EVALUATION OF SELECTED HERBS TO ENHANCE SHELF LIFE OF GHEE AGAINST OXIDATIVE DETERIORATION

A

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IN

DAIRY CHEMISTRY

BY

KAPADIYA DHARTIBEN BIPINBHAI

M. Tech. (DAIRY CHEMISTRY)

DAIRY CHEMISTRY DEPARTMENT

SHETH M. C. COLLEGE OF DAIRY SCIENCE

ANAND AGRICULTURAL UNIVERSITY

ANAND – 388 110 (GUJARAT) INDIA

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Name of Student	Name of Major Advisor
Ms. Kapadiya Dhartiben Bipinbhai	Dr. K. D. Aparnathi

DAIRY CHEMISTRY DEPARTMENT
SHETH M. C. COLLEGE OF DAIRY SCIENCE
ANAND AGRICULTURAL UNIVERSITY
ANAND – 388 110, GUJARAT

ABSTRACT

Ghee under ambient conditions of storage undergoes oxidative deterioration. Addition of synthetic antioxidants is common approach to extend shelf-life of ghee, but use of synthetic antioxidants which posse potential health risk necessitated attention towards natural antioxidants. Herbs are considered very good sources of natural antioxidants, but very limited work has been reported for utilization of herbs as a possible antioxidant in ghee. Therefore, the present study was contemplated to evaluate potential of common herbs as a natural antioxidant in ghee. The study was planned with work in to 6 phases: (1) assessment of compatibility of the common herbs in ghee, (2) evaluation of herbs for antioxidant activity in ghee, (3) selection of stage in preparation of ghee for addition of herbs, (4) optimization of rate for addition of the selected herbs in ghee, (5) combinations of the selected herbs as antioxidants in ghee, (6) comparison for effectiveness of the selected herbs as an antioxidant for ghee in comparison with permitted synthetic antioxidant (BHA) and analysis of ghee samples for quality parameters prescribed under FSSAI regulations and AGMARK standards.

Total 15 herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi) being very common in use were shortlisted for study. On treating ghee with 0.5 per cent (w/w) herb, all the ghee samples were found acceptable, though characteristic aroma of the herbs was perceived. Total phenolic content (mg GAE/100 g) of these dried herbs was 0396.67 ± 16.99, 3653.33 ± 18.86, 1740.00 ± 58.88, 1140.00 ± 32.66, 1913.33 ± 57.35, 1613.33 ± 65.99, 0673.33 ± 24.94, 1673.33 ± 24.94, 1266.67

± 57.35, 1846.67 ± 67.99, 0660.00 ± 28.28, 1030.00 ± 69.76, 1640.00 ± 32.66, 1106.67 ± 73.64 and 1863.33 ± 49.22 respectively. Similarly, radical scavenging activity (% inhibition) of these dried herbs was 63.52 ± 1.47, 87.31 ± 0.77, 86.64 ± 0.52, 79.40 ± 0.76, 84.07 ± 0.93, 83.62 ± 1.13, 61.37 ± 0.13, 16.39 ± 0.77, 79.72 ± 0.82, 65.24 ± 1.44, 37.05 ± 1.21, 83.20 ± 1.29, 85.76 ± 0.28, 28.41 ± 0.89 and 39.24 ± 1.46 respectively as measured by DPPH assay.

To screen herbs for their effect on oxidative stability of ghee, the herbs were tested at 0.5 per cent in treatment of ghee and ghee was monitored for changes in peroxide value and flavour score by Schaal oven test at 80°±2°C for 12 days. Among 15 shortlisted herbs dill, drumstick leaves, jequirity, lemon grass, shatavari and tulsi proved prooxidant with strong negative effect on shelf-life of ghee, whereas, betel leaves, curry leaves and liquorice appeared promising for use as an antioxidant in ghee. The remaining herbs showed the antioxidant potential, but much lower than the promising herbs.

In evaluation for stages in ghee preparation process 3 stages (initial stage of heat clarification i.e. in melted butter, final stage of heat clarification i.e. near 105°C temperature and after the heat clarification i.e. in hot ghee) were identified to add herbs by monitoring changes in peroxide value and flavour score of ghee on storage at 80°±2°C for 12 days. The third stage (hot ghee) was used in 1st phase and found inconvenient, since necessitate re-filtration. Hence initial stage of heat clarification and final stage of heat clarification were tested for betel leaves, liquorice and curry leaves. For addition of betel leaves and curry leaves in treatment of ghee final stage of heat clarification (i.e. near 105°C temperature) found more effective, whereas, for liquorice initial stage of heat clarification (i.e. in melted butter) was more effective.

For optimizing rate for addition of selected herbs (betel leaves, curry leaves and liquorice) in ghee concentration range of 0.1 to 0.4 per cent at an interval of 0.1 per cent was tested by treating ghee at respective selected stages of addition and monitoring changes in peroxide value and flavour score of ghee on storage at 80°±2°C for 22 days. The rate of 0.3 per cent was found optimum for all the 3 selected herbs in treatment ghee.

To study possible additive, synergistic or antagonistic effect of combining selected herbs on antioxidant activities binary blend (betel leaves + curry leaves, betel leaves +

liquorice and curry leaves + liquorice) as well as ternary blend (betel leaves + curry leaves + liquorice) were applied. Each herb was added at the optimized rate (i.e. @ 0.3%) and selected stage in preparation of ghee. Simultaneously, ghee samples treated with each individual herbs as well as control ghee were also prepared and monitored for changes in peroxide value and flavour score on storage at 80°±2°C for 29 days. The combination of betel leaves and liquorice was most effective in controlling peroxide formation and flavour deterioration in ghee during the storage. However, betel leaves alone was also found efficient for the purpose.

Finally, performance of betel leaves and blend of betel leaves with liquorice as an antioxidant in ghee was compared with BHA (permitted synthetic antioxidant) by monitoring changes in peroxide value, carbonyl value, radical scavenging activity and flavour score on storage at 80°±2°C for 34 days and also at 35°±2°C for 180 days. The fresh samples of ghee were also analysed for quality standards (moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and baudouin test) as prescribed under FSSAI as well as AGMARK and evaluated for sensory attributes (flavour, texture, colour and appearance and overall acceptability). The betel leaves alone and its blend with liquorice were significantly more effective compared to BHA for controlling development oxidative rancidity and other associated changes in ghee during storage. All samples of ghee fulfilled requirement of all the quality parameters prescribed under FSSAI and AGMARK standards. Similarly, samples of ghee treated with betel leaves alone or in combination with liquorice were very well acceptable in sensory evaluation.

The study suggests that addition of betel leaves (0.3%) along with liquorice (0.3%) and even betel leaves alone (0.3%) during preparation of ghee help to reduce oxidative rancidity in ghee against oxidative deterioration during storage.

SHETH M. C. COLLEGE OF DAIRY SCIENCE

ANAND AGRICULTURAL UNIVERSITY

ANAND – 388 110, GUJARAT

Dr. K. D. Aparnathi
Professor & Head
Dairy Chemistry Department,
Sheth M. C. College of Dairy Science,
Anand Agricultural University,
Anand – 388 110
Gujarat, India.

CERTIFICATE

This is to certify that the thesis entitled "**EVALUATION OF SELECTED HERBS TO ENHANCE SHELF LIFE OF GHEE AGAINST OXIDATIVE DETERIORATION**" submitted by **Kapadiya Dhartiben Bipinbhai** (Reg No: 04-2491-2014) in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** 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 **(K. D. Aparnathi)**

Date: / /2017 **Major Guide**

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Place: Anand ***(Kapadiya Dhartiben Bipinbhai)***

Date: / /2017

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SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BIS	Bureau of Indian Standards
C. D.	Critical difference
C. V.	Coefficient of Variation
*	Significant
NS	Non-significant
g	Gram(s)
kg	Kilogram
l	Liter(s)
mg	Milligram
min	Minute(s)
ml	Milliliters
mm	Millimeter
N	Normality
NaOH	Sodium hydroxide
SEm	Standard error of mean
µ	Micron
TPC	Total phenolic content
TS	Total Solids
%	Per cent
BR reading	Butyro-Refractometer reading
PV	Polenske value
RM	Reichert Meissl
mM	Micro molar
FFA	Free fatty acid

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 is a complex mixtures of triacylglycerol, together with small quantity of free fatty acids, phospholipids, sterols, hydrocarbons, carbonyl compounds, fat soluble vitamins (A, D, E, and K), carotenoid pigments, moisture and traces of elements like copper and iron. On an average ghee contains 99.0-99.5% fat and less than 0.5% moisture. Ghee is an indigenous milk product. It enjoys a supreme status in Indian dietary, being a rich source of energy, fat soluble vitamins, essential fatty acids and pleasing flavour. In India, ghee is considered as an excellent medium for cooking (Bhatted and Singh, 2002). This fat rich dairy product is highly regarded for its typical pleasing flavour and cooked taste.

Ghee deteriorates through the process of rancidification which may occur through hydrolytic and/or oxidative routes (Kuchroo and Narayanan, 1973). The onset of rancidity in ghee is mainly due to the oxidation of unsaturated glycerides leading to development of peroxides and/or due to hydrolysis of glycerides resulting in increased levels of free fatty acids (FFA) (Amr, 1991). Hydrolytic rancidity can be prevented to a great extent by proper heat treatment of raw materials before manufacture of ghee so as to inactivate the lipolytic enzymes (Rangappa and Achaya, 1974). Oxidative rancidity is the major pathway by which ghee undergoes deterioration. This is referred to as autoxidation because the rate of oxidation increases as the reaction proceeds under usual processing and storage conditions. Oxidative deterioration, once initiated, is essentially a self-catalysed reaction. Oxidative deterioration of ghee is one of the major factors that limit the storage life of ghee (Mehta, 2006; Puravankara *et al.*, 2000).

Due to its low moisture (0.5%) content, ghee has better shelf life than other indigenous dairy products. However, it undergoes oxidative deterioration that not only affects the economic value of product but also spoils its appetising flavour, making it

unpalatable and generating potentially toxic compounds (lipid peroxides, hydroxyl fatty acids, carbonyl compounds such as malonaldehyde, cyclic monomers, dimers, polymers, polycyclic aromatic compounds and oxidised sterols) (Rangappa and Achaya 1974; Mehta *et al.*, 2015).

Consumption of such products leads to diarrhea, poor growth rate, promotion of tumor growth and carcinogenic properties (Sanders, 1989). Therefore, oxidation of milk fat results in a number of adverse effects on its quality. These in tern determine the storage stability of ghee. Therefore, constant research endeavors are made to extend the shelf-life of ghee by various approaches. Several workers have done exhaustive work to improve the stability of ghee against autoxidation through feeding specific feed to milch animals (Rama Murthy and Narayanan, 1972; Tandon, 1977), altering processing parameters (Rama Murthy *et al*., 1968; Singh *et al*., 1979), using proper packaging materials and storage conditions (Chauhan and Wadhwa, 1987), adding milk components (Gupta *et al*., 1979; Bhatia *et al*., 1978), adding synthetic antioxidants (Kuchroo and Narayanan, 1972; Chatterjee, 1977), incorporating natural antioxidants from edible plant materials, spices and condiments, aromatic herbs, etc. (Ahmad *et al*.,1960; Amr, 1990). One of the most common approaches is addition of antioxidants.

However, scientific studies have shown that application of synthetic antioxidants in foods may cause damage to liver and have been responsible for carcinogenesis (Parmar *et al.,* 2013). Synthetic antioxidants may cause health hazards such as teratogenic, carcinogenic and mutagenic effects in experimental animals (Hathway, 1966; Pawar *et al.*, 2012). These reasons have directed the attention towards the use of edible plant resources as safer and also consumer demand for natural food ingredients has resulted in extensive research on naturally occurring antioxidants. Recently, the use of natural antioxidants in the food industries has increased rapidly and consequently many related studies have been reported (Jeong *et al.,* 2004). Numerous herbs have the potential to retard lipid oxidation during storage of foods which is usually mediated through their intrinsic antioxidant activity and the addition of herb and spice extracts in milk and milk products is evolving rapidly (Pokorny and Korczak, 2001; Pawar *et al.*, 2012).

Herbs have been used as food and as medicine for centuries. Since the beginning of human civilization, herbs have been an integral part of the society, valued both for

their culinary and medicinal properties. The phenolic compounds or polyphenols, secondary vegetal metabolites, constitute a wide and complex array of phytochemicals that exhibit antioxidant action and consequently a beneficial physiological effect. Their ability to delay lipid oxidation in foodstuffs. Phenolic phytochemicals are known to exhibit several health beneficial activities such as antioxidant, anti-inflammatory, antitumor and antimicrobial (Al-juhaimi, and Ghafoor, 2011).

The phenolic compound in herbs act as antioxidants because of their redox properties which allow them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators (Parmar *et al.*, 2013). Antioxidant activity can be derived using them either singly or in combination, and some even act synergistically to prevent the oxidation process (Pokorny and Korczak, 2001).

Therefore, the present study was contemplated with a view to evaluate the potential of dried herbs (coarse size) as a natural antioxidant for preventing oxidative rancidity in ghee. Keeping this idea as a central goal, the study was planned with the following objectives.

