotton tract areas in Gujarat (FSSAI, 2011). As per AGMARK standards BR reading of ghee at 40°C should be 40.0 to 43.0 for areas other than cotton tract areas in Gujarat. Thus, BR reading of samples of ghee treated with herbs in present study was slightly higher than that of control ghee samples.

4.4.3 RM Value

RM value of different ghee samples varied on an average from 28.16 to 29.15. The difference in RM value of different ghee samples was statistically non-significant (P>0.05). The results revealed that the treatment of ghee with herbs (ajwain leaves, betel leaves and curry leaves) had no significant effect on RM value of ghee.

The RM value of ghee is reported to vary from 25.7-39.1 (Achaya, 1949). As per FSSAI standards RM value of ghee should be minimum 24.0 for areas other than cotton tract areas in Gujarat (FSSAI, 2011). As per AGMARK standards RM value of ghee should be minimum 28 for areas other than cotton tract areas in Gujarat. Thus, RM value of different ghee samples of obtained in present study was in general agreement with reported values and within limits of the standards prescribed by both FSSAI (2011) and AGMARK (1991).

4.4.4 Polenske Value

The Polenske value of ghee samples varied on an average from 1.77 to 1.82 under different treatments (Achaya, 1949). The Polenske value of all the samples of ghee was statistically at par, there was no significant difference in Polenske value of different samples of ghee. As per FSSAI and AGMARK standards, Polenske value of ghee should be 1 to 2. Thus, Polenske value of different ghee samples of obtained in present study was in general agreement with reported values and within limits of the standards prescribed by both FSSAI (2011) and AGMARK (1991).

4.4.5 FFA Content

The FFA content of ghee samples varied on an average from 0.22 to 0.25 per cent oleic acid under different treatments. The FFA content of all the samples of ghee was statistically at par, there was no significant difference in FFA content of different samples of ghee.

FFA content of ghee is reported to vary from 0.197 to 0.280 (Sharma, 1981; Purohit, 2011). Bector and Narayanan (1977) observed that ghee with higher FFA deteriorated faster than the ghee with lower FFA content. As per FSSAI standards FFA content of ghee should not be more than 3.0 per cent (FSSAI, 2011). According to AGMARK, free fatty acid contains should not be more than 1.4 for special grade ghee, 2.5 for general grade ghee and 3.0 for standard grade ghee. Thus, the level of FFA content different samples of obtained in present study was in general agreement with reported values and within limits of the standards prescribed by both FSSAI (2011) and AGMARK (1991).

4.4.6 Baudouin Test

According to FSSAI and AGMARK standards, baudouin test should be negative in ghee. In the present Baudouin test was found negative for all ghee samples including control ghee and ghee treated with ajwain leaves, betel leaves and curry leaves. Thus, all the ghee samples of fulfilled requirement of Baudouin test as prescribed by under FSSAI (2011) and AGMARK (1991) standard.

4.4.7 Peroxide Value

Since fresh butter was used in making ghee, the samples of ghee did not form peroxides in it. The peroxide Value of all the samples of ghee was statistically at par, there was no significant difference in peroxide value of different samples of ghee. Jain (2015) studied the effect of selected spices and BHA on changes in peroxide value of ghee during storage at 35±2°C and he did not get peroxide value on 0 day.

4.5 EFFECT OF STORAGE ON SELECTED PROPERTIES OF GHEE

The all ghee samples were stored at 40±2°C and evaluated for cholesterol content, peroxide value, free fatty acid and sensory evaluation at the 5 days of interval. Total four replications were taken and results are described at below.

4.5.1 Effect of Herbs on Cholesterol Content of Ghee during Storage at 40±2°C

The samples were analyzed for cholesterol content as described in Section 3.7 and results are shown in Table 4.12

The cholesterol content of control ghee sample on 0 day was 219.13 mg/100 g fat that was non-significantly decreased to 217.07 mg/100 g on 30th days of storage. Similarly the ghee samples treated with ajwain leaves have cholesterol content reduced from 207.06 to 205.72 mg/100g on 20th days of storage and then increased to 207.58 mg/100g on 30th days of storage. However, the changes in cholesterol content of the ghee treated with ajwain leaves were statistically non-significant. Similarly, ghee samples treated with betel leaves and curry leaves have also shown marginal changes in cholesterol content and that were even statistically non-significant during storage. Thus, from the above data, it is cleared that there was no significant effect on cholesterol content of the ghee samples during storage at 40 ±2°C.

4.5.2 Effect of Herbs on Peroxide Value of Ghee during Storage at 40±2°C

For evaluating the effect of on oxidative changes in ghee during storage at 40±2°C all the samples of ghee were analysed for peroxide value at an interval of 5 days up to 30 days. The results obtained for changes in peroxide value of ghee during storage at 40±2°C are presented in Table 4.13.

The changes in peroxide values of ghee samples during storage at 40±2°C were non-significant but period of storage was significant ($P<0.05$). The interaction between period of storage as well as herbs used in treatment of ghee was also non-significant ($P>0.05$). The interaction effect indicated that different herbs used in treatment of ghee as well as period of the storage did not differed significantly from each other over a period of storage. Thus, it became evident that the effect of different herbs used in treatment of ghee and period of storage were not dependent on each other.

4.5.3 Effect of Herbs on FFA (% Oleic Acid) Content of Ghee during Storage at 40±2°C

The results obtained for changes in FFA content of ghee during storage at 40±2°C are presented in Table 4.14

Table 4.12 Changes in cholesterol content of ghee on Storage at 40±2°C

Herb used	Changes (%) in cholesterol content (mg/100gm) of ghee upon storage at 40±2°C														
	0th day		5th day		10th day		15th day		20th day		20th day		30th day		Mean (Herbs)
	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	Changes	Content	Changes	
Ajwain leaves	207.06 ±0.87	-.50	207.56 ±1.01	- 4.41	205.90 ±1.65	- 5.01	207.63 ±1.56	- 4.29	205.72 ±1.21	- 4.36	206.51 ±1.03	- 4.89	207.58 ±0.80	- 4.37	206.85
Betel leaves	202.20 ±1.27	- 7.72	204.85 ±0.65	- 5.66	204.88 ±2.50	- 5.48	206.77 ±1.46	- 4.69	205.76 ±0.83	- 4.34	205.43 ±0.53	- 4.93	206.31 ±0.93	- 4.61	205.21
Curry leaves	199.55 ±0.83	- 8.93	201.54 ±1.49	- 7.19	200.60 ±2.55	- 7.45	200.55 ±3.47	- 7.55	200.90 ±0.62	- 6.59	202.60 ±0.98	- 6.69	202.65 ±0.59	- 6.64	201.20
Control	219.13 ±1.66	-	217.14 ±0.95	-	216.75 ±0.47	-	216.94 ±1.33	-	215.09 ±0.90	-	217.13 ±0.52	-	217.07 ±0.87	-	217.03
Mean (Storage Period)	206.98		207.77		207.03		207.27		206.86		207.97		208.40		

Source of variation	Herbs	Storage Period	Storage Period x herbs
SEm	0.520	0.687	1.375
CD	1.46	NS	NS
CV%	1.32		