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

(b) To evaluate the compatible herbs for their antioxidant activity in ghee.

(c) To select the stage and rate of addition of selected herbs in ghee.

(d) To compare combinations of selected herbs as antioxidants in ghee.

(e) To compare effectiveness/ antioxidant potential of the selected herbs in ghee with permitted synthetic antioxidant (BHA).

(f) To analyse the ghee samples for parameters required under FSSAI and AGMARK.

CHAPTER 2

REVIEW OF LITERATURE

Ghee is an anhydrous milk fat and occupies a prominent place in the Indian diet. Ghee, a major dairy product, is the usual Indian name for clarified butter fat. It has an important place in the diet of the people of the Indian sub-continent due to its good flavour, pleasant aroma and granular texture.

2.1 GHEE

Chemically ghee (99.5% 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). Ghee has excellent nutritive, medicinal and therapeutic properties. Milk fat is regarded as good for enhancing grace, memory power, grasping power and power to control senses. It has power of digestion and metabolism (Bhatted and Singh, 2002). A major portion of ghee is utilised for culinary purposes and more preferably as a frying medium to prepare various dishes. For culinary purposes, milk fat is the only animal fat universally acceptable by both 'vegetarian' and 'non-vegetarian' population in India (Patel *et al.,* 2014).

Ghee has better shelf life than other indigenous dairy products due to its low-moisture content. Ghee deterioration and loss of ghee flavour may occur mainly through hydrolytic and/or oxidative rancidification mechanisms (Kuchroo and Narayanan, 1973). However, it undergoes oxidative deterioration that not only affects the economic value of product but also spoils its appetising flavour, making it unpalatable and generating potentially toxic compounds (Rangappa and Achaya, 1974; Mehta *et al.,* 2015).

2.2 RANCIDITY IN RELATION TO NUTRITION

Oxidized fat contains several classes of compounds which have toxic effects. These toxic compounds may be categorized as lipid peroxides, hydroxyl fatty acids and carbonyl compounds like malonaldehyde, cyclic monomers, dimers, polymers, polycyclic aromatic compounds and oxidized sterols (Logani and Davies, 1980;

Nawar, 1996). Acute effect of consuming oxidized fat is diarrhea. The other groups of compounds responsible for acute adverse biological effects are secondary lipid oxidation products. Tissue congestion, fatty degeneration and neurosis were more severe in mice dosed with autoxidized methyl linoleate containing secondary oxidation products than with methyl linoleate hydroperoxides (Patel *et al.,* 2013). Chronic effects of consuming oxidized fat have been summarized by Sanders (1989) which include diarrhea, poor growth rate, myopathy, hepatomegaly, steatites, yellow fat disease, haemolytic anaemia and secondary deficiency of vitamin A and E. Long term effects associated with the consumption of oxidized fats are initiation and promotion of tumor growth. These are mainly because of fatty acid hydroperoxides which are mutagenic (MacGregor *et al*., 1985) and malonaldehydes which is both mutagenic and carcinogenic (Shamberger *et al*., 1974).

The new point of concern is the involvement of lipid peroxides in atherogenesis leading to atherosclerosis. Further, the oxidation products of cholesterol have also shown to be atherogenic in experimental animals (Imai *et al*., 1981). Oxidized cholesterol may be carcinogenic or may promote tumor growth (Patel *et al.,* 2013). It has been suggested that high incidence of coronary heart disease amongst Asian Indian men of Indian descent in Britain may be related to their intake of oxidized cholesterol in ghee (Jacobson, 1987).

2.3 MECHANISM OF DEVELOPMENT OF OXIDATIVE RANCIDITY

Edible fats containing unsaturated molecules are susceptible to attack by molecular oxygen. This process is referred to as lipid oxidation and can give rise to undesirable volatile flavour compounds, potentially toxic oxidation products, and a general deterioration in the quality of the fat. Fat oxidation is influenced by a range of parameters including light exposure, temperature, presence of pro-oxidant metals, presence of antioxidant compounds and the degree of unsaturation of the fat. The volatile flavour products of lipid oxidation include aldehydes, ketones, alcohols, esters, lactones and hydrocarbons, of which the unsaturated aldehydes and ketones are primarily responsible for undesirable oxidized (rancid) flavours. Lipid auto-oxidation is a chain reaction involving initiation, propagation and termination stages. Oxidation of unsaturated fatty acids results in the formation of odourless, tasteless intermediate products known as fatty acid hydroperoxides. These compounds are unstable and can

degrade via a variety of pathways, ultimately yielding volatile flavour products. Inhibiting the progress of lipid oxidation in foods, including milk and milk products, is a key factor in maintaining quality and extending shelf life.

Oxidation plays a fundamental role in the reduction of the quality of fats, oils and many other basic ingredients in food or animal feed. Oxidative rancidity is the major type of rancidity that occurs in ghee. It is caused by the action of oxygen present as air at surface of ghee or in dissolved form. Since the phenomenon of oxidative deterioration once initiated is essentially a self-catalyzed reaction, it is referred to as autoxidation (Nawar, 1996).

The free radical autoxidation is best described in Figure 2.1 (in terms of initiation, propagation and termination reactions). The initiation can occur by the action of external energy sources such as heat, light or high energy radiation or by chemical initiation involving metal ions or metalloproteins such as haem.

The classical route depends on the production of free radicals ($R^{\cdot}$) from lipid molecules (RH) by their interaction with oxygen in the presence of a catalyst. The free radical ($R^{\cdot}$) produced in the initiation step can then react to form a lipid peroxy radical ($ROO^{\cdot}$) which can react further to give the hydroperoxide (ROOH). The reaction of propagation steps also provides further a free radical $R^{\cdot}$, making it a self-propagating chain process. In this way a small amount of catalyst e.g. copper ions, can initiate the reaction, which then produce many hydroperoxide molecules, which ultimately breakdown to a variety of saturated and unsaturated compounds to giving rise to oxidized flavours. The self-propagating chain can be stopped by termination reactions, where two similar or dissimilar radicals combine to give products which do not feed the propagating reaction (Hamilton, 1989).

The hydroperoxides formed in the autoxidation of unsaturated fatty acids are unstable and readily decompose. The main products of hydroperoxides decomposition are saturated and unsaturated aldehydes. Frankel (2005) had indicated the production of primary oxidation and secondary oxidation products with time during autoxidation which is shown in Figure 2.2.

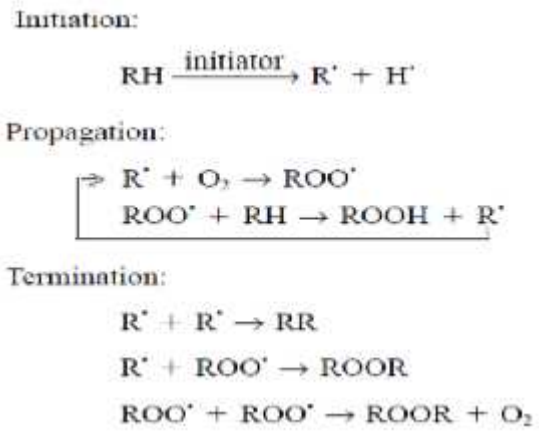


Figure 2.1: Autooxidation of fatty acids.

The mechanism suggested for the formation of aldehydes involves cleavage of the isomeric hydroperoxide to the alkoxy radicals, which undergone carbon to carbon fission to form aldehydes (Frankel *et al*., 1961). In addition to aldehydes, other secondary products of lipid oxidation such as unsaturated ketones, saturated and unsaturated alcohols, saturated and unsaturated hydrocarbons and semi aldehydes have been observed in the decomposition of hydroperoxides of oxidized lipid system (Mehta *et al.*, 2015).

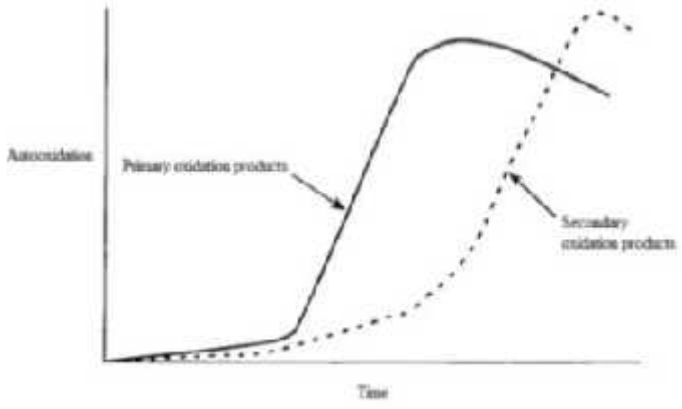


Figure 2.2: Theoretical development of primary and secondary oxidation products as a function of time in lipid oxidation (Frankel, 2005).

Oxidation plays a fundamental role in the reduction of the quality of fats and oils. Initially a lipid radical is formed in this oxidation process, which requires a significant amount of energy. Therefore, autoxidation is strongly accelerated by increased temperatures. Once the lipid radical is formed it will react with other radicals such as triplet oxygen. For singlet oxidation the reaction does not proceed via a radical type

reaction. The singlet state oxygen reacts directly with the unsaturated fatty acid via a concerted `ene' addition mechanism.

A significant amount of research data is available on the impact of autoxidation caused by the most abundant and stable form of oxygen: triplet oxygen ($^3O_2$). However, an alternative oxidation process occurs when food components are exposed to a light source, even at low temperatures. Many molecules are able to absorb energy from light after which this energy is transferred to an oxygen molecule. This process leads to the conversion of the most abundant triplet oxygen into the more reactive singlet oxygen ($^1O_2$) (Dyck, 2010). The intermediates in both the cases, however, are lipid hydroperoxides only. These are odourless and tasteless compounds which readily decompose to small molecules yielding off-flavours (Frankel, 1980).

Oxygen is thus inserted at either end carbon of a double bond, which is shifted to yield an allylic hydroperoxide in the trans configuration. The oxidation by singlet oxidation or photo-oxidation proceeds by a different mechanism than free radical autoxidation and has been recognized as an alternative to the free radical chain reaction (Korycka-Dhal and Richardson, 1980). According to this mechanism, oleate produces a mixture of 9- and 10- hydroperoxides, linoleate produces a mixture of 9-, 10-, 12-, and 13-hydroperoxides and linolenate produces a mixture of 9-, 10-, 11-, 12-, 13-, 15- and 16-hydroperoxides. Once the initial hydroperoxides are formed, the free radical chain reaction prevails as the main mechanism (Frankel, 2005).

Metals in traces are believed by some workers to be essential to initiate the autoxidation reaction. These react with molecular oxygen and give it a negative charge, making its further reaction with unsaturated fats (Heaton and Uri, 1961). They appear to function by increasing the rate of formation of free radicals. Metal ions may react with molecular oxygen to form radical ions which can initiate chain reactions. Direct reaction of metal with fatty substrate to yield free radicals may also occur. Metals which show high catalytic activity, such as copper and cobalt abstract hydrogen from the unsaturated substrate directly, while metals like iron and nickel do so from the hydroperoxides formed. All these metals have an inner incomplete group of electron, hence they can pass into a higher valence state but still retain the power to react further with oxygen which they transfer to the substrate, thus oxidizing it, while themselves reversing to the lower valence oxidation state (Uri, 1961).

2.4 INHIBITION OF LIPID OXIDATION

Optimum oxidative stability can be achieved by minimizing exposure of lipids and lipid-containing food products to air, light and higher temperatures during processing and storage. Theoretically, the most elegant way of preserving fatty foods from oxidative spoilage is to remove all oxygen from the food during manufacture and from the packaging container. Modern packaging material and equipment, allows inert-gas vacuum packaging, but residual oxygen levels of less than 1% are extremely difficult to obtain in a production environment (Loliger, 1991). The free radical chain process of autoxidation can be retarded by two categories of inhibitors: chain-breaking inhibitors and preventive inhibitors. Radical scavengers usually donate one electron to the unpaired electron of the free radical and thus reduce it (Frankel, 1980).

2.5 ANTIOXIDANTS

Mehta (2006) defined antioxidant as a molecule which remove radicals from the system either by reacting with them to yield harmless products or by disrupting free radical chain reactions. In broader terms, Halliwell and Gutteridge (1995) defined an antioxidant as "any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or inhibit oxidation". This emphasizes the source of oxidative damage in the characterization of an antioxidant (Halliwell *et al*., 1995). Krinsky (1992) defined biological antioxidants broadly as compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations". In general, oxidizable substrates include lipids, proteins, carbohydrates and DNA.

Antioxidants can be classified according to the mechanism of action to reduce lipid oxidation into two groups: - First is the primary (chain-breaking) antioxidant that acts as hydrogen donors to the lipid free radical formed during the lipid oxidation (Figure 2.3) and rearrange to a stable conformation. The primary antioxidants (AH) react with lipid peroxyl radicals ($ROO^{\bullet}$) and convert them to more stable, antioxidant radicals ($A^{\bullet}$). The primary antioxidant is able to scavenge lipid radicals, e.g.:

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

Antioxidant radicals are stable due to delocalization of the unpaired electron around a phenol ring and cannot easily react with fatty acids. They are able to terminate radical chain process by reacting with radicals, e.g. these free radical interceptors react with

peroxy radicals (ROO•) to stop chain propagation; thus, they inhibit the formation of peroxides.