- : indicates decreased in cholesterol content in (%)
+ : indicates increased in cholesterol content in (%)

Table 4.13 Changes in Peroxide value of Ghee on storage at 40±2°C

Herb used	Changes in peroxide value(meq of O2 per kg fat) of ghee upon storage at 40±2°C							
	0th day	**5th day**	**10th day**	**15th day**	**20th day**	**20th day**	**30th day**	**Mean (Herbs)**
Ajwain leaves	0.00±0.00	0.10±0.01	0.25±0.01	0.44±0.01	1.34±0.01	1.28±0.05	1.35±0.03	0.66
Betel leaves	0.00±0.00	0.11±0.01	0.26±0.01	0.46±0.01	1.24±0.01	1.35±0.03	1.38±0.09	0.68
Curry leaves	0.00±0.00	0.11±0.01	0.27±0.00	0.45±0.01	1.20±0.12	1.28±0.06	1.30±0.04	0.65
Control	0.00±0.00	0.12±0.00	0.29±0.01	0.47±0.01	1.26±0.02	1.30±0.04	1.40±0.04	0.69
Mean(Storage Period)	0.00	0.11	0.26	0.45	1.23	1.30	1.35	

Source of variation	**Herbs**	**Storage Period**	**Storage Period x herbs**
SEm	0.012	0.016	0.033
CD	NS	0.046	NS
CV%	9.66		

Table 4.14 Changes in FFA content of Ghee on storage at 40±2°C

Herb used	Changes FFA content (% oleic acid) of ghee upon storage at 40±2°C							
	0th day	**5th day**	**10th day**	**15th day**	**20th day**	**20th day**	**30th day**	**Mean (Herbs)**
Ajwain leaves	0.23±0.00	0.25±0.00	0.29±0.00	0.32±0.01	0.37±0.09	0.39±0.00	0.44±0.00	0.32
Betel leaves	0.22±0.00	0.25±0.00	0.27±0.00	0.32±0.01	0.38 ±0.10	0.46±0.00	0.48±0.00	0.31
Curry leaves	0.23±0.01	0.25±0.00	0.26±0.01	0.29±0.01	0.34±0.09	0.43±0.01	0.48±0.01	0.31
Control	0.25±0.01	0.27±0.00	0.31±0.01	0.36±0.01	0.42 ±0.11	0.47±0.01	0.51±0.01	0.34
Mean (Storage Period)	0.23	0.25	0.28	0.32	0.25	0.44	0.48	

Source of variation	Herbs	Storage Period	Storage Period x herbs
SEm	0.013	0.018	0.036
CD	NS	0.05	NS
CV%	22.08		

FFA content of control ghee sample on 0 day was 0.25 per cent oleic acid that significantly increased to 0.51 per cent oleic acid on 30th days of storage. Similarly the ghee samples treated with ajwain leaves was increased FFA content from 0.23 to 0.44 per cent oleic acid during 30th days of storage. Ghee samples treated with betel leaves have also shown increase in FFA content from 0.22 to 0.48 per cent oleic acid. Ghee samples treated with curry leaves have also shown increase in FFA content from 0.23 to 0.48. The changes in FFA content of ghee samples during storage at 40°±2°C were non-significant but period of storage was significant (P<0.05). The interaction between period of storage as well as herbs used in treatment of ghee were non-significant (P>0.05). The interaction effect indicated that different herbs used in treatment of ghee as well as period of the storage did not differed significantly from each other over a period of storage. Thus, it became evident that the effect of different herbs used in treatment of ghee and period of storage were not dependent on each other.

4.5.4 Effect of Herbs on Flavour Score of Ghee during Storage at 40±2°C

The results obtained for changes in flavour score of ghee during storage at 40±2°C are presented in Table 4.15

Flavour score of control ghee sample on 0 day was 8.89 that significantly decreased to 8.00 on 30th days of storage. Similarly the flavour score of ghee samples treated with ajwain leaves was decreased from 8.16 to 7.23 during 30th days of storage. The flavour score of ghee samples treated with betel leaves as well as curry leaves have also shown decreasing trends over a period of storage. The flavour score of ghee samples during storage at 40±2°C was significant (P<0.05). Moreover, the interaction between period of storage as well as herbs used in treatment of ghee were also non-significant (P>0.05). The interaction effect indicated that different herbs used in treatment of ghee as well as period of the storage did not differed significantly from each over a period of storage.

The above results indicated that all samples of ghee prepared in this study fulfilled requirement of all the quality parameters prescribed under FSSAI and AGMARK standards. The use of ajwain leaves, betel leaves and curry leaves did not affect parameters of quality (moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and Baudouin test) as prescribed under FSSAI and AGMARK standard.

Table 4.15 Changes in flavour score of Ghee on storage at 40±2°C

Herb used	Changes in flavour score of ghee upon storage at 40±2°C							
	0th day	**5th day**	**10th day**	**15th day**	**20th day**	**20th day**	**30th day**	**Mean (Herbs)**
Ajwain leaves	8.16±0.09	8.08±0.06	7.98±0.11	7.70±0.04	7.25±0.14	7.25±0.14	7.13±0.13	7.65
Betel leaves	8.10±0.07	8.20±0.12	7.98±0.17	7.53±0.05	7.25±0.14	7.25±0.10	7.19±0.12	7.64
Curry leaves	8.01±0.04	7.93±0.06	7.73±0.19	7.48±0.05	7.13±0.13	7.48±0.06	7.00±0.00	7.47
Control	8.89±0.05	8.83±0.02	8.75±0.12	8.60±0.08	8.13±0.13	8.60±0.07	8.00±0.00	8.47
Mean (Storage Period)	8.28	8.25	8.10	7.83	7.44	7.42	7.32	

Source of variation	Herbs	Storage Period	Storage Period x herbs
SEm	0.038	0.051	0.101
CD	0.108	0.142	NS
CV%	2.59		

This study, therefore, entails to conclude that addition of herbs (ajwain leaves, betel leaves and curry leaves) help to reduce cholesterol content of the ghee. The polyphenol/flavonoid compounds content of herbs might be responsible for lowering down the cholesterol content of the ghee. Addition of herbs into ghee was more effective than initial stage as well as final stage of heat clarification of butter on cholesterol lowering ability in of ghee. Addition of ajwain leaves, betel leaves and curry leaves in ghee in the form of coarse particle at the rate of 0.50 per cent with heating at 60°C for 30 min. was found best in reducing the cholesterol content without affecting the flavour score of ghee. Ghee samples incorporated with ajwain leaves, betel leaves and curry leaves fulfilled the legal requirements for quality standards prescribed under FSSAI and AGMARK.

CHAPTER- 5

SUMMARY AND CONCLUSION

It is common knowledge that cholesterol ensures stability of cell membranes, necessary for production of different steroid hormones and vitamin D, takes significant part in the activity of cerebrum synapse and immune system, including cancer prevention. The high cholesterol level causes atherosclerosis that associated with cardiovascular diseases such as strokes, heart attacks, and peripheral vascular diseases. There are various methods and /or processes available in the literature for removal of cholesterol content in butter oil and foods in general. They are generally categorized into physical, chemical, biological and complexation processes. These processes are costly and have side effects due to use of chemicals as well as remove fat soluble vitamins. It causes loss to nutritive value of ghee. Other way to lower down the cholesterol level in body is using cholesterol lowering drugs (e.g. statins) but this leads to various side effects. The plants flavonoids with antiartherosclerotic activity gained much attention and were proven to reduce the risk of atherosclerosis *in vitro* and *in vivo* based on different animal models. The flavonoids compounds also exhibit lipid lowering effects and anti-inflammatory and antiatherogenic properties. There is need to develop natural way to reduce cholesterol by using herbs in the product itself. Till date, there is no report available to evaluate herbs for its cholesterol lowering capacity in ghee.

The present study was undertaken to evaluate the effect of selected herbs on cholesterol level in ghee.

The first phase of study dealt with the compatibility of herbs added in ghee. This was followed by screening of herbs for cholesterol lowering property. The selected herbs, then, studied for optimization of processing parameters (*viz*. stage of addition, form of addition, rate of addition and duration of heating exposures) and evaluating its effect on cholesterol level. Final ghee samples were subjected for its common physico-chemical properties.

5.1 Compatibility Assessment of Herbs Used in Ghee

In selection of herbs for cholesterol lowering capacity, the first and foremost point for consideration was the compatibility of the herbs to be used for study. The compatibility assessment of the herbs used in ghee was carried out by sensory evaluation using flavour score. Flavour of any edible substance is the most important sensory attribute, which significantly affecting acceptance or rejection of the product. The total seven commonly used herbs namely ajwain leaves, betel leaves, mint, coriander leaves, fenugreek leaves, liquorice and curry leaves were selected for the assessment of their compatibility in ghee. They were added at the rate of 0.5 per cent (w/w) in ghee. The resultant ghee samples were subjected to sensory evaluation using flavour score for their acceptability using 9 point hedonic scale. The samples of ghee considered as unacceptable when flavour score became < 5 in sensory evaluation.

The result indicated that the flavour score of all ghee samples were varies from 5.92 to 8.50. The flavour score of the ghee samples added with fenugreek leaves was lowest having range from 5.56 to 6.50. The flavour score of control ghee sample varies from 8.31-8.69 representing highest score. The average flavour score of ghee samples added with betel leaves, licorice and curry leaves were much closed. The flavour score of ghee samples obtained by sensory evaluation were >5.0 in all treated ghee samples. All the herbs were considered as compatible in flavour score and all seven herbs were selected for further study.

5.2 Evaluation of the Herbs for their Cholesterol Lowering Ability in Ghee

In the present study, the above listed seven compatible herbs were incorporated (@ 0.5 per cent) in ghee for evaluating cholesterol lowering ability in ghee. Ghee sample without addition of any herbs was used as a control sample. The resultant samples were subjected to cholesterol estimation. It was found that ajwain leaves, betel, fenugreek, curry leaves and liquorice were able to lower down the cholesterol content of the ghee. The per cent reduction in cholesterol contents observed in ghee samples in descending order were betel leaves (10.53 per cent) > ajwain leaves (4.63 per cent) > curry leaves (4.50 per cent) > and licorice (0.96 per cent). Though licorice have ability to lower down the cholesterol content of the ghee samples it gave bit green color to the ghee sample and it was not carried forward for further study. The three herbs namely betel leaves, ajwain leaves and curry leaves were ability of reduced

cholesterol content as well as compatible in ghee and these three herbs were selected for further study.

5.3 Optimization of the Herbs for Application in Ghee

To optimize the application of herbs (*viz* ajwain leaves, betel leaves and curry leaves) in ghee, criteria namely stage of addition of herbs in ghee, form of addition of herbs in ghee and rate of addition of herbs in ghee were worked out.

The herbs (@ 0.5 per cent) were added into ghee in three different stages: Stage 1: Initial stage of heat clarification (in melted butter), Stage 2: Final stage of heat clarification after reaching temperature near to 105°C and Stage 3: In hot ghee at 90°C and allow it to cool at 60°C. The cholesterol content of the control ghee samples varies from 210.80 to 238.4 mg/100 gm with an average of 219.50 mg/100 gm of ghee in stage 1. The cholesterol content of ghee samples treated with ajwain leaves, betel and curry leaves were 221.42, 188.88 and 194.48 mg/100 gm of ghee samples respectively. The highest cholesterol reduction was observed in betel leaves (14.16 per cent) whereas ghee treated with ajwain leaves slightly increased the cholesterol content during study of stage 1. In stage 2, the cholesterol contents were marginally reduced in all the treated ghee samples. The per cent reduction in cholesterol content of ghee obtained in stage 2 were very closed and highest reduction was found only 5.97 per cent in ghee when treated with curry leaves i.e. reduced from 218.90 mg/100 gm (control) to 205.15 mg/100 gm (curry leave). When data observed in the treated ghee samples obtained from stage 3, the cholesterol contents were significantly reduced. The average cholesterol content was 212.68 mg/100 gm that decreased to 175.93 mg/100 gm in ghee added with ajwain leaves. Thus, highest reduction found in ajwain leaves (17.46 per cent) > betel leaves (16.99 per cent) > curry leaves (11.77 per cent). Among the all stages, the maximum reduction in cholesterol contents were observed in stage 3 and hence stage 3 was selected for further study. The flavour score of the treated samples of ghee were acceptable in all the stages. The highest reduction of cholesterol content and high flavour scores was observed in Stage 3 and hence, Stage 3 was carried forward for further study.

The selected herbs were added in the form of pieces, paste, coarse and fine particles at the rate of 0.5 per cent of each in ghee samples. The data on cholesterol content of ghee samples indicated that the form of addition of herbs was significant ($P < 0.05$). It

was found that the per cent reduction in cholesterol contents of ghee when treated with ajwain leaves in the form of coarse particles was highest (6.98 per cent). Similarly, highest reduction in cholesterol content was also observed in both betel leaves (5.75 per cent) and curry leaves (14.7 per cent) when added in the form of coarse particles as compared to other three forms. From the data, it is cleared that when coarse particles added in ghee gave highest flavour score in all treated ghee samples as compared to other forms of herbs. Thus, it is cleared that among the all forms of herbs, the maximum reduction in cholesterol content as well as highest flavour score was observed in ghee added with coarse particles and hence coarse particles was selected for further phase.

The selected herbs in the form of coarse particles were added at the rate of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 per cent in ghee. When ajwain leaves, betel leaves and curry leaves were added at the rate of 0.5 per cent in ghee samples, the maximum reduction of cholesterol content were obtained as 4.25 per cent, 4.54 per cent and 9.55 per cent respectively as compared to other rate of addition in ghee. Among three herbs used, the maximum reduction in cholesterol content was found in ghee added with curry leaves. Similarly flavour score was also acceptable when these three herbs added @0.5 per cent in the ghee.

The selected herbs were added according to results obtained from the selected stage in the form of coarse particles at the rate of 0.5 per cent.