$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA$

The most effective antioxidants interrupt the free radical chain reaction and usually contain aromatic rings capable of donating $H^{\bullet}$ to the free radical formed during lipid oxidation. The formed antioxidant radical is stabilized by delocalization of the unpaired electron around the phenol ring to form a stable resonance hybrid (Figure 2.4).

Second is the secondary (preventive) antioxidant, in opposite to the primary antioxidants, do not break free radical chain or do not convert free radicals into stable molecules. Secondary antioxidant act through various mechanisms to slow the rate of oxidation reactions, as: reducers and chelators of metal ions, provide H to primary antioxidants, decompose hydroperoxide to nonradical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers (Gad and Sayd, 2015).

Tsuchihashi *et al*. (1995) proposed that the antioxidant potency is determined by several factors such as intrinsic chemical reactivity of the antioxidant toward the radical, site of generation and reactivity of the radicals, site of antioxidant, concentration and mobility of the antioxidant at the microenvironment, stability and fate of antioxidant-derived radical, and interaction with other antioxidants.

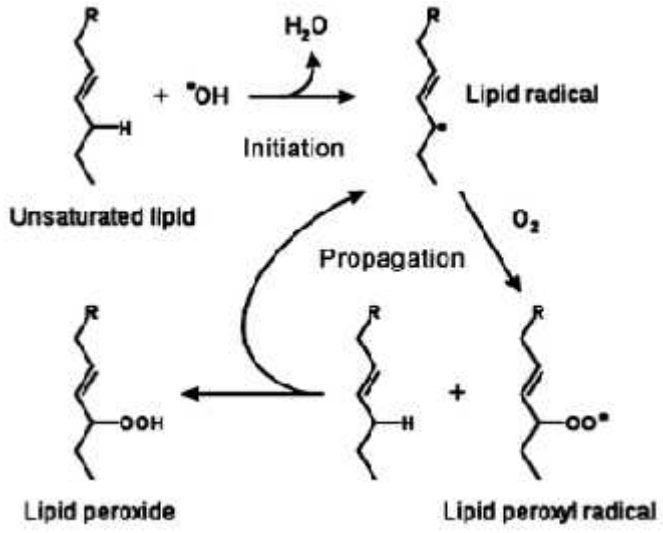


Figure 2.3: Unsaturated lipid oxidation formed lipid free radical.

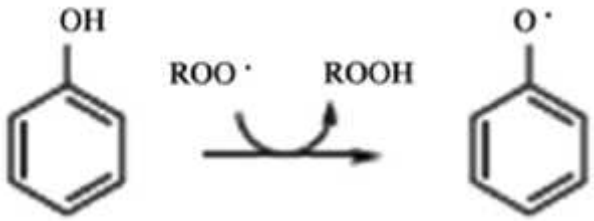


Figure 2.4: The formed antioxidant radical is stabilized by delocalization of the unpaired electron around the phenol ring to form a stable resonance hybrid.

Lipids are generally susceptible to oxidation, which produces undesirable volatile compounds and causes detrimental flavour effects in foods. Moreover, reactive oxygen species such as hydroxyl (-OH•), or peroxyl (ROO•) radicals, formed in human tissue cells by many endogenous and exogenous causes, produce extensive oxidative damage, which in turn, may contribute to aging, cancer, and other human diseases (Aruoma, 1999; Reaven and Witzum, 1996). To control and reduce oxidative damage, nature makes use of several types of compounds, known as antioxidants, which react rapidly with free radicals at one of the stages of an oxidative sequence, to retard or decrease the extent of oxidative deterioration (Krinsky, 1992). Several workers have done exhaustive work to improve the stability of ghee against autoxidation through feeding specific feed to milch animals (Rama Murthy and Narayanan, 1972; Tandon, 1977), altering processing parameters (Rama Murthy *et al*., 1968; Singh *et al*., 1979), using proper packaging materials and storage conditions (Chauhan and Wadhwa, 1987), adding milk components (Gupta *et al*., 1979; Bhatia *et al*., 1978), adding synthetic antioxidants (Kuchroo and Narayanan, 1972; Chatterjee, 1977), incorporating natural antioxidants from edible plant materials, spices and condiments, aromatic herbs, etc. (Ahmad *et al*., 1960; Amr, 1990). To prevent or retard the oxidative deterioration of foods, natural or synthetic antioxidants have been used as additives in fats and oils.

Polyphenols are very active in this respect and the radical-scavenging activities of gallates, nordihydroguairetic acid and flavonoids arise from this process. Aromatic amines inhibit the autoxidation via the same electron-transfer mechanism. Quinones (vitamin K, ubiquinone) are also chain-breaking inhibitors (antioxidants) of autoxidation acting as electron-acceptor antioxidants by competing with oxygen for alkyl radicals. Alkyl radicals react extremely rapidly with oxygen under atmospheric conditions. Peroxide decomposers such as thioethers, methionine and thiodipropionic

acid and its esters prevent the formation of free radicals for initiation of new chain reactions (Frankel, 1980).

2.5.1 Mechanism of Antioxidants

Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $^{\cdot}O_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentrations (Nawar, 1996).

To prevent the oxidation, antioxidant acts effective way by acting at different stages of lipid oxidation such as initiation, propagation, and termination. To formulate an effective antioxidant, it must prevent chemical reactions in Figure 2.5, shown with an X mark, to proceed. To be an effective antioxidant, the formulation must contain a combination of antioxidants (e.g., a free radical scavenger and an oxygen scavenger) (Shahidi, 2015).

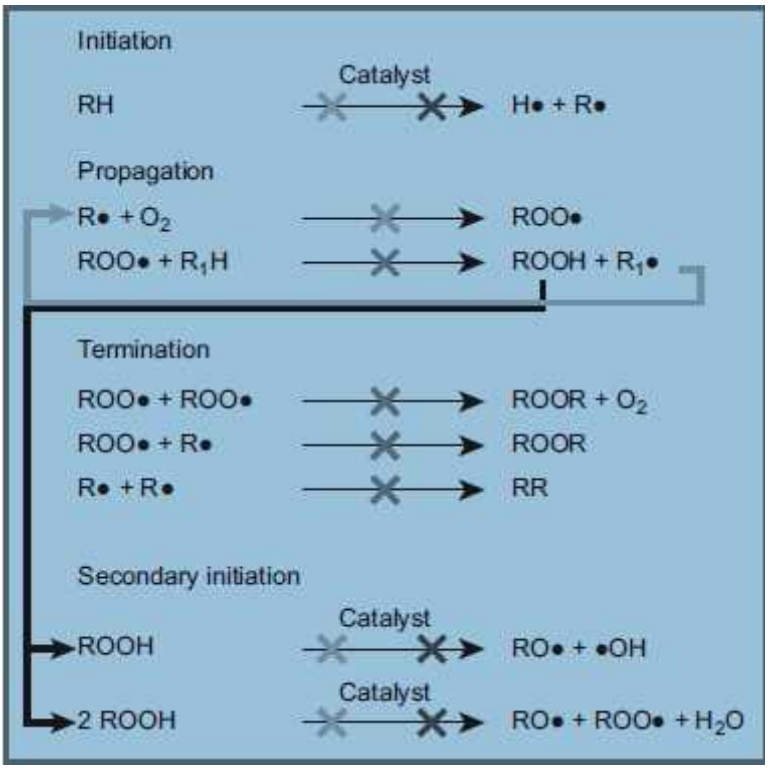


Figure 2.5: Specific sites where antioxidants can be effectively applied to slow down or inhibit oxidation of fats and oils, as shown by an X mark. (1) Initiation

stage: employ oxygen scavengers and/or chelating agents (2) Propagation stage: use oxygen and/or free radical scavengers (3) Termination stage: apply free radical scavengers.

2.5.2 Synthetic Antioxidants Reported for Ghee

Consumers are concerned about the safety of their food and about potential effects of synthetic additives on their health. Despite the superior efficacy, low cost, and high stability of synthetic antioxidants in foods, the suspicion that these compounds may act to promote carcinogenicity has led to a decrease in their use.

Synthetic antioxidants are monohydric or polyhydric phenols with various ring substitutions. These phenolic antioxidants perform the function of capturing free radicals. Use of these compounds is very well recognized. Some of the examples are butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DOG), etc. BHA and BHT are strongly lipophilic and are used extensively in oil-in-water emulsions. BHA and BHT are also typically used together in mixtures, acting synergistically (Nawar, 1996). The addition of the antioxidants to food is usually restricted to 0.02 % on fat basis (AOAC, 1997). For addition in ghee BHA is permitted at the rate of 0.02 % under FSSAI (2011).

Synthetic (BHA 0.02% and TBHQ 0.02%) antioxidants incorporated ghee was found effective in inhibiting the development of oxidative degradation products such as peroxide value, conjugated dienes and free fatty acid (Purohit, 2011). Sampath and Anantkrishnan (1957) reported that the addition of butylated hydroxy anisole (BHA) and ethyl gallate (EG) in small quantities greatly reduced the peroxide development and loss of vitamin A in ghee.

Kuchroo and Narayanan (1972) studied the effect of some synthetic antioxidants, namely propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DOG), butylated hydroxyl anisole (BHA) and butylated hydroxy toluene (BHT), singly (0.005-0.02 %) or in combination of two (mixture not exceeding 0.02 %) with or without phospholipids, on oxidation of ghee. The results revealed that the efficiency of antioxidants decreased in the sequence PG > OG > DOG > BHT > BHA and on addition of mixtures of two antioxidants (0.01 % each), the protective factor decreased in the sequence: BHA+PG > BHT+PG > BHA+BHT > BHT+OG > BHA+OG > BHT+DOG > BHA+DOG. Among the mixtures of antioxidants studied,

only BHA + PG and BHA+ BHT gave a synergistic effect. The addition of phospholipids either to an individual or mixture of antioxidants increased protective factor. Among the antioxidants studied phospholipids gave synergistic effect only with BHA+ PG and BHT+PG mixture. Further, Kuchroo and Narayanan (1973) found BHT to be more effective than BHA over 12 months of storage period.

BHA was found to be more capable of retarding oxidative degradation in ghee than the aqueous and ethanolic extracts of herbs (vidarikand, shatavari and ashwagandha) (Pawar *et al.*, 2014). Chatterjee (1977) observed that under commercial conditions, BHA (0.01 %) could improve the aroma, flavour and shelf life of ghee irrespective of season of production. Gupta *et al.* (1979) found that antioxidant potentialities of certain compounds tested were in order: hydroquinone > catechol > resorcinol. The strong effect of hydroquinone could be due to formation is a stable quinone which can terminate the chain reaction.

Lal and Narayanan (1980) concluded 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. The stability of antioxidants decreased with increase in storage period of ghee (Lal and Narayanan, 1980; Sree and Lal, 1990). The losses of antioxidants observed during storage might be ascribed to the scavenging of antioxidants by the free radicals formed during autoxidation of ghee (Sree and Lal, 1990).

Rao *et al*. (1984) observed that addition of BHA or BHT (0.02 %) or BHA + BHT (0.01 % each) had a retarding effect on the development of both free fatty acids and peroxide values in buffaloes' ghee during storage at room temperature (18-36ºC). All the three antioxidant treatments had a similar effect upon free fatty acid development but peroxide development was retarded to a greater extent by BHT or BHA plus BHT than by BHA. Pande and Verma (1989) studied the efficacy of BHA, BHT and Tertiary butyl hydroquinone (TBHQ) as antioxidants in ghee for different storage periods. They found that when ghee added with these antioxidants was stored in amber coloured plastic bottles, BHA exhibited better antioxidant property in the first month of storage while BHT and TBHQ had similar antioxidant effect after the end of 60 days and only TBHQ exhibited maximum effect after 90 days of storage. Thus, they indicated that TBHQ could be used for long term storage of ghee.

2.5.2.1 Health significance

Synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are widely used in many foods. However, their use has been questioned because of issues related to toxicity and carcinogenicity. These substances may be inappropriate for chronic human consumption because of their possible toxic properties for human health and the environment (Purohit, 2011). Continuous use of synthetic antioxidants may cause health hazards such as carcinogenic and mutagenic effects in experimental animals (Hathway, 1966; Maeura *et al.*, 1984). For this reason, considerable attention has been given to the application of natural antioxidants in foods, because of their potential nutritional and therapeutic effects.

2.5.3 Natural Antioxidants Reported for Ghee

Consumers are concerned about the safety of their food and about potential effects of synthetic additives on their health. Despite the superior efficacy, low cost, and high stability of synthetic antioxidants in foods, the suspicion that these compounds may act to promote carcinogenicity has led to a decrease in their use (Namiki, 1990).

The use of plant products as food and medicinal remedies since ancient times is partially attributed to the biological efficacy of secondary metabolites that possess antioxidant activities such as phenolic compounds, vitamins C and E, and carotenoids. Phenolic compounds, which are derived from the shikimate and phenylpropanoid pathways, constitute a diverse and ubiquitous class of plant secondary metabolites characterised by an aromatic ring and one or more hydroxyl groups (Robards *et al*., 1999).