The various heating intervals affected the cholesterol content as well as flavour score of the ghee samples. This was worked out using various time of intervals *viz*. 15 min, 30 min and 45 min at 60 °C. The duration of 30 min was considered as optimum in maximum reduction of cholesterol content in treated ghee samples. The highest cholesterol reduction was found as 6.11, 6.72 and 11.84 per cent in ajwain leaves, betel leaves and curry leaves treated ghee respectively when heated at 60 °C for 30 min. as compared to heating interval of 15 and 45 min. Similarly the flavour score of the treated ghee samples were also in acceptable limit when samples heated at 60 °C for 30 min. as compared to heating interval of 15 and 45 min.

5.4 Physico-Chemical Properties of Herb added Ghee

The physico-chemical properties (such as moisture, BR reading, RM and PV values, free fatty acids, peroxide value and baudouin test) of ghee samples treated with ajwain leaves, betel leaves and curry leaves were worked out. However, the results indicated

all the samples of ghee were statistically at par when compared with the control ghee sample. The average moisture content, RM value, PV value and FFA various from 0.18 to 0.23 per cent, 28.16 to 29.15, 1.77 to 1.82 and 0.22 to 0.25 per cent oleic acid respectively in all ghee samples. BR reading of samples of ghee treated with herbs in present study was slightly lower than that of control ghee samples. Since fresh butter was used in making ghee, the peroxide values were not detected in these samples. Baudouin test was found negative for all ghee samples.

5.5 Effect of Storage on Selected Properties of Ghee

The all ghee samples were stored at 40±2°C and evaluated for cholesterol content, peroxide value, free fatty acid and sensory evaluation at the 5 days of interval.

The cholesterol content of control ghee sample on 0 day was 219.13 mg/100 g fat that was non-significantly decreased to 217.07 mg/100 g on 30th days of storage. Similarly the ghee samples treated with ajwain leaves have cholesterol content reduced from 207.06 to 205.72 mg/100g on 20th days of storage and then increased to 207.58 mg/100g on 30th days of storage. However, the changes in cholesterol content of the ghee treated with ajwain leaves were statistically non-significant. Similarly, ghee samples treated with betel leaves and curry leaves have also shown marginal changes in cholesterol content and that were even statistically non-significant during storage. Thus, from the above data, it is cleared that there was no significant effect on cholesterol content of the ghee samples during storage at 40±2°C.

The changes in peroxide values of ghee samples during storage at 40±2° were non-significant but period of storage was significant ($P<0.05$). The interaction effect indicated that different herbs used in treatment of ghee as well as period of the storage did not differed significantly from each other over a period of storage. FFA content of control ghee sample on 0 day was 0.25per cent oleic acid that significantly increased to 0.51 per cent oleic acid on 30th days of storage. Similarly, FFA content of the ghee samples treated with ajwain leaves, betel leaves and curry leaves were increased from 0.23 to 0.44 per cent, 0.22 to 0.48 per cent, 0.23 to 0.48 per cent oleic acid respectively during 30th days of storage. The changes in FFA content of ghee samples during storage at 40±2°C were non-significant but period of storage was significant ($P<0.05$). Flavour score of control ghee sample on 0 day was 8.89 that significantly decreased to 8.00 on 30th days of storage. Similarly the flavour score of

ghee samples treated with ajwain leaves, betel leaves and curry leaves leaves have also shown decreasing trends over a period of storage. The flavour score of ghee samples during storage at 40±2°C was significant ($P<0.05$).

The above results indicated that all samples of ghee prepared in this study fulfilled requirement of all the quality parameters prescribed under FSSAI and AGMARK standards. The use of ajwain leaves, betel leaves and curry leaves did not affect parameters of quality (moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and Baudouin test) as prescribed under FSSAI and AGMARK standard.

This study, therefore, entails to conclude that addition of herbs (ajwain leaves, betel leaves and curry leaves) help to reduce cholesterol content of the ghee. Addition of ajwain leaves, betel leaves and curry leaves in ghee in the form of coarse particle at the rate of 0.50 per cent with heating at 60 °C for 30 min. was found best in reducing the cholesterol content without affecting the flavour score of ghee. Ghee samples incorporated with ajwain leaves, betel leaves and curry leaves fulfilled the legal requirements for quality standards prescribed under FSSAI and AGMARK.

REFERENCES

Achaya, K. T. (1949). Rancidity in indian butterfats (Ghee). *Biochemical Journal*, *44*(5), 561.

Achaya, K. T. (1997). Ghee, vanaspati and special fats in India. *Lipid Technologies and Applications*, Marcel Dekker Inc. New York, pp.369-390.

Agmark(1991). Agricultural produce (Grading and Marketing Rules) (AGMARK). Principal rules published in 1938 and Fifth amendment published vide G.S.R. 694 dated 27th November 1991 appeared on pages 2814 – 2817 in the Gazette of India, part II, section 3, sub-Section (i) dated 14-12-1991.

Ahn, J., & Kwak, H. S. (1999). Optimizing cholesterol removal in cream using β- cyclodextrin and response surface methodology. *Journal of Food Science*, 64(4), 629-632.

Akalln, A. S., & Tokusoglu, O. (2003). A potential anti-carcinogenic agent: Conjugated Linoleic Acid (CLA). *Pakistan Journal of Nutrition*, 2(2), 109-110.

Al-Juhaimi, F., & Ghafoor, K. A. (2011). Total phenols and antioxidant activities of leaf and stem extracts from coriander, mint and parsley grown in Saudi Arabia. *Pakistan Journal of Botany*, 43(4), 2235-2237.

Al-Okbi, S. Y., Fadel, H. H., & Mohamed, D. A. (2015). Phytochemical constituents, antioxidant and anticancer activity of Mentha citrata and Mentha longifolia. *Research journal of Pharmaceutical Biological and Chemical Sciences*, 6(1), 739-751.

Aneja, R. P., Mathur, B. N., Chandan, R. C., & Banerjee, A. K. (2007). Technology of Indian milk products, Published by: Dairy India Yearbook New Delhi-110092, India.

Angelo, P. M., & Jorge, N. (2008). Antioxidant evaluation of coriander extract and ascorbyl palmitate in sunflower oil under thermoxidation. *Journal of the American Oil Chemists' Society*, 85(11), 1045-1049.

Anilakumar, K. R., Nagaraj, N. S., & Santhanam, K. (2001). Effect of coriander seeds on hexachlorocyclohexane induced lipid peroxidation in rat liver. *Nutrition Research*, 21(11), 1455-1462.

AOAC. (2000). Cholesterol in foods. Official Methods of Analysis, 17th ed. Vol.2, Chapter 45, 82-84.

AOAC. (2005). Cholesterol in foods. Official Methods of Analysis, 18th ed. Chapter 45, 100-102.

Aristova, V.P. & Bekhova, E.K. 1976. Cholesterol in milk and milk products. *Trudy, Vsesoyuznyi Nanchnoissledova-tel'skii Institut Malochoi Promyshlennosti*. 42, 45 (cf. DSA 1977(39), 2748).

Arul, J., Boudreau, A., Makhlouf, J., Tardif, R., & Grenier, B. (1988). Distribution of cholesterol in milk fat fractions. *Journal of Dairy Research*, *55*(3), 361-371.

Bachman, K. C., Lin, J. H., & Wilcox, C. J. (1976). Sensitive colorimetric determination of cholesterol in dairy products. *Journal-Association of Official Analytical Chemists*, 59(5), 1146-1149.