The empirical use of natural compounds as antioxidants is very old. Natural antioxidants are found in almost all plants, microorganisms, fungi and even in animal tissues. The majority of natural antioxidants are phenolic compounds, except tocopherols. The most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids and carotenoids (Pokorny and Korczak, 2001). These are excellent antioxidant at low concentrations, but at high concentrations its ability to reduce metal initiators can actually lead to a prooxidant effect (Frankel, 1995). Extensive research has been dedicated to identification of antioxidants from various natural sources. Ascorbic acid and tocopherols are the most important commercial

natural antioxidants. Other sources of natural antioxidants include carotenoids, flavonoids, amino acids, proteins, protein hydrolysates, Maillard reaction products, phospholipids, and sterols. Numerous naturally occurring phenolic antioxidants have been identified in plant sources and vegetable extracts. Enzymes also play important roles as antioxidants.

A favorable trend towards natural products has developed due to reports from medicinal centres regarding the potential teratogenic, carcinogenic and mutagenic effects of synthetic antioxidants in experimental animals including primates. Hence, due to increased reservations such as government regulations and toxicity of using synthetic antioxidants, the use of naturally occurring antioxidants hold good promise (Hathway, 1966). The complied literature on edible plant and milk materials used for protecting ghee against oxidative deterioration is given below:

Ghee residue is obtained as a by-product during making. It is a dark brown or brown material left after filtration of ghee. Narayanan *et al.* (1966) found that cows' and buffaloes' ghee prepared from cream or butter by heating to 120^0C had a bland flavour and contained only traces of phospholipids and the rest being left in the ghee residue. Therefore, a systematic study was undertaken by Rama Murthy *et al.* (1969) to see the effect of addition of different concentrations of ghee residue on phospholipids free ghee. In such studies, ghee residue having 6.012 % phospholipids was added at different levels (0 to 50 %) to phospholipids free ghee and stored at 37^0C for varying periods. It was observed that in control samples (phospholipids free ghee without ghee residue), the peroxides appeared at an early stage of storage (2 months) than those samples with added ghee residue. For instance, the peroxide values (millimoles of peroxide per kg fat) in buffalo ghee samples at 6 months of storage were, control (8.4), ghee with 1.0 % ghee residue (1.6), ghee with 2.0 % ghee residue (1.1) and ghee with 5.0 % ghee residue (0.2). It was also observed that ghee samples with 5.0 % ghee residue showed better oxidative stability than those having lower levels of ghee residue. It was further noted that in all the control samples, the rate of increase of peroxide value was considerably higher in buffalo ghee (0.3, 2.8 and 8.4 at 2, 3 and 6 months of storage respectively). The higher peroxide values in control samples of buffalo ghee as compared to cow ghee were interpreted to be due to the differences in the natural antioxidants content like tocopherol in cows' and buffaloes' ghee.

Santha and Narayanan (1978) reported that the antioxidant property of ghee residue was dependent on the temperature of clarification, and method of preparation of ghee. With increase in temperature of clarification the antioxidant property decreased. The antioxidant efficiency of ghee residue obtained by different methods of preparation decreased in the following sequence: creamery butter ghee residue> desi butter ghee residue> direct cream ghee residue. The lipid fraction especially the acetone-insoluble fraction of ghee residue had the greatest antioxidant effect (Pagote and Bhandari, 1988). Santha and Narayanan (1979) reported that phospholipids, the lipid constituents of ghee residue had maximum antioxidant property followed by α-tocopherol and vitamin A. Among the non-lipid constituents, the amino acids- proline, lysine, cysteine hydrochloride and tryptophan showed antioxidant property. The addition of lactose, glucose, galactose and their interaction products with protein and phospholipids to ghee increased the oxidative stability of ghee (Pagote and Bhandari, 1988). Hence, it was concluded that the antioxidant property of ghee residue is due to its above mentioned various constituents.

Bhatia *et al.* (1978) isolated phospholipids from sunflower seed, groundnut seed and cotton seed and added to ghee. The antioxidant potentiality of whole phospholipids from these sources was in order: sunflower > groundnut > soybean > cotton seed. This was in order of decreasing phosphatidyl ethanolamine content. Gupta *et al.* (1979) isolated lecithin and phenolic compounds from gram seeds (*Cicer arietinum*). They observed that phospholipids from this source could be good antioxidant for ghee.

Kaur *et al.* (1982) compared the seed phosphatides and synthetic compounds as antioxidants for cow and buffalo ghee. They found that antioxidant efficiency of sunflower seed oil phosphatides and synthetic compounds were in order: phosphatidyl ethanolamine > propyl gallate > palmitoylascorbate > BHA > phosphatidyl choline. The authors concluded that seed phospholipids were more effective than many synthetic antioxidants in controlling oxidative and lipolytic deterioration of ghee during storage.

Dinesh *et al.* (2000) isolated the antioxidant principles namely phenolics and phospholipids from MSKP (Dried mango seed kernel powder) using organic solvents. These compounds were dissolved in ghee to prepare phenolic and phospholipids extracts separately and in combination. Addition of extract in combination was more effective than individual extract. Moreover the phenolics were more effective than

phospholipids in prolonging the induction period of ghee. Addition of extracts either individually or in combination at a level of 5 % or above were more effective in increasing the stability of ghee than addition of BHA at a 0.02 % level. It was concluded that the phenolic compounds in MSKP seemed to be the main anti-oxidative compounds which along with phospholipids gave the maximum stabilizing effect to ghee against oxidative deterioration.

Ghee with added clove, green tea and coriander extracts showed higher induction period as compared to ghee containing vidarikand, ashwagandha, shatavari or BHA (Patel *et al.*, 2014). Betel and curry leaves when added at 1.0 per cent level to ghee showed higher resistance to oxidative deterioration than BHA and BHT mixture (Patel and Rajorhia, 1979). Ethanol extracts of peanut skins, pomegranate peels and olive pomace gave good antioxidant activity during accelerated oxidative incubation of ghee. The results revealed that ethanolic extracts, at a concentration of 200 ppm, could be used to retard fat auto-oxidation (El-Shourbagy and El-Zahar, 2014).

Asha *et al.* (2015) evaluated the antioxidant activities of BHA and orange peel powder extract in ghee stored at different storage temperatures (T1:6±2°C; T2: 32±2°C; T3:60±2°C) for a period of 21 days. Ghee incorporated with orange peel extract showed stronger activity in quenching DPPH radicals and least development of peroxide value, TBA and FFA than ghee incorporated with BHA and control.

Tomato seed powder added at 5.0 % level in ghee inhibited oxidation and ensured its stability practically to the same extent as 0.01 % of BHT or BHA (Guleria *et al.*, 1983). Jain (1996) elucidated the effect of addition of antioxidant principles of onion (*Allium cepa*) skin via pre-extract on the oxidative stability of ghee. The anti-oxygenic compounds of onion skin were extracted into methanol and dried. The dried material was mixed with ghee at a rate of 0.5 % (w/v). Addition of such extracts at different levels was found to be almost at par with addition of BHA at 0.02 % in protecting ghee.

Kaur *et al.* (2001) studied the use of Sorghum (*Sorghum bicolor* L.) grain powder in enhancing the oxidative stability of ghee. Direct addition of Sorghum grain powder (SGP) at different levels in ghee was elevated the phospholipids as well as water extractable phenolic compounds of ghee. The results also revealed that addition of SGP at a level of 1 % (w/v) and above have higher effect than the addition of

permitted level of BHA. The proactive action of SGP in ghee could be attributed to the transfer of phospholipids and phenolic compounds present in SGP.

Mehta (2006) reported that addition of methanol pre-extract of de-husked ragi powder (DRP) at the rate of 0.1%, 0.25% and 0.5% resulted in a corresponding increase (over control) in phospholipids content and water extractable phenolics content of ghee. The anti-oxygenic indices calculated from the induction periods of ghee samples stored at 80 ± 2^0C in comparison with sample of ghee added DRP gave better result than control sample in order to prevent oxidative rancidity. This result suggested that the phospholipids and the phenolic compounds of DRP transferred to ghee enhance its oxidative stability.

2.6 HERBS AS A SOURCE OF NATURAL ANTIOXIDANTS

The mere mention of natural antioxidants brings about an association with herbs, because they are the most important targets in the search for natural antioxidants from the point of view of safety. Man has used them not only for flavouring foods but also for antiseptic and medical properties since the prehistoric era. Herbs have been used for flavour, colour and preservation of foods and beverages for several hundred years. Herbs have also been used for medicinal purposes for a very long time. They are also one of the best sources of natural antioxidants because they contain potent compounds that have been shown to impart antioxidative effects in food (Carlsen *et al.,* 2010). They possess antioxidant activity because they contain effective antioxidant compounds (hydroxyl chavicol, eugenol, linalyl acetate, triterpenes, flavonoids, limonene, polyphenolics, coumarins, saponins, ascorbic acid and beta carotene).

Herbs are derived from plants and are considered among the best sources of natural antioxidants (Schwarz *et al*., 2001; Suhaj, 2006; Abbasi *et al*., 2016). Herbs are the green, fresh and dried leaves from temperate plants, while spices are the flowers, fruit, seeds, bark, and roots of tropical plants that typically provide more pungent and strong flavours than herbs.

Polyphenols constitute a large group of naturally occurring substances in the plant kingdom, which include the flavonoids. The plant phenolics are commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts. These substances have considerable interest in the field of food chemistry, pharmacy and medicine due to a wide range of favourable biological effects including antioxidant

properties. The antioxidant property of phenolics is mainly due to their redox properties. They act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelators (Cook and Samman, 1996). Evidence suggests that intake of antioxidant nutrients from food sources offers health advantages (Aruoma, 1998).

The essential oils from a number of herbs were also studied for antioxidative activity, e.g. coriander, dill, tulsi, betel leaves, lemon grass, centella and ajmapan. Although the compounds in the essential oils such as linalool from mint, eugenol from tulsi, caryophyllene from centella possess antioxidant activity, the aromatic character of these compounds limits the use of the essential oils as antioxidants in foods (Aruoma, 1999). From time immemorial, herb extract were used for preserving the quality of soybean oil, beef, meat, poultry, fish, and lard. Simple phenols, polyphenolics and phenolic acid derivatives are the antioxidants that are common to all plant sources. However, fortification by herbal extract in dairy products is a newly emerged area (Bandoyopadhyay *et al.,* 2008).

Amr (1990) studied the effect of addition of four aromatic herbs on oxidative stability of ghee made from Ewe's milk. Aromatic herbs namely, rosemary (*Rosmarinus officinalis*), sage (*Artemisia herballa*), fennel (*Foeniculum vulgare*) and rue (*Ruta graveatons*) were added at 7.5 % level to the ghee, only rosemary showed an antioxidative effect equivalent to that of BHA+ BHT (1:1, 250 ppm). All these herbs had an antioxidant effect at least for first 24 h of storage.

Betel and curry leaves when added at 1.0 % level to ghee showed higher resistance to oxidative deterioration than BHA and BHT mixture. The antioxidative properties of betel and curry leaves were attributed to phenolic compounds, predominantly hydroxyl chavicol (Patel and Rajorhia, 1979). These leaves also contained some ascorbic acid which might work as synergist (Sethi and Aggarwal, 1956). When betel, curry and drumstick leaves were added at 1.0 and 3.0 % levels to ghee, which was subsequently stored for 12 m at ambient temperature, only curry leaves could protect ghee from hydrolytic rancidity and none could prevent oxidative deterioration (Thakar *et al*., 1984).

Coriander extract and oleoresin were significantly effective in retarding the oxidative deterioration of ghee relative to control. However, these were less effective than

butylated hydroxyanisole (BHA). During deep fat frying, steam distilled extracts showed higher antioxidant activity compared to oleoresin and BHA (Patel *et al.*, 2013). Pawar *et al.* (2012) compared the potential of *Asparagus racemosus* (shatavari) extract with natural (rosemary, green tea) and synthetic (butylated hydroxyanisole, tert-butyl hydroquinone) antioxidants, in ghee using accelerated oxidation tests.

Sharma (1997) isolated the antioxidant principles of Tulsi (*Ocimum sanctum* Linn.) leaves via a pre-extraction. The anti-oxygenic compounds of Tulsi leaves were extracted into methanol and then vacuum dried. The dried materials were further fractionated into water insoluble fraction which was then treated with mixture of silica gel and charcoal and designated at SCF. Addition of SCF pre-extract at the level of 0.6% (w/v) was found to be more effective than the addition of BHA at the level of 0.02%. The phenolic compounds appeared to be the main contributory factors in enhancing the oxidative stability of ghee.

The antioxidant activities of vidarikand (*Pueraria tuberosa*), shatavari (*Asparagus racemosus*) and ashwagandha (*Withania somnifera*) extracts (aqueous and ethanolic) were evaluated and compared with BHA using β-carotene bleaching assay, DPPH assay and Rancimat method (Pawar *et al.*, 2014). The ethanolic extracts showed more antioxidant activity (β-carotene–linoleic acid model system and DPPH system) than their aqueous counterparts. Antioxidant activity of the herbs decreased in the order vidarikand > ashwagandha > shatavari.

Patel *et al*. (2014) evaluated antioxidant potential of ashwagandha, clove, coriander, green tea, shatavari and vidarikand extracts as compared to BHA. Clove, coriander and green tea extracts showed significantly higher antioxidant activity than vidarikand, shatavari and ashwagandha extracts. Ghee with added clove, green tea and coriander extracts showed higher induction period as compared to ghee containing vidarikand, ashwagandha, shatavari or BHA.

Houhoula *et al*. (2003) studied the effect of oregano on the oxidative stability of cottonseed oil during frying of potato chips. The storage stability of potato chips was also monitored. Two forms of oregano were used in this study: ground oregano and an ethanol extract. These were added to the oil before frying. The ground oregano and the ethanol extract decreased the amount of conjugated dienes, polar compounds, dimeric and polymerized triacylglycerols and the *p*-anisidine value of the frying oil.

Both ground oregano and ethanol extract depressed the formation of peroxide and of conjugated dienes in the oil of potato chips, thus increasing their shelf life.

The antioxygenic compounds from two varieties of Tulsi (Ocimum sanctum Linn. 'Sri Tulsi' and 'Krishna Tulsi') leaves powder were extracted with methanol. After vacuum drying they were fractionated into water soluble fraction (WSF) and water insoluble fractions (WIF), The WSF carried pro-oxidant principles whereas the WIF exhibited good antioxygenic properties. WIF was further treated with silica gel and charcoal mixture to remove colour-Imparting pigments and was designated as silica gel charcoal treated fraction (SCF). The SCF fractions from 'Sri' and 'Krishna TUlsi' leaves powder respectively contained on dry matter basis the total phenolics of 107.53 mg/g and 154.13 mg/g with negligible amounts of phospholipids. 'Krishna Tulsi' leaves exhibited slightly higher antioxygenic activity as compared to 'Sri Tulsi' leaves. The addition of SCF pre-extract of 'Krishna Tulsi' leaves powder at a level of 0.6% (w/v) into creamery butter ghee was found almost equally effective as that of BHA at a level of 0.02% In preventing autoxidation for a period of 8 days (192 h) at 80 ± 2°C storage or until the peroxide value of 5 meq of peroxide oxygen was reached. The phenolics present in the 'Tulsi' leaves appeared to be the main contributory factors in extending the oxidative stability of ghee (Merai *et al.,* 2003).

2.6.1 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).

Oueslati *et al.* (2016) estimated total phenolic content of aqueous and ethanolic extracts (70%) of *Trachyspermum ammi* and found that the total phenolic content in aqueous extract of the *T. ammi*, as estimated by the Folin-Ciocalteu method, had the highest level (162.17 mg GAE per g DW) followed by the ethanolic extract (89.9 mg GAE per g DW). Gruyal (2014) evaluated DPPH activity of methanolic extract of *Coleus aromaticus* and showed that it scavenged the radicals at 55.62%.

2.6.2 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).

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).

Jamal *et al.* (2010) studied forty types of Malaysian medicinal plants and found that *Piper betel* L. had phenolic content of 8986.67 mg per liter GAE. Jaiswal *et al.* (2014) collected six different variety of betel leaf [Banarasisafeda (PA), Calcutta (PB), Cuttack (PC), Desibagla (PD), Maharashtra (PE) and Sofia (PF)] from Uttar Pradesh, West Bengal, Odisha, Maharashtra and Bihar and were extracted using five solvents (80% methanol, 80% ethanol, 80% acetone, 80% ethyl acetate and distilled water) to determine total phenol content and antioxidant activity. The TPC for six variety betel leaf extract in five different solvents were found in the range of 0.29 to 2.62 mg GAE per g dw for PA, 0.08 to 2.59 mg GAE per g dw for PB, 0.09 to 2.38 mg GAE per g

dw for PC, 0.07 to 2.87 mg GAE per g dw for PD, 0.16 to 2.07 mg GAE per g dw for PE and 0.04 to 1.16 mg GAE per g dw for PF. The DPPH scavenging activity for six variety betel leaf extract in five different solvents were found in the range of 7.20 to 133.5 mg CE per g dw for PF, 127.5±0.25 mg CE/g dw for PE, 77.71±1.62 for PD, 74.96±1.8 for PC, 55.37±1.25 for PB and 2.48 mg CE/g dw for PA. The highest level of DPPH activity was observed for 80% ethanol (for PC, PD, PE and PF variety) and 80% methanol extract. Tamuly *et al.* (2013) obtained IC_{50} value of 36.45 µg per ml for methanol: water (1:1) extract of *P. betel*.

2.6.3 Centella

The *Centella asiatica* L. belongs to the family Apiaceae or Umbelliferae, a small creeping perennial herbal plant that flourishes in wet areas of Malaysia, Indonesia, India, and other parts of Asia including China. The herb is also known as pegaga in Malaysia, Indian pennywort and Gotu Kola in Europe and America, mandookaparni in India, pegagan or kaki kuda in Indonesia, Luei Gong Gen or Tung Chain in China. There are several types of *Centella asiatica* that can be found in Malaysia such as Pegaga Cina or Nyonya, Pegaga Daun Lebar, Pegaga Salad and Pegaga Renek. *Centella asiatica* is used in Indian Ayurvedic medicine and in herbal medicine in Malaysia and China, and other part of Asia for hundreds of years (Ullah *et al.,* 2009). Besides being used as a traditional and alternative medicine, Centella is commonly used in these countries as vegetables and drinks as in tea or juice (Brinkhaus *et al.,* 2000).

It contains high total phenolic contents which contributed by the flavonoids such as quercetin, kaempherol, catechin, rutin, apigenin and naringin (Suntornsuk and Anurukvorakun, 2005) and volatile oils such as caryophyllene, farnesol and elemene. Centella leaves are reported to give antioxidant effect using 3 pathways: superoxide free radical activity (86.4%), inhibition of linoleic acid peroxidation (98.2%) and radical scavenging activity (92.7%) (Hasim, 2011).

Mohankumar *et al.* (2017) investigated total phenolic content of selected medicinal plants from Kanyakumari district in which *Centella Asiatica* had highest phenolic content (17.1 mg GAE per g). Leaves of *Centella asiatica* collected from three districts of Assam were analysed for phenolic content and antioxidant activity by Upadhyaya and Saikia (2012) found total phenol content of the ethanolic extracts

between 199.5 and 277 mg per g dry material. DPPH activity was between 73.7% and 88.2% in the ethanolic extract of *C. asiatica*.

2.6.4 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 parts used are ripe, dry fruits (seeds) (ground or whole), essential oil, herb, and oleoresin. The leaves are used fresh (whole or chopped) and dried (whole and crushed). Coriander is one of the most important herbs in the world and is regularly used in the Indian diet.

The major constituent of the oil is D -linalool (55–90%), neryl acetate, γ -terpinene, camphor, α -pinene, and geranyl acetate (Nejad *et al.,* 2010). The major compounds in the plants are tocopherols, carotenoids and chlorophylls and sugars, ascorbic acid, phenolics, flavonols, and anthocyanins (Dias *et al.,* 2011).

Several researches concluded that volatile components in essential oils, from both seeds and leaves of coriander inhibited the growth of a range of micro-organisms (Delaquis *et al.,* 2002) and inhibition of lipid peroxidation (Anilakumar *et al.,* 2001; Tanabe *et al.,* 2002). 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.

Farah *et al.* (2015) evaluated the total phenolic content and antioxidant activity of ethanolic extracts coriander (*Coriandrum sativum*) plants grown in Saudi Arabia. They obtained 0.83 ± 0.02 g GAE per 100 g phenolic content and 89.82% DPPH activity from coriander extract. Paul *et al.* (2013) found total phenolic content of were 63.22±3.59, 62.86±12.81 and 54.17±6.15 mg per g equivalent to gallic acid in coriander using ethanolic extraction, water extraction and petroleum ether extraction respectively.

2.6.5 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 hepato-protective 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% DPPH activity. Devatkal *et al.* (2012) determined antioxidant effect of aqueous extracts of curry leaves in raw chicken patties. Curry leaves extract had total phenolic content of 59.2±0.90 mg per g GAE and 61.4±1.3 % DPPH free radical scavenging activity.

2.6.6 Dill

Dill, *Anethum graveolens*, is a common, aromatic garden herb, known for its culinary and medicinal properties. *Anethum graveolens* (Linn.), commonly known as European dill, is a plant, native to Orient; and Mediterranean - countries and is also found growing wildly in various parts of Africa, Asia, Europe and South Russia. It is cultivated in England, Germany, and United States of America and the cultivated variety is often known as ’garden dill’. The Indian dill plant, usually known as ’sowa’, is indigenous to the Indian subcontinent and is cultivated throughout India.

The essential oil of dill contains carvone, eugenol, linalyl acetate, anethofuran, limonene and coumarins (Isbilir and Sagiroglu, 2011). Stankevicius *et al.* (2010) analysed the phenolic content and DPPH-free radical scavenging capacity of dill leaves (*Anethum graveolens*). The content of phenolic compounds was found in ethanolic extracts of dill (1399.8±2.4) mg of GAE per 100 g of dry plant material and DPPH activity was 51.7%. Abbasi *et al.* (2016) studied phenolic content and antioxidant activity of the aqueous extract of *Anethum graveolens* and showed that dill had 163±3.8 mg GAE per g total phenolic content and 64-86% DPPH activity.

2.6.7 Dodi

Leptadenia reticulata locally known as "Paaledaag" among the Irula tribes, belongs to Asclepiadaceae family, is a branched twining shrub distributed in tropical and sub-tropical parts of Asia. They are mainly found in the sub-Himalayan tracts of Punjab to Sikkim, Khasi hills, Utter Pradesh, Gujarat and throughout the Deccan peninsula up to an altitude of 900 m, particularly in hedges. *L. reticulata* known as Jivanti in Ayurveda (George *et al.*, 2015).

Khasawneh *et al.* (2011) identified the polyphenolic compounds of dodi in various plant extracts by HPLC analysis method. Six polyphenolic compounds (gallic acid, vanillic acid, caffeic acid, epicatechin, trans-cinnamic acid and quercetin-3-β-D-glucoside) were identified in various plant extracts (ethanol, ethyl acetate, n-butanol and water).

Hewageegana *et al.* (2014) studied proximate analysis and standardization of leaves: *Leptadenia reticulata* (retz) wight and they found that the mean total polyphenolic content of methanolic extract of *L. reticulata* was 55.6±0.50 mg GAE per g extract. Phyto-pharmacological evaluation of *Leptadenia reticulata* was carried out by George *et al.* (2015). They extracted the leaves in methanol and found that phenolic content and IC_{50} value were 40 µg GAE per gm and 220 µg per ml respectively in methanolic extract.

2.6.8 Drumstick leaves

Moringa oleifera Lam (Moringaceae) (also known as drumstick tree, horseradish tree) is cul- tivated throughout Pakistan, especially in southern Punjab the tree is widely grown near the houses to sit in the shade. Every part of this miraculous tree is full of nutrition and fresh, tender pods are cooked and eaten as a vegetable, leaves are used for animal feeding and manufacturing of many herbal medicines. The leaves of *Moringa oleifera* contain up to 8% antioxidants on dry matter basis.

Wangcharoen and Gomolmanee (2011) who reported that leaves had higher contents of phenolic compounds (including gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin) and flavonoids (including glucosides, rutinosides, malonylglycosides and acetylglycosides of kaempferol, quercetin and isoramnethin).

Abdulkadir *et al.* (2015) determined the total phenolic content and antioxidant activity of drumstic leaves (*Moringa oleifera* Lam.) in methanolic extract. They found that drumstick extract possessed mean percentage inhibition (DPPH) of 66.85±1.20% and contained 32.83±1.38 mg GAE per g total phenolic content. The antioxidant activity of the leaves of *Moringa oleifera* were investigated by Pakade *et al.* (2013). Fresh *M. oleifera* samples were harvested in April 2011 from two growing areas – Limpopo (LF) and Atteridgeville (AF) – in South Africa. The leaf samples were collected from trees that were planted in different years (2006, 2009 and 2010) and labelled as such, that is, 2006LF, 2009LF, 2010LF and 2010AF. A total of four moringa leaf samples were investigated by extracting their leaves by methanol as a solvent. The DPPH activity for four different moringa samples were found to be in the range of 51.6±15.2 to 59.8±1.7 (percentage of the DPPH concentration left in solution) for 2006LF, 2009LF, 2010LF and 2010AF respectively.

2.6.9 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.

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% DPPH activity. Devatkal *et al.* (2012) determined the total phenolic content and antioxidant activity of aqueous extract of fenugreek leaves in raw chicken patties. Fenugreek leaves extract contained 52.2±0.85 mg per g GAE phenolic content and 64.2±0.78 % DPPH activity.

2.6.10 Jequirity leaves

Abrus precatorius (L.) (Fabaceae family) is native to India and found throughout the tropical regions of the world. It is used in traditional medicine for the treatment of the wide range of ailments. *Abrus precatorius* is a woody twinning plant with characteristic toxic red seeds with black mark at the base. It is native to India, at altitudes up to 1200 m on the outer Himalayas. It is now naturalized in all tropical countries (Bhatia *et al.,* 2013).