Basch, E., Ulbricht, C., Kuo, G., Szapary, P. & Smith, M. (2003). Therapeutic application of fenugreek. *Alternative Medicine Review*, 8(1), 20-27

Bector, B. S., & Narayanan, K. M. (1975). Carbonyls in thermally oxidized ghee. *Indian journal of dairy science*.

Benabdallah, A., Rahmoune, C., Boumendjel, M., Aissi, O., & Messaoud, C. (2016). Total phenolic content and antioxidant activity of six wild Mentha species (Lamiaceae) from northeast of Algeria. *Asian Pacific Journal of Tropical Biomedicine*, 6(9), 760-766.

Bhatted, S., & Singh, G. (2002). A national seminar on "Role of pure ghee in health and nutrition- exploding myths. Jointly organized by Indian Dairy Association Gujarat Chapter, Anand and Gujarat Co-operative Milk Marketing Federation Ltd., Anand and J S Ayurveda College, Nadiad, pp. 19-22.

Bindal, M. P., Wadhwa, B. K., Lal, D., Rai, T. & Aggarwal, P. K. (1994).Removal of cholesterol from milk and milk products: Application of biotechnical processes. *NDRI Annual Report.* pp. 98-99.

Bindal, M. P., & Jain, M. K. (2014). Estimation of total cholesterol in ghee prepared from milk of cows and buffaloes.

Bloor, W. R. (1916). The determination of cholesterol in blood. *Journal of Biological Chemistry*, 24(3), 227-231.

Borradaile, N. M., Carroll, K. K., & Kurowska, E. M. (1999). Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids*, 34(6), 591-598.

Boudreau, A., & Arul, J. (1993). Cholesterol reduction and fat fractionation technologies for milk fat: an overview. *Journal of dairy science*, 76(6), 1772-1781.

Bradley Jr, R. L. (1989). Removal of Cholesterol from Milk Fat Using Supercritical Carbon Dioxide1. *Journal of Dairy Science*, 72(10), 2834-2840.

Bragagnolo, N. (2009). Cholesterol and cholesterol oxides in meat and meat products. *Handbook of muscle foods analysis*, 187-219.

Igara, C. E., Omoboyowa, D. A., Ahuchaogu, A. A., Orji, N. U., & Ndukwe, M. K. (2016). Phytochemical and nutritional profile of Murraya Koenigii (Linn) Spreng leaf. *Journal of Pharmacognosy and Phytochemistry*, 5(5), 7.

Carroll, R. M., & Rudel, L. L. (1981). Evaluation of a high-performance liquid chromatography method for isolation and quantitation of cholesterol and cholesteryl esters. *Journal of lipid research*, 22(2), 359-363.

Chandrasekara, N., & Shahidi, F. (2011). Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels, and testa. *Journal of Agricultural and Food Chemistry*, 59(9), 5006-5014.

Christodoulou, S., HUNG, T. V., Trewhell, M. A., & Black, R. G. (1994). Enzymatic degradation of egg yolk cholesterol. *Journal of food protection*, 57(10), 908-912.

Cook, N. C., & Samman, S. (1996). Flavonoids chemistry, metabolism,cardioprotective effects, and dietary sources. *The Journal of nutritional biochemistry*, 7(2), 66-76.

Davidson, S. (1990). Low-cholesterol milk and eggs. *Rural Research*, (148), 27-28.

De, S. (2004), "Outlines of Dairy Technology", 19th edition, Oxford publishing Company, New Delhi.

Dehal, S., Freier, T., Young, W., Hartman, A., & Beitz, C. (1991). *Fat and Cholesterol Reduced Food: Technologies and Strategies*, Haberstroh C, Moms C. (Eds.), Portflio Publishing Company, Woodlands, p 203-220.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International journal of food microbiology*, 74(1-2), 101-109.

Devatkal, S. K., Thorat, P. R., Manjunatha, M., & Anurag, R. K. (2012). Comparative antioxidant effect of aqueous extracts of curry leaves, fenugreek leaves and butylated hydroxytoluene in raw chicken patties. *Journal of food science and technology*, 49(6), 781-785.

Dias, M. I., Barros, L., Sousa, M. J., & Ferreira, I. C. (2011). Comparative study of lipophilic and hydrophilic antioxidants from in vivo and in vitro grown Coriandrum sativum. *Plant foods for human nutrition*, 66(2), 181-186.

Duncan, I. W., Culbreth, P. H., & Burtis, C. A. (1979). Determination of free, total, and esterified cholesterol by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, 162(3), 281-292.

Dunkley, W. L. (1982). Reducing fat in milk and dairy products by processing. *Journal of Dairy Science*, 65, 454–458.

El-sherif, M. A., El-sherif, G. M., & Tolba, K. H. (2014). Extraction and identification of natural antioxidants from liquorices (Glycyrrhiza glabra) and carob (Ceratonia siliqua) and its application in El-Mewled El-Nabawy sweets (Sesames and Folia). *World Academy of Science, Engineering and Technology, International Journal of Pharmacological and Pharmaceutical Sciences*, 1(8), 256-263.

Farah, H., Elbadrawy, E., & Al-Atoom, A. A. (2015). Evaluation of anti-oxidant and antimicrobial activities of ethanolic extracts of parsley (Petroselinum erispum) and coriander (Coriandrum sativum) plants grown in saudi arabia. *Int. J. Adv. Res*, 3, 1244-1255.

Fletouris, D. J., Botsoglou, N. A., Psomas, I. E., & Mantis, A. I. (1998). Rapid determination of cholesterol in milk and milk products by direct saponification and capillary gas chromatography. *Journal of Dairy Science*, 81(11), 2833-2840.

FSSAI (2011). Food Safety and Standards (Food Products Standards and Food Additives) Regulations. Food Safety and Standard Authority of India, Ministry of Health and Family Welfare, Government of India, New Delhi.

Fuhrman, B., Volkova, N., Kaplan, M., Presser, D., Attias, J., Hayek, T., & Aviram, M. (2002). Antiatherosclerotic effects of licorice extract supplementation on hypercholesterolemic patients: increased resistance of LDL to atherogenic modifications, reduced plasma lipid levels, and decreased systolic blood pressure. *Nutrition*, 18(3), 268-273.

Fuke, Y., & Matsuoka, H. (1974). Cholesterol content and identification of foreign fats in processed cheeses. *Journal of Japanese Society of Food and Nutrition*.

Ganguli, N. C., & Jain, M. K. (1973). Ghee: Its chemistry, processing and technology. *Journal of Dairy Science*, 56(1), 19-25.

Geetha, k. (2013). *Studies on Anti-hyperlipidemic and Anti-atherosclerotic Activities of Selected Indian Medicinal Plants.*(Doctoral thesis, Andhra University, Visakhapatnam, India).

Gerard, K. A., & Roberts, J. S. (2004). Microwave heating of apple mash to improve juice yield and quality. *LWT-Food Science and Technology*, *37*(5), 551-557.

Ghasi, S., Nwobodo, E., & Ofili, J. O. (2000). Hypocholesterolemic effects of crude extract of leaf of Moringa oleifera Lam in high-fat diet fed Wistar rats. *Journal of Ethnopharmacology*, 69(1), 21-25.