Phenolic compounds that possess antioxidant activity are known to be mainly phenolic acids and flavonoids (Wojdylo *et al.,* 2007). It was observed that most of the plant parts showed the presence of Gallic acid and Catechol, while Vanillin, Caffeic acid and p-Coumaric acid were found in leaves and stem. The methanol extract showed high amount of Catechol around 6.3 µg/g to 1309.0 µg/g of dry weight. The leaves of *Abrus precatorius* contain precol, abrol, glycyrrhizin triterpenoids and alkaloids (Jain *et al.,* 2015).

Gul *et al.* (2013) studied total phenolic content of jequirity leaves. They prepared four different extracts using different solvent such as hexane, ethyl acetate, ethanol and water. The Total phenolic content for four different jequirity leaves extract were found in the range of 1.65±0.22 to 25.48±0.62 GAE mg per g. Jain *et al.* (2015) estimated the polyphenolic content and antioxidant activity of different parts of *Abrus precatorius.* They found that methanolic extract of *Abrus precatorius* leaves contained 4.80 mg GAE per g polyphenolic content and 68% DPPH radical scavenging activity.

2.6.11 Lemon grass

Lemongrass (*Cymbopogon citratus*) is a widely used herb in tropical countries, especially in Southeast Asia. Its oil is a yellow or amber liquid containing about 75-85% of aldehydes, chiefly citral, geranial and neral. It is used in aromatherapy. Some of the reported phytochemicals are essential oils that contain citral α, citral β, nerol geraniol, citronellal, terpinolene, geranyl acetate, myrecene and terpinol methylheptenone. Two triterpenoids, cymbopogone and cymbopogonol and flavones identified as luteolin and its 6-C-glucoside have also been isolated from leaves of *C. Citratus* (Hasim *et al*., 2015).

The plant also contains reported phytochemicals such as flavonoids and phenolic compounds, which consist of luteolin, isoorientin 2'-O-rhamnoside, quercetin, kaempferol and apiginin. The compounds identified in *C. citratus* are mainly terpenes, alcohols, ketones, aldehyde and esters. Gruyal (2014) showed that methanolic extract of *Cymbopogon citratus* scavenged the radicals by 75.38% and contained 44.05±0.147 mg GAE per g phenolic content. Hasim *et al.* (2015) prepared extracts of lemongrass leaves (*Cymbopogon citratus*) using 30, 70 and 96% ethanol and determined the total phenolic content and antioxidant activity of the extracts. The highest total phenol content was in the 30% ethanolic extract of 50.017 GAE mg per g and the best IC_{50} value was obtained in the 70% ethanol extract of 79.444 mg per l.

2.6.12 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).

It shows a variety of pharmacological activities, including antiulceric, antioxidative, antiallergic and antiviral (El-sherif *et al.,* 2011). Tupe *et al.* (2013) evaluated the radical-scavenging activity and total phenolic content of liquorice extract (methanolic). They found that liquorice contained 71.29±4.67 mg GAE/g total phenolic content and 87.64±0.7% DPPH activity. The DPPH-radical-scavenging

activity of the methanolic extracts of licorice from different habitats determined by Karahan *et al.* (2016). The IC_{50} values of the extracts were found to be between 588±0.86 µg/ml and 2190±1.73 µg/ml.

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

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

Total phenolic content of six wild menthe species (*M. aquatica, M. arvensis, M. piperita, M. pulegium, M. rutendifolia and M. villosa*) from Algeria determined by Benabdallah *et al.* (2016). The methanol extract of Algerian mints contained total phenolic content in the range of 14.66±0.30 mg GAE per g dw to 43.21±1.09 mg GAE per g dw. The highest free radical scavenging activity (34.21%) was observed by Al-juhaimi and Ghafoor (2011) for the diethyl ether extract of mint leaves. Gruyal (2014) evaluated the free radical scavenging capacity of *Mentha arvensis* extract (methanol) which scavenged radicals at 93.48%.

2.6.14 Shatavari

Asparagus racemosus is an indigenous medicinal plant of the family Liliaceae (Pawar *et al.*, 2014) is important for its sapogenin content (Subramanian and Nair, 1968), the precursor of many pharmacologically active steroids. This species occurs widely throughout the tropical and subtropical regions. Several authors (Subramanian and

Nair, 1968; Pawar *et al.*, 2014; Sachan *et al.*, 2012) have shown that the species from different localities often differ in their chemical constituents and contents.

The active components of *Asparagus racemosus* are steroidal glycosides, saponins, polyphenols, flavonoids, galactose and vitamins. Other active compounds such as diosgenin and quercetin-3 glucuronide are also present in the leaves. Shatavari is also known to possess antioxidant activity in vivo condition as well as in animal models. *Asparagus racemosus* is reported to have immunostimulant and antioxidant activities in laboratory animals.

Pawar (2011) studied the effect of herb extract (*Asparagus racemosus*/shatavari) incorporation on storage stability of ghee and he found the phenolic content of aqueous and ethanolic extract of shatavari was found to be 14.49±0.44 and 24.99±0.74 mg GAE per g extract, respectively. He also found the radical-scavenging activity of ethanolic and aqueous of shatavari was 62.89±0.34% and 40.17±0.44% respectively. Kaur and Mondal (2014) evaluated antioxidant activity of alcoholic extracts of *Asparagus racemosus* by DPPH free radical method. *Asparagus racemosus* leaves showed 65%.

2.6.15 Tulsi

Tulsi (*Ocimum sanctum*) of family Labiateae is known as “Queen of plants”. *Ocimum sanctum* Linn, (Labiatae) commonly known as “Tulsi” in Hindi is a medicinal plant commonly grown in India.

The leaves of *Ocimum sanctum* contain 0.7% volatile oil comprising about 71% eugenol and 20% methyl eugenol. The oil also contains carvacrol and sesquiterpine hydrocarbon caryophyllene. Fresh leaves and stem of *Ocimum sanctum* extract yielded some phenolic compounds (antioxidants) such as cirsilineol, circimaritin, isothymusin, apigenin and rosameric acid and appreciable quantities of eugenol. Two flavonoids, viz., orientin and vicenin from aqueous leaf extract of *Ocimum sanctum* have been isolated. Ursolic acid, luteolin, apigenin, apigenin–7-O-glucuronide, luteolin-7-Oglucuronide, orientin and molludistin have also been isolated from the leaf extract. *Ocimum sanctum* also contains a number of sesquiterpenes and monoterpenes viz., bornyl acetate, camphene, campesterol, cholesterol, stigmasterol and sitosterol. The fixed oils of *Ocimum sanctum* revealed the presence of five fatty acids such as stearic, palmitic,

oleic, linoleic, linolenic acids. It is a good source of beta carotene, vitamin C and calcium.

It is good source of linalool, eugenol, methyl chavicol and cineole. Presence of eugenol contributes to its antioxidant property and is also thought to be responsible for inhibition of lipid peroxidation (Gupta *et al.,* 2014). Essential oils of tulsi have antibacterial, antifungal and antiviral properties (Tewari *et al.,* 2014).

Basak *et al*. (2014) assessed antioxidant and anti-inflammatory activities of extracts with different polarities (hexane, dichloromethane, ethyl acetate, ethanol and methanol) obtained from *Ocimum sanctum* leaves. Total phenolics content of tulsi leaves extracts was varied from 8.8±0.3 to 127.3±1.6 mg GAE per g dw. Mitra *et al.* (2014) determined the scavenging activity of aqueous tulsi leaf (*Ocimum sanctu*m) extract. The aqueous TLE exhibited over 59.94% scavenging activity of DPPH radical at a concentration of 1 µg per ml. In work carried out by Kaur and Mondal (2014) on antioxidant activity of alcoholic extracts from *Osmium sanctum* leaves showed 74.73% inhibition of DPPH radical.

2.7 COMBINATION EFFECT OF HERBS

Synergistic actions between synthetic only, natural and synthetic, and natural antioxidants have been observed (Moure *et al.,* 2001). This effect is defined as the combined action which results in increased antioxidant potential more than that expected from a mere additive effect. Yi *et al.* (1991) observed that α-tocopherol and ascorbic acid acted highly synergistically with each other in a fish oil/lecithin/water system, requiring a minimum of 0.01-0.02% ascorbic acid. Protective effects against caffeic acid autoxidation in the presence of ascorbic acid were observed by Cilliers and Singleton (1990).

Chu and Hsu (1999) observed a two or three times higher oxidative stability index for peanut oil when mixtures of antioxidants were used. Mixtures of tocopherol and carotene as well as mixtures with other substances (ascorbic acid and lecithin), which have been reported to enhance the antioxidant activity (Moure *et al.,* 2001). Synergy among the different classes of polyphenols was observed in red wine (Ghiselli *et al.,* 1998).

Interactive effects between flavonoids and phenolic acids. However, the simultaneous presence of some compounds may present lower antioxidant activity than expected; in

this way antagonist effects were observed between ellagic acid and catechin (Meyer *et al*., 1998; Heinonen *et al.,* 1998). The authors suggested the possible existence of hydrogen bonding between carbonyls in ellagic acid and o-dihydroxyl groups in catechin. The PV for the oil, subjected to oxidation in the presence of added pure polyphenols, ranged from 84 meq/kg when p-coumaric acid was added to 39 meq/kg when protocatechuic acid was used. Other reasons could be the synergistic effects of the different phenolic compounds. Since there is no single antioxidant that can scavenge all kinds of radicals or that performs optimally for all lipid products, mixtures of antioxidants resulting in a synergistic effect are preferred for preventing free radical-induced diseases. Combined use of antioxidants will probably be desirable (Moure *et al.,* 2001). Some antioxidants, such as vitamins E and C, are known to have synergistic interactions through their recycling mechanisms, whereby the combination of compounds has a better antioxidant activity than the sum of separate activities (Niki, 1987). The use of synergistic mixtures of antioxidants allows a reduction in the concentration of each and also increases the antioxidant effectiveness with respect to the activity of the separate components although, even in widely used and commercialized extracts, such as rosemary, the antioxidative behaviour and synergistic actions of most of the compounds remain unknown (Cuvelier *et al.,* 1996).

The beneficial effects of using mixtures of antioxidants were summarized by Saucier and Waterhouse (1999) as: (1) advantages of their different effectiveness (2) minimalisation of solubility or colour problems presented by individual compounds (3) better control and accuracy of application (4) complete distribution or solution of antioxidants and chelating agents.

Betel and curry leaves when added at 1.0 % level to ghee showed higher resistance to oxidative deterioration than BHA and BHT mixture. The anti-oxidative properties of betel and curry leaves were attributed to phenolic compounds, predominantly hydroxyl chavicol (Patel and Rajorhia, 1979). These leaves also contained some ascorbic acid which might work as synergist (Sethi and Aggarwal, 1956).

Gupta *et al.* (2014) evaluated the synergistic antioxidant activity of tea with ginger, black pepper and tulsi and found that all the combinations (tea and ginger; tea and black pepper; tea and tulsi; tea, ginger and black pepper; tea, black pepper and tulsi; tea, ginger and tulsi; tea, ginger, black pepper and tulsi) exhibited radical scavenging

activity for aqueous and methanolic extract. Among all the mixtures, the mixture of methanolic extract of different herbs, the best result is obtained in the synergistic combination; a (tea: ginger). This clearly shows that the amount of antioxidant compounds present in methanolic extract are more than the aqueous extracts of above herbs. The results showed that selected polyherbal combinations of all the extracts with tea were found to produce best antioxidant activity in comparison to their individual extracts.

2.8 METHODS FOR DETERMINING PHENOLIC COMPOUNDS AND ANTIOXIDANT POTENTIAL OF HERBS

Crude extracts of herbs, spices and other plant materials rich in phenolics are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The widely used method for estimation of total polyphenol content is Folin-Ciocalteu colorimetric method (Nadeem *et al.,* 2013; El-sherif *et al.,* 2011). The quantification is done with respect to the standard curve of gallic acid and the results are expressed as gallic acid equivalents (GAE), mg per 100 g of dry weight.

A desirable method for evaluating the antioxidant activity of a compound should be rapid, reproducible, should require small amounts of chemicals and should not be influenced by the physical properties of the compound (Gray, 1978). The most commonly used method to assess antioxidant activity is DPPH assay. The relatively stable organic radical, DPPH, has been widely used in the determination of antioxidant activity of single compounds, as well as of different plant extracts (Hasim *et al.,* 2015; Abbasi *et al.,* 2016). The DPPH scavenging assay is based on electron donation of antioxidants to neutralize DPPH radical. In DPPH assay, the purple chromogen radical 2,2-diphenyl- 1-picrylhydrazyl ($DPPH^{\bullet}$) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 517 nm, which is proportional to concentration of free radical scavenger added to DPPH solution (Boligon *et al.,* 2014).