Gu, Y. F., Chen, Y., & Hammond, E. G. (1994). Use of cyclic anhydrides to remove cholesterol and other hydroxy compounds from fats and oils. *Journal of the American Oil Chemists' Society*, 71(11), 1205-1209.

Gulcin, I. (2006). Antioxidant and antiradical activities of L-carnitine. *Life sciences*, 78(8), 803-811.

Guthrie, N. (2007). Effect of citrus flavonoids and tocotrienols on serum cholesterol levels in hypercholesterolemic subjects. *Alternative Therapies in Health and Medicine*, 13(6), 44.

Huber, W., Molero, A., Pereyra, C. D., & Martinez De La Ossa, E. (1996). Dynamic supercritical CO2 extraction for removal of cholesterol from anhydrous milk fat. *International Journal of Food Science & Technology*, 31(2), 143-151.

Hurst, W.J., Aleo, Z.M.D., and Martin Jr., R.A. 1983. High performance liquid chromatography analysis of cholesterol in milk. *Journal of Dairy Science*, 66(8), 2192-2194.

Igara, C. E., Omoboyowa, D. A., Ahuchaogu, A. A., Orji, N. U., & Ndukwe, M. K. (2016). Phytochemical and nutritional profile of *Murraya koenigii* (Linn) Spreng leaf. *Journal of Pharmacognosy and Phytochemistry*, 5(5), 07-09.

Igual, M., García-Martínez, E., Camacho, M. M., & Martínez-Navarrete, N. (2011). Changes in flavonoid content of grapefruit juice caused by thermal treatment and storage. *Innovative Food Science & Emerging Technologies*, 12(2), 153-162.

Irina, I., & Mohamed, G. (2012). Biological activities and effects of food processing on flavonoids as phenolic antioxidants. In *Advances in applied biotechnology*, Prof. Marian Petre (Ed.)ISBN: 978-953-307-820-5, InTech.

IS:3508 (1966). Indian Standards, Methods for Sampling and Test for Ghee (Butterfat). New Delhi: Bureau of Indian Standards.

Ishtiaque, S., Naz, S., Soomro, N., Khan, K., & Siddiqui, R. (2015). Antioxidant activity and total phenolics content of extracts from Murraya koenigii (curry leaves), Laurus nobilis (bay leaves), and Camellia sinensis (tea). *Quaid-E-Awam University Research Journal of Engineering, Science & Technology*, 14(2), 20-25.

Isomaa, B. O., Almgren, P., Tuomi, T., Forsén, B., Lahti, K., Nissen, M., & Groop, L. (2001). Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes care*, 24(4), 683-689.

Itoh, T., Tamura, T., & Matsumoto, T. (1973). Sterol composition of 19 vegetable oils. *Journal of the American Oil Chemists Society*, 50(4), 122-125.

Jain, P. G., Patil, S. D., Haswani, N. G., Girase, M. V., & Surana, S. J. (2010). Hypolipidemic activity of Moringa oleifera Lam., Moringaceae, on high fat diet induced hyperlipidemia in albino rats. *Revista Brasileira de Farmacognosia*, 20(6), 969-973.

James, R., Zheng X., & Najla, G. (2007). Effect of citrus flavonoids and tocotrienols on serum cholesterol levels in hypercholesterolemic subjects. *Alternative Therapies in Health and Medicine*, 13(6), 44.

Guthrie, N. (2007). Effect of citrus flavonoids and tocotrienols on serum cholesterol levels in hypercholesterolemic subjects. *Alternative Therapies in Health and Medicine*, 13(6), 44.

Jenness, R., & Patton, S. (1959). *Principles of dairy chemistry*. John Wiley; New York.

Karahan, F., Avsar, C., Ozyigit, I. I., & Berber, I. (2016). Antimicrobial and antioxidant activities of medicinal plant Glycyrrhiza glabra var. glandulifera from different habitats. *Biotechnology & Biotechnological Equipment*, 30(4), 797-804.

Kassaian, N., Azadbakht, L., Forghani, B., & Amini, M. (2009). Effect of fenugreek seeds on blood glucose and lipid profiles in type 2 diabetic patients. *International Journal for Vitamin and Nutrition Research*, 79(1), 34-39.

Kaufmann, W., Frede, E., Precht, D., Timmen, H., Biernoth, G., & Merk, W. (1982). Fractionation of butterfat by extraction with supercritical CO2. *Milchwissenschaft*.

Khanal, R. C., & Olson, K. C. (2004). Factors affecting conjugated linoleic acid (CLA) content in milk, meat, and egg: A review. *Pakistan Journal of Nutrition*, 3(2), 82-98.

Kovacs, M. I. P., Anderson, W. E., & Ackman, R. G. (1979). A Simple Method for the Determination of Cholesterol and Some Plant Sterols in Fishery- Based Food Products. *Journal of Food Science*, 44(5), 1299-1301.

Kumari, O. S., & Rao, N. B. (2015). Phyto Chemical Analysis of Piper Betel Leaf Extract. *WJPPS*, *4*, 699-703.

Kwak, H. S., Jung, C. S., Seok, J. S., & Ahn, J. (2003). Cholesterol removal and flavor development in Cheddar cheese. *Asian Australasian Journal of Animal Sciences*, 16(3), 409-416.

Kwak, H. S., Nam, C. G., & Ahn, J. (2001). Low cholesterol Mozzarella cheese obtained from homogenized and b-cyclodextrin-treated milk. *Asian-Australasian Journal of Animal Sciences*, 14, 268-275.

Lad, D. (1998). Vasant. *The Complete Book of Ayurvedic Home Remedies. New York City: Harmony Books*.

Lalitha, K. R., & Dasttje, N. (1953). Keeping quality of ghee. 1. Effect of nature of milk, method of preparation, temperature of melting and antioxidants on the keeping quality. *Indian Journal of Dairy Science*, 6, 147-168.

Lalwani, A., Sinha, A. & Raza, M. T. (2014). Comparative Analysis of Antioxidant Properties of Curry Leaves and *Euphorbia helioscopia*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences,* 5(5), 456-460.

Lanzani, A., Bondioli, P., Mariani, C., Folegatti, L., Ventunni, S., Fedeli, E. & Barreteau, P. (1994). A New Short-Path Distillation System Applied to the Reduction of Cholesterol in Butter and Lard. *Journal of American Oil Chemists' Society*, 71, 609-614.

Larsen, J. E., & Froning, G. W. (1981). Extraction and processing of various components from egg yolk. *Poultry Science*, 60(1), 160-167.

Lee, D. K., Ahn, J., & Kwak, H. S. (1999). Cholesterol removal from homogenized milk with β-cyclodextrin. *Journal of dairy science*, 82(11), 2327-2330.

Li, S., &Parish, E. (1998). The chemistry of waxes and sterols. In Akoh CC, Min DB. Editors. Food Lipids. New York: Dekker Inc. pp 89–114.

Makwana, T. (2007). Development of a method For Cholesterol Estimation in Milk Fat Using Non Enzymatic Diagnostic kit. pp 8-23.

Micich, T. J. (1990). Behavior of polymer-supported digitonin with cholesterol in the absence and presence of butter oil. *Journal of agricultural and food chemistry*, 38(9), 1839-1843.

NajgebauerLejko, D., Grega, T., Sady, M. & Domagala, J. (2009). The quality and storage stability of butter made from sour cream with addition of dried sage

Nejad Ebrahimi, S., Hadian, J., & Ranjbar, H. (2010). Essential oil compositions of different accessions of Coriandrum sativum L. from Iran. *Natural product research*, 24(14), 1287-1294.

Newkirk, R., & Sheppard,J. (1981). HPLC determination of cholesterol in foods. *Association of Official Analytical Chemists*, 64, 54-57.

Nicoliy, M. C., Anese, M. & Parpine, M. (1999). Influence of processing on the antioxidant properties of fruit and vegetables. *Trends in Food Science and Technology*, 10(3), 94-100.

Oakenfull DG & Sidhu GS. (1991). Cholesterol reduction. Commonwealth Scientific and Industrial Research Organization, assignee. Int. Pat. No. WO 91/11114.

Oakenfull, D. G., Pearce, R. J. & Sidhu, G. S. (1991). Low-Cholesterol Dairy Products. *The Australian Journal of Dairy Technology*, 46(2), 110-112.

Odriozola-Serrano, I., Soliva-Fortuny, R., & Martín-Belloso, O. (2008). Phenolic acids, flavonoids, vitamin C and antioxidant capacity of strawberry juices processed by high-intensity pulsed electric fields or heat treatments. *European Food Research and Technology*, 228(2), 239.

Odriozola-Serrano, I., Soliva-Fortuny, R., Hernández-Jover, T., & Martín-Belloso, O. (2009). Carotenoid and phenolic profile of tomato juices processed by high intensity pulsed electric fields compared with conventional thermal treatments. *Food Chemistry*, 112(1), 258-266.

Oh, H. I., Chang, E. J., & Kwak, H. S. (1998). Conditions of the removal of cholesterol from milk by treatment with saponin. *Korean Journal of Dairy Science (Korea Republic)*.

Ong, C. P., Lee, H. K., & Li, S. (1990). Supercritical fluid extraction and chromatography of cholesterol in food samples. *Journal of Chromatography A*, 515, 509-513.

Ostlund, R., (2007). Phytosterols, cholesterol absorption and healthy diets. *Lipids*, 42(1), 41-45.

Pantulu, P. C., Bhimasena Rao, M., Sethu Rao, D., & Anantakrishnan, C. P. (1975). Application of Liebermann-Burchard reaction to the unsaponifiable portion of milk lipids as a criterion for the quantitative determination of cholesterol in milk and milk products. *Milchwissenschaft (Germany, FR)*.

Paradkar, M. M., & Irudayaraj, J. (2002). Determination of cholesterol in dairy products by infrared techniques: 2. FT- NIR method. *International Journal of Dairy Technology*, 55(3), 133-138.

Parodi, P. W. (1999). Conjugated linoleic acid and other anticarcinogenic agents of bovine milk fat. *Journal of Dairy Science*, 82(6), 1339-1349.

Patel, S., & Rajorhia, S. (1979). Antioxidative role of curry (Murrya koenigi) and betel (*Piper betel*) leaves in ghee. *Journal of Food Science and Technology*, 16(4),158-160.

Patton, S., & Jensen, R. (1976). *Biomedical Aspects of Lactation*. UK: Pergamon Press Ltd.

Paul, N., Sen, K., Karim, F., Hasan, N. & Faruque, A. (2013). Phytochemical and biological investigations of *Coriandrum sativum* (cilantro) leaves. *International Journal of Innovative pharmaceutical sciences and research*, 1(1), 170-184.

Prasad, R., & Pandita, N. N. (1987). Variations in the cholesterol content of butter fat. *Indian Journal of Dairy Science*,45(6), 165-173.

Premanath, R., Sudisha, J., Lakshmi Devi, N., & Aradhya, S. M. (2011). Antibacterial and anti-oxidant activities of fenugreek (Trigonella foenum graecum L.) leaves. *Journal Medicinal Plants Resereach*, 5(6), 695-705.

Punwar, J. K. (1975). Gas-liquid chromatographic determination of total cholesterol in multicomponent foods. *Journal of Association of Official Analytical Chemists*, 58(4), 804-810.

Vijaykumar, P. A. (2011). *Effect of herb extract (withania somnifera/ashwagandha) incorporation on storage stability of ghee* (Doctoral dissertation, NDRI, Karnal).

Rajorhia, G.S. (1993). Ghee: encyclopedia of food science. In: Hui, Y.M. Ed. Food Technology and Food Nutrition. Academic Press, London.

Rasineni, G. K., & Reddy, A. R. (2008). Free radical quenching activity and polyphenols in three species of Coleus. *Journal of Medicinal Plants Research*, 2(10), 285-291.

Rastogi, R. P., & Mehrotra, B. N. (1994). Compendium of Indian medicinal plants published by central drug research institute, Lucknow and national institute of sciences communication and information resources, New Delhi. 6, 395-398.

Reinhold, J. G., & Shiels, E. M. (1936). The Determination of Blood Cholesterol: I. A Comparison of Standard Colorimetric Methods and A Modified Method with Gravimetric Determination of Digitonin Precipitates. *American Journal of Clinical Pathology*, 6(1), 22-30.

Riccomini, M. A., Wick, C., Peterson, A, Jimenez-Flores, R. and Richardson, T. (1990) Cholesterol removal from cream and anhydrous milk fat using saponins. J. Dairy Sci. 73(1), 107

Risdian, C., Widowati, W., Mozef, T., Wargasetia, T. L., & Khiong, K. (2011). Free Radical Scavenging Activity of Ethanolic Leaves Extract and Its Different Solvent Fractions of Piper betle L. In Vitro. *Indonesian Journal of Cancer Chemoprevention*, 2(1), 141-145.

Roschlau, P. V., Bernt, E., & Gruber, W. (1974). Enzymatische bestimmung des gesamt-cholesterins im serum. *Clinical Chemistry and Laboratory Medicine*, 12(9), 403-407.

Rudel, L., & Morris, D. (1973). Determination of cholesterol using o phthalaldehyde. *Journal of Lipid Research*, 14(3), 364-366.

Roshan, A., Verma, N. K., Kumar, C. S., Chandra, V., Singh, D. P., & Panday, M. K. (2012). Phytochemical constituent, pharmacological activities and medicinal uses through the millenia of Glycyrrhiza glabra Linn: a review. *International Research Journal* of *Pharmacy*, *3*(8), 45-55.

Sadovoy, V. V., Aralina, A. A., & Shchedrina, T. V. (2013). Computer simulation of the mechanism of interaction of red grape flavonoids with cholesterol. *Russian Agricultural Sciences*, 39(4), 370-372.

Sawale, P. D., Patil, G. R., Singh, B., Arvind, K., & Ghule, K. (2012). Potential application of milk and milk products as carrier for herbs and nutraceuticals. *Current Topics in Nutraceuticals Research*, 10(2), 101.

Schlimme, E. (1990). Removal of cholesterol from milk fat. *European Dairy Magazine*, (4), 12-21.

Seth, R., & Singh, A. (1994). Removal of cholesterol from milk using β-cyclodextrin and preparation of milk products from such treated milk. *NDRI Annual Report*, 95.