2.9 METHODS TO MEASURE LIPID OXIDATION

The application of lipid oxidation methods involves measuring lipid oxidation after the sample has been oxidized under standardized conditions to a suitable endpoint (Frankel, 1993). The sensory analysis is one of the most sensitive methods available but it is not practical for routine analyses and generally lacks reproducibility. The scoring by taste or odor panellists may vary greatly from laboratory to laboratory. Therefore, chemical methods are developed to improve reproducibility, sensitivity, and quantitativeness (Gray, 1978). Analysis of volatiles by gas chromatography is closely related to flavor evaluation and is therefore, the most suitable method for comparison with the results of sensory panel tests (Frankel, 1993).

The methods reported to monitor oxidative deterioration of various fats and oils are based on chemical changes taking place in the primary and secondary stages of oxidative deterioration. The first compounds formed during the primary stage of the oxidation process are peroxides, especially hydroperoxides, which in turn can generate secondary oxidation products, including aldehydes, ketones, hydroxyl compounds, epoxides and polymers (Mehta *et al.*, 2015). Peroxide value is a common method to measure lipid oxidation and its use is limited to the initial stages of oxidation (Gray, 1978).

The carbonyl compounds, including aldehydes and ketones, are the secondary oxidation products generated from degradation of hydroperoxides and are suggested to be the major contributors to off-flavours associated with the rancidity of many food products. The intensity of undesirable sensory notes has been positively correlated with the content of carbonyl compounds formed through lipid autoxidation reactions. The carbonyl compounds present have the greatest impact on flavour owing to their low flavour thresholds (Shahidi and Zhong, 2005). The spectrophotometric method is based on the absorbance of the quinoidal ion, a derivative of aldehydes and ketones. This ion is formed from the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl moiety of an aldehyde or ketone, followed by the reaction of the resulting hydrazone with alkali (Ronald, 2001).

The capacity of antioxidants to quench DPPH radical in ghee was determined before and after accelerated oxidation tests (Espin *et. al.*, 2000). Ethyl acetate was used as a better solvent for hydrophobic compounds.

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.

3.1.2 Glasswares

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

3.1.3 Equipments

Equipments used include 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 Instument, Germany), water bath (SAIF surgical & scientific equipment, Anand), butyro-refractometer (ATAGO, Japan), centrifuge (Labline, Ahmedabad), vortex mixer (Labline, Ahmedabad) and spectrophotometer (SYSTRONICS, Ahmedabad).

3.1.4 White Butter

White butter was procured from a commercial dairy plant of Amul Dairy, Anand.

3.1.5 Herbs

Fourteen herbs including ajwain leaves (*Coleus aromaticus*), betel leaves (*Piper betle*), centella (*Centella asiatica*), coriander leaves (*Coriandrum sativum*), curry leaves (*Murraya koenigii*), dill leaves (*Anethum graveolens*), dodi (*Leptadenia reticulate*), fenugreek leaves (*Trigonella foenum graecum*), jequirity leaves (*Abrus precatorius*), lemon grass (*Cymbopogon citratus*), liquorice (*Glycyrrhiza glabra*), mint (*Mentha citrata*), shatavari (*Asparagus racemosus*) and tulsi (*Ocimum sanctum*) were brought

from Directorate of Medicinal and Aromatic Plants Research, BACA, AAU, Anand, Gujarat, India. One herb was brought from the local market namely drumstick leaves (*Moringa oleifera*) in dried form. The herbs were cleaned to remove dirt and damage parts then the herbs were vaccum tray dried in anubhav dairy except the sample (drumstick leaves – already purchased in dried form), AAU, Anand. After drying, samples were converted into coarse size particles and transferred in to zip lock plastic cover, then put in air tight plastic bottle and stored at refrigeration temperature.

3.2 EVALUATION OF HERBS FOR THEIR ANTIOXIDANT POTENTIAL

3.2.1 Preparation of Extracts

0.5 g powder of each herb was treated with 10 ml of methanol-water (8:2, v/v) in a shaking water bath at 35°C for 24 h as described by Song *et al*. (2010). The mixture was then centrifuged at 4,000 rpm for 10 min. The supernatant (herb extract) was recovered for the determination of the total phenolic content and radical scavenging activity (using DPPH). All the experiments were carried out in triplicate.

3.2.2 Analysis of Total Phenolic Content

Total phenolic content of herb extracts were analysed by Folin-Ciocalteu reagent according to the procedure described by Singleton and Rossi (1965) with some modification.

0.05 ml sample for all herbs extract except betel leaves (0.03ml) was taken in a test tube and volume was made up to 1 ml with distilled water. To this 0.5 ml each of diluted Folin Ciocalteau reagent (1:1) and 10 ml 7.5 % sodium carbonate solution were added. The contents were mixed using vortex mixer. This was incubated under dark at room temperature for 30 min. For blank preparation 1 ml of distilled water was taken instead of sample. The absorbance of the samples were measured against blank at 750 nm using spectrophotometer.

For standard curve preparation known concentration of gallic acid (10-100 µg/ml) was prepared and volume was made up to 1 ml with distilled water and there after treated in the same way as sample. The result was expressed in terms of mg of GAE per 100 gm of dried herb.

3.2.3 Radical-Scavenging Activity by DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Assay

The radical-scavenging activity of herb extracts was determined as the ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals according to the procedure of Brand Williams *et al*. (1995) with some modification. The method in brief is discussed below.

0.05 ml of sample (herb extract) was taken in a test tube and the volume was made up to 1 ml with methanol. 3 ml of 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 37^{o}C for 15 min. in a water bath. The control was prepared by taking 1 ml methanol and 3 ml of 0.1 mM methanolic DPPH solution. Methanol was used (as blank) for the baseline correction. Absorbance of the control and samples were measured at 517 nm using a spectrophotometer against blank (methanol). Radical-scavenging activity was expressed as the inhibition percentage and was calculated using the following formula.

Radical-scavenging activity (% inhibition) = [(Ac – A)/Ac] x 100

Where, Ac = Absorbance of control and A = Absorbance of sample

3.3 PREPARATION OF GHEE

White butter was procured from a commercial dairy plant of Amul Dairy, Anand. Ghee was prepared by creamery butter method. The 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). Ghee prepared in stainless steel vessel (pan) is shown in Figure 3.1.

Figure 3.1: Ghee in stainless steel vessel.

3.3.1 Compatibility Assessment of Herbs for Use in Ghee

Ghee was prepared as per the procedure discussed in section 3.3 and prepared ghee was divided into sixteen portions of 100g each. To portion 1 nothing was added which served as control. To the remaining fifteen portions, above listed herbs (section 3.1.5) i.e. ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi were added at the rate of 0.5% (w/w) respectively. The mixtures of ghee and herbs were thoroughly mixed using glass rod and allowed to stand at 80^{o}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 (in terms of flavour) for acceptability. Three replications were conducted for evaluating compatibility of selected herbs as additive in ghee. Ghee samples added with fifteen herbs and control ghee sample are shown in Figure 3.2.

Figure 3.2: Ghee samples added with fifteen herbs and control sample.

3.3.2 Evaluation of Compatible Herbs for Antioxidant Activity in Ghee

The samples of ghee with compatible herbs were prepared as per the details described in section 3.3.1. The samples of ghee were stored in incubator at 80º±2ºC (accelerated condition). Oxidative changes taking place in ghee were monitored by analysing the ghee samples for peroxide value when fresh and after an interval of three days. Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. The storage study was continued for a period of 12 days where almost all the samples of ghee became unacceptable in sensory evaluation. The colour characteristic of ghee samples was also observed when samples were fresh (on 0 day) and after storage (at the end of 12 days). Herbs giving better

stability against oxidative deterioration of ghee and relatively better compatibility were selected for further study. Three replications were conducted for evaluating the compatibility of herbs in ghee. In this phase curry leaves, betel leaves and liquorice were selected as per the results obtained.

3.3.3 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 (betel leaves, curry leaves and liquorice) were added at two different stages during manufacture of ghee.

(1) Addition at the initial stage of heat clarification of butter (in melted butter)
(2) Addition at the final stage of heat clarification of butter (105^{o}C temperature)

For evaluating two different stages in preparation of ghee (initial stage of clarification and final stage of clarification) for treatment with the selected herbs, the sample of butter (120 g) was taken in to each of seven of 500 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 centre of the sand bath. The sand bath was heated by gas fired burner with flame at the centre. When butter was melted completely betel leaves, curry leaves and liquorice was added separately in 3 beakers. In another 3 samples of melted butter betel leaves, curry leaves and liquorice was added separately when their temperature reached to 105°C (nearer to heat clarification). Out of 7 samples of the butter kept for heat clarification to prepared ghee, one sample was not treated with any herb to serve as a control. The heating was continued till temperature reached to 120°C.

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 cloth, ghee was collected in 150 ml glass beakers and stored in incubator at 80°±2°C for 12 days. To prepare 7 samples of ghee simultaneously under similar and uniform heating conditions sand bath was used.

The ghee samples were analysed for peroxide value when fresh and at an interval of 2 days. Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. Total four replications were conducted. The stage for addition of herbs giving better stability against oxidative deterioration of ghee was selected for further study. Betel leaves and curry leaves were giving better oxidative stability at the final stage of clarification of butter fat into ghee whereas liquorice was giving better oxidative stability at the initial stage of clarification of butter

fat into ghee.

3.3.4 Optimization of Rate for Addition of Herb in Ghee

The selected herbs (betel leaves, curry leaves and liquorice) were added according to results obtained from the selected stage at the rate of 0.1, 0.2, 0.3 and 0.4% (expected yield of ghee from butter). The herb treated ghee was prepared as per the procedure described in section 3.3.3. The sample of ghee without addition of herb was also prepared to serve as control. All the 13 ghee samples were stored in an incubator at 80º±2ºC. The ghee samples were analysed for peroxide value when fresh and at an interval of 2 days for 12 days then after at an interval of 1 day till flavour score of all most all the samples went below acceptable level (*i.e.* 22 days). Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. Total three replications were conducted.

3.3.5 Combinations of the Selected Herbs as Antioxidants in Ghee

Combination of betel leaves and curry leaves, betel leaves and liquorice, curry leaves and liquorice, curry leaves, betel leaves and liquorice were added at the selected stage and optimised rate into the butter. Betel leaves, curry leaves and liquorice were added individually at the selected stage and optimised rate into the butter. The herb treated ghee was prepared as per the procedure described in section 3.3.3. The sample of ghee without addition of herb was also prepared to serve as control. All the ghee samples were stored in an incubator at 80º±2ºC (accelerated condition). The ghee samples were analysed for peroxide value when fresh and at an interval of 2 days then after at an interval of 1 days till flavour score of all most all the samples went below acceptable level (*i.e.* 29 days). Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. Total three replications were conducted. Combination of betel leaves with liquorice and betel leaves alone were selected for comparison with BHA.

3.3.6 Comparison of Herbs with Synthetic Antioxidant (BHA)

In this phase, ghee samples were prepared with betel leaves and combination of betel leaves with liquorice at the selected stage and optimized rate on the basis of expected yield of ghee from butter as per procedure described in section 3.3.3. Simultaneously sample of ghee was prepared with butylated hydroxyl anisole (BHA) at the rate of 0.02% (w/w). The sample of ghee without addition of herb served as a control. One part of ghee was stored in incubator at 35°±2°C and another part was stored at 80°±2°C. Total three replications were conducted for each sample.

Fresh ghee samples were analysed for quality standards and evaluated for sensory attributes. All the samples of ghee stored at elevated temperature (80°±2°C) as well as at normal room temperature (35°±2°C) and regularly monitored for effect of antioxidants on oxidative changes taking place in the ghee. Oxidative changes taking place in ghee were monitored by analysing the ghee samples for peroxide value, carbonyl value and radical scavenging activity (DPPH assay) when fresh and after an interval of 2 days till flavour score of all most all the samples went below acceptable level (*i.e.* 34 days). Simultaneously, the samples were also monitored for changes in flavour score by sensory evaluation using 9-point hedonic scale. The change in colour characteristic of ghee samples was also monitored by visual observation when samples were fresh (on 0 day) and after storage (at the end of 12 days).

3.4 SENSORY EVALUATION

All the samples of ghee made in laboratory were evaluated for their sensory characteristics on a 9 point hedonic scale by a panel of 9 judges. The samples were evaluated for their flavour. The 9 point hedonic scale score card for sensory analysis is given in Table 3.1.

Table 3.1: 9 point hedonic scale used to evaluate flavour score of ghee

Score Card

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:

3.5 EVALUATION OF OXIDATIVE STABILITY OF GHEE

3.5.1 Determination of Peroxide Value

The peroxide value of ghee was determined by the method (Iodometric method) as described in IS: SP: 18 (part XI) (1981).

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 and one volume of chloroform) was added. The contents were heated to boil within 30 sec in a pre-heated boiling water bath and allowed to boil for not more than 30 sec. The contents in test tube 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 1ml of starch indicator (0.5%), near to end point. A blank was also performed without using ghee sample.

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

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

Where,

T = Volume in milliliters of 0.002 N sodium thiosulphate

W = Weight in g of sample

3.5.2 Determination of Carbonyl Value

The carbonyl value of ghee was determined as per the method (Ronald, 2001). The method is described below.

The ghee sample (0.05–0.08 g) was weighed into a 50 ml volumetric flask to which is added 5 ml of benzene, 3 ml of 4.3% trichloroacetic acid solution in benzene, and 5.0 ml of 0.05% dinitrophenylhydrazine in benzene were added. The volumetric flask was stoppered and heated for 30 min at 60°C in a water bath, and then cooled to room temperature. To develop the color, 10 ml of 4% potassium hydroxide in ethanol was added, and the solution was diluted to 50 ml with absolute ethanol. After 10 min, the absorbance was read against a reagent blank prepared in the same manner, but without the ghee sample. The determination of saturated and unsaturated carbonyl contents

from the absorbencies at the convenient wavelengths of 430 and 460 nm as follows:

CU = (3.861 x A460 - 3.012 x A430) / 0.854

CS = 3.861 x A460 - 2.170 x CU

CT = CU + CS

Where,

A_{460} and A_{430} are the sample absorbance at 460 and 430 nm, respectively and C_U,

C_S and C_T are the unsaturated, saturated and total carbonyl contents, respectively. The carbonyl value was expressed as µmol carbonyl/g sample.

3.5.3 Radical Scavenging Activity of Ghee Samples by DPPH Assay

The capacity of antioxidants to quench DPPH radical in ghee was determined before and after accelerated oxidation tests (Espin *et al.,* 2000). Ethyl acetate was used as a better solvent for hydrophobic compounds. The method in brief is as follows:

0.2 ml of ghee sample was added to 3.8 ml of ethyl acetate to obtain 4 ml of the mixture, followed by addition of 1 ml of DPPH (12.18×10-5 mol/L) solution in ethyl acetate (total volume, 5 ml). After elapse of 10 min. of addition of reagents, absorbance was measured at wavelength 517nm. The reference sample used contained 1 ml of DPPH solution and 4 ml ethyl acetate. Radical-scavenging activity was expressed as percentage inhibition and was calculated using the following formula:

Radical-scavenging activity ((% inhibition) = [(Ac – A)/Ac] x 100

Where

Ac = Absorbance of control, A = Absorbance of sample

3.6. ANALYSIS OF GHEE FOR QUALITY STANDARDS

The fresh samples of ghee prepared were analysed for quality standards such as moisture content, free fatty acid content, Reichert Meissl value, Polenske value, Butyro-Refractometer reading at 40^{o}C, and Baudouin Test. The details of the procedures for these parameters are discussed below.

3.6.1 Determination of Moisture

Moisture content of ghee samples was determined by the method as described in IS: SP: 18 (part XI) (1981). 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

W = Weight in g of empty dish

3.6.2 Determination of Free Fatty Acids (FFA)

The free fatty acids content of ghee was determined by the method as described in IS: SP: 18 (part XI) (1981). 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 (% 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

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

Reichert-Meissl (RM) and Polenske Value was determined as per the method described in IS: SP: 18 (part XI) (1981). Accurately 5.0 g of sample was weighed in to

a Polenske flask and then saponified with 20.0 g of glycerol and 2.0 ml of 50% (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 15ºC 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

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

Butyro-Refractometer (BR) reading of different ghee samples were determined by the method described in IS: SP: 18 (part XI) (1981). Before determining the BR reading of a sample, the temperature of the samples were brought to 40.0°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.

3.6.5 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).

3.7 STATISTICAL ANALYSIS

The results were analysed using Analysis of Variance (ANOVA). All tests were conducted at the 5% 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 were used for analysis.

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

Where,

Y_{ij} = response due to the i^{th} 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.

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

Where,

Y_{ijk} = Response from the j^{th} 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 j^{th} and k^{th} level.

CHAPTER 4

RESULTS AND DISCUSSION

Clarified butterfat, known as ghee in India, is usually prepared from cow or buffalo milk or combination thereof. Ghee is a rich source of energy, fat soluble vitamins, essential fatty acids and pleasing flavour (Bhatted and Singh, 2002). Development of rancidity in ghee during storage has also been reported which reduces the shelf life of the product resulting in alteration of major quality parameters such as colour, flavour, aroma and nutritive value. Negative health implications of oxidised lipids have been testified by many reports (Purohit, 2011; Hathway, 1966; Maeura *et al.,* 1984). These in turn determine the storage stability and are of paramount importance from economic viewpoints. Therefore, constant research endeavours are made to extend the shelf life by various approaches. One of the most common approaches is addition of antioxidants.

Antioxidants can be classified according to their source as natural or synthetic. Use of synthetic antioxidants, however, is the target of safety issues, prompting the search for natural antioxidants that can act individually or synergistically with other additives to replace the synthetic ones (Shahidi, 2000). It is generally accepted that natural antioxidants are safer than synthetic antioxidants. Consequently, in recent years many attempts have been made for search of natural antioxidant compounds that can properly serve the demand of consumers and needs of the food manufacturers.

Herbs have been used as food and as medicine for centuries. Since the beginning of human civilization, herbs have been an integral part of the society, valued both for their culinary and medicinal properties. Herbs are not just valuable in adding flavour to foods. Their antioxidant activity also helps to preserve foods from oxidative deterioration, increasing their shelf life. There has been increasing research in the role of herbs as natural preservatives. Various herbs have been recognized for their antioxidant activity and used throughout the past as an alternative approach to preserve foods. Spices and herbs, in addition to contributing taste and aroma to foods, contain variety of bioactive substances including antioxidants. The phenolic content in herbs act as antioxidant due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators (Nawar, 1996). Many herbs are known to exhibit antioxidant activity in food lipids (Pokorny and Korczak,

2001). However, there is a limited work reported for exploiting antioxidant potential of herbs by addition in ghee.

Keeping in view the forgoing resume, present study was planned to study the antioxidant properties of fifteen selected herbs to extend the shelf life of ghee. The study was divided into six phases. In the first phase, compatibility of fifteen very commonly consumed herbs as an additive in ghee was assessed. In the second phase, compatible herbs were evaluated for their antioxidant activity in ghee. In the third phase, suitable stage for addition of herbs in ghee preparation was studied. In fourth phase, optimization of rate for addition of herbs was carried out. In fifth phase effectiveness of selected combination of herbs was studied. In the final phase, comparison of the antioxidative behaviour of the selected herbs in ghee with permitted synthetic antioxidant (*i.e.* BHA) was made and samples of ghee were also analysed for the quality standards as prescribed by FSSAI (2011) and AGMARK (1991).

4.1 COMPATIBILITY ASSESSMENT OF HERBS FOR USE IN GHEE

There is a big difference between the preparation of synthetic antioxidants and natural antioxidants for application in food products and processing. Synthetic antioxidants are produced as pure substances of constant composition, and are applied as such or in well-defined mixtures with other pure substances. Application is thus relatively easy, requiring no substantial modifications of the recipe and processing conditions. On the contrary, natural antioxidants are available from raw materials of variable composition. Both the content of active substances (usually a mixture of several compounds) and the content of various other compounds, either inactive or possessing negligible activities, depend on the plant variety, agro technology, climatic conditions, degree of ripeness, and many other factors. Their composition should be determined in every batch, and if necessary, the procedure of their preparation or application, and the amount added to food products should be adapted according to analytical results (Pokorny and Korczak, 2001).

Many other food components possessing antioxidant activities are used in their natural form, such as spices. Spices and herbs are used as antioxidants in different forms: (1) as is, i.e., the plant material, whole or ground; (2) as an extract; (3) as an oleoresin; and (4) as isolated bioactive compounds with active components standardized at specific concentration for consistent effectiveness (Embuscado, 2015). The preliminary

processing of such food components may be drying (in case of leaves or stems), milling of dried material (such as seeds), or some other mechanical treatment. Several ground spices (added in the amount of 5 %) were found to be active in sunflower oil, especially, sage, sumac and thyme. The content of active antioxidants in natural materials is usually rather low so that large additions would be necessary to obtain a significant improvement in stability against oxidation. However, such large additions could have a negative effect on the flavour or functional properties of the product. Extraction using edible oil or fat is a very simple method. Natural material containing antioxidants, such as herbs and spices, is mixed with fats and/ or oils, and the mixture is left at room temperature or at a moderately increased temperature (in case of solid fats, such as pork, lard, beef tallow or cooking fats) for a defined time, for example overnight, with or without stirring. The mixture is then filtered and the fat or oil containing dissolved antioxidants is used directly in food preparation (Pokorny and Korczak, 2001). Therefore, in present study for treatment of ghee with selected herbs, direct extraction of the herbs in ghee was followed.

Synergism and antagonism are very important in a mixture such as that of plant extracts for their effectiveness in action (Pokorny and Korczak, 2001). It has been reported that that the antioxidant activity of herbs and spices reduced in extracts prepared from an equivalent amount of herbs or spice as opposed to that prepared from the whole spice. These observations confirming that the wide range of compounds acting together are important as antioxidants in the plant material, mainly due their synergistic effect in antioxidant activity (Suhaj, 2006). Herbs contain complex mixtures of volatile constituents biosynthesized by plants and the interactions between these components may lead to antagonistic, additive or synergistic effects. Some studies have demonstrated that whole essential oils usually have higher activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity (Tsao, 2015; Juiz *et al*., 2016). Therefore, in present study it was decided to use dried herbs (coarse size form) for treatment of ghee.

In selection of herbs for enhancing the shelf life of ghee, the first and foremost point for consideration was the compatibility of the herbs to be used for study. Since, herbs were to be incorporated during preparation of ghee, some preliminary work required to assess their compatibility in ghee. In total fifteen commonly used herbs namely ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves,

fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi were selected for the assessment of their compatibility in ghee.

Ghee was prepared as per the procedure described in section 3.3.3 and divided in to sixteen parts. Fifteen different herbs as listed above were added separately at the rate of 0.5 per cent (w/w) to each sample of ghee. The mixtures of ghee and herbs were thoroughly mixed and allowed to stand at 80^{o}C for 30 min for the extraction of phenolic compounds into the ghee. The samples of ghee treated with herbs were filtered through 6 layers of muslin cloth. The ghee sample without addition of any herb was used as a control sample. All the samples of ghee were for flavour and colour.

4.1.1 Flavour of Ghee up on Treatment with Different Herbs

Flavour (aroma and taste) of any edible substance is the most important sensory attribute, which significant 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. Therefore, samples of ghee treated with different herbs were given to judges of sensory panel to evaluate the samples of ghee for acceptability of their flavour in terms. The result of the acceptability is presented in the Table 4.1.

Table 4.1: Acceptability of ghee for flavour up on treatment with different herbs

Sr. No.	Herb used		Remark*
	Common name	Scientific name	
1	- (Control)	-	Acceptable
2	Ajwain leaves	*Coleus aromaticus*	Acceptable
3	Betel leaves	*Piper betle*	Acceptable
4	Centella	*Centella asiatica*	Acceptable
5	Coriander leaves	*Coriandrum sativum*	Acceptable
6	Curry leaves	*Murraya koenigii*	Acceptable
7	Dill	*Anethum graveolens*	Acceptable
8	Dodi	*Leptadenia reticulate*	Acceptable
9	Drumstick leaves	*Moringa oleifera*	Acceptable
10	Fenugreek leaves	*Trigonella foenum graecum*	Acceptable
11	Jequirity leaves	*Abrus precatorius*	Acceptable
12	Lemon grass	*Cymbopogon citratus*	Acceptable
13	Liquorice	*Glycyrrhiza glabra*	Acceptable
14	Mint	*Mentha citrata*	Acceptable
15	Shatavari	*Asparagus racemosus*	Acceptable
16	Tulsi	*Ocimum sanctum*	Acceptable
***** Acceptable or Unacceptable			

The results indicated that all the samples of ghee treated with herbs were found acceptable for their flavour in sensory evaluation by members of the panel of the judges. None of the sample was found unacceptable among all samples of herb treated ghee.

Patel and Rajorhia (1979) noticed that when betel (*Piper betel*) and curry (*Murraya koeniji*) leaves were added to butter during clarification in to 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. Therefore, acceptability of the betel leaves and curry leaves treated ghee samples for flavour in sensory evaluation was very well in corroboration with results reported by these authors. No work is reported in the literature regarding acceptability of the remaining herbs treated ghee samples or even herbs other than those used in the present study. Therefore, results of the present study for other herbs treated ghee samples cannot be compared with published literature.

4.1.2 Colour of Ghee up on Treatment with Different Herbs

Colour and appearance of the product is the first sensory attribute come across in acceptance or rejection of the product. Therefore, samples of ghee treated with different herbs were examined by visual observation for their colour. The colour of ghee samples obtained after treatment with different herbs is presented in the Figure 4.1.

It was evident from the examination of the Figure 4.1 that the control ghee sample (without treatment of any herb) acquired golden yellow colour. The most of the ghee samples treated with different herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, fenugreek leaves, lemon grass, liquorice, mint, shatavari and tulsi) also acquired yellow to golden yellow colour, almost similar to that of the control ghee sample. However, in some cases variation in colour of ghee samples was observed when treated with herbs. Particularly, when ghee samples were treated with drumstick leaves or jequirity leaves. The drumstick leaves and jequirity leaves treated sample acquired dark olive green colour and greenish yellow colour respectively, instead of usual golden yellow colour. Such green discoloration of the ghee samples might be attributed to leaching of chlorophylls from the herbs.