Sharma, H. M. (1990). Butter oil (ghee)–Myths and facts. *Indian J Clin Pract*, *1*, 31-2.

Sharma, R., Nath, B. S., & Lal, D. (1999). Approaches for cholesterol removal from milk fat: An overview. *Indian Journal of Dairy and Biosciences*, 10, 138-146.

Sharma, R. S. (1981). Ghee: a resume of recent researches. *Journal of food Science and Technology*.

Shingla, K. M., & Mehta, B. M. (2018). Cholesterol and its oxidation products: occurrence and analysis in milk and milk products. *International Journal of Health, Animal Science and Food Safety*, 5(1).

Singh, I., & Gupta, M. P. (1982). Physicochemical characteristics of ghee prepared from goat milk [clarified butter, India]. *Asian Journal of Dairy Research*.

Snehlata, H. S., & Payal, D. R. (2012). Fenugreek (Trigonella foenum-graecum L.): an overview. *International Journal of Current Pharmaceuticle Reviews and Research*, 2(4), 169-87.

Srinivasan, M.R. (1976). Ghee making in the tropical countries and possibilities of its industrial production. *Indian Dairyman*, 28, 279.

Sserunjogi, M. L., Abrahamsen, R. K., & Narvhus, J. (1998). A review paper: current knowledge of ghee and related products. *International Dairy Journal*, 8(8), 677-688.

Steel, R. G., & Torrie, J. H. (1986). *Principles and procedures of statistics: a biometrical approach*. McGraw-Hill.

Sugino, K., Terao, J., Murakami, H., & Matsushita, S. (1986). High-performance liquid chromatographic method for the quantification of cholesterol epoxides in spray-dried egg. *Journal of Agricultural and Food Chemistry*, 34(1), 36-39.

Sweeney, J. P., & WeBirauch, J. L. (1976). Summary of available data for cholesterol in foods and methods for its determination. *Critical Reviews in Food Science & Nutrition*, 8(2), 131-159.

Tanabe, H., Yoshida, M., & Tomita, N. (2002). Comparison of the antioxidant activities of 22 commonly used culinary herbs and spices on the lipid oxidation of pork meat. *Animal Science Journal*, 73(5), 389-393.

Thilagavathi, S., & Hariram, N. (2016). *Coleus aromaticus* benth synthesis of potentially nanomedicine as high nutritive value of human health and immunomodulatory. *International Journal of Science and Research Methodology*, 4(4), 18-38.

Thirumalai, T., Tamilselvan, N., & David E. (1990). Hypolipidemic activity of Piper betel in high fat diet induced hyperlipidemic rat. *Journal of Acute Disease*, 14, 131-135.

Tirtha, S.S. (1998). *The Ayurveda Encyclopedia*. Bayville, NY: *Ayurveda* Holistic Center Press, New York.

Tsui IC. 1989. Rapid determination of total cholesterol in homogenized milk. J Assoc Off Anal Chem 72(3), 421-424.

Tupe, R. S., Kemse, N. G. & Khaire, A. A. (2013). Evaluation of antioxidant potentials and total phenolic contents of selected Indian herbs powder extracts. *International Food Research Journal*, 20(3), 1053-1063.

Tylkin, X.B., Grishina, V.E. & Suidovskii, A.N. 1975. Photometric investigation of the contents of fat soluble vitamins and cholesterol in dairy products. *Tovarovedenie*. 8, 42 (cf. DSA 1976(38), 1879).

Uddin, M. F., Uddin, S. A., Hossain, M. D., & Manchur, M. A. (2015). Antioxidant, Cytotoxic and Phytochemical Properties of the Ethanol Extract of Piper Betle Leaf. *International Journal of Pharmaceutical Sciences and Research*, 6(10), 4252.

Vanzetti, G. (1964). Methodes photometriques de dosage du cholesterol dans le serum. *Clinica Chimica Acta*, 10(5), 389-405.

Vijaykumar, P. A. (2011). *Effect of herb extract (withania somnifera/ashwagandha) incorporation on storage stability of ghee* (Doctoral dissertation, NDRI, Karnal).

Vina, S. Z., & Chaves, A. R. (2008). Effect of heat treatment and refrigerated storage on antioxidant properties of pre- cut celery (Apium graveolens L.). *International Journal of Food Science & Technology*, 43(1), 44-51.

Vyshemirskii FA, Silin VM, Poyarkova GS, Vergelesov VM, Rashevskaya T. (1977). Trudy Vsessoyuzuyi Nauchnoissle-dovatel'skii Institut Maslodel'noi Syrodel'noi Promushlemmosti, 21(62).

Wani, S. A., & Kumar, P. (2016). Fenugreek: A review on its nutraceutical properties and utilization in various food products. *Journal of the Saudi Society of Agricultural Sciences*, 13(3), 118-125.

Watanabe, K. H., Aihara, H., & Nakamura R. (1989). Properties of the purified extracellular cholesterol oxidase from Rhodococcus equi No. *23*. *Journal Agriculture and Food Chemistry*, 37(4), 1178-1182.

Windous, A. (1910). Uber die quantitative bestimmung des cholesterins undder cholesterinester in einigen normalen und pathologischen nieren. *Journal of Physiological Chemistry*. 65, 110.

Winston, J. (1999). Health-promoting properties of common herbs1, 2. *The American Journal of Clinical Nutrition*, 70 (3), 491-499.

Wrezel, P. W., Krishnamurthy, R. G., & Hasenhuettl, G. L. (1992). *U.S. Patent No. 5,128,162*. Washington, DC: U.S. Patent and Trademark Office.

Xiansheng, W., Hung, T. V., Drew. P. *G.* & Versteeg, K. (1990). Enzymatic Degradation of Cholesterol in Milk. *Australian Journal of Dairy Technology*, 45(2), 50-52.

Xu, X. H., Li, R. K., Chen, J., Chen, P., Ling, X. Y. & Rao, P. F. (2002). Quantification of cholesterol in foods using non-aqueous capillary electrophoresis. *Journal Chromatography B*, 768(2), 369-373.

Yadav, S. K., & Sehgal, S. (1995). Effect of home processing on ascorbic acid and β-carotene content of spinach (Spinacia oleracia) and amaranth (Amaranthus tricolor) leaves. *Plant foods for human nutrition*, 47(2), 125-131.

Yen G. C. & Tsai, L. J. (1995). Cholesterol removal from a Lard-Water Mixture with β-Cyclodextrin. *Journal of Food Science,* 60, 561-565.

Yu, J., Ahmedna, M., & Goktepe, I. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food chemistry*, 90(1-2), 199-206.

Zielinski, H., Michalska, A., Amigo-Benavent, M., del Castillo, M. D., & Piskula, M. K. (2009). Changes in protein quality and antioxidant properties of buckwheat seeds and groats induced by roasting. *Journal of agricultural and food chemistry*, *57*(11), 4771-4776.

APPENDIX

SCORE CARD FOR SENSORY EVALUATION OF GHEE.

Hedonic rating	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Sensory score card for ghee on 9 point hedonic scale

Sensory attribute	1	2	3	4	5
Flavour					

Comment if any:

Date: Name of panelist: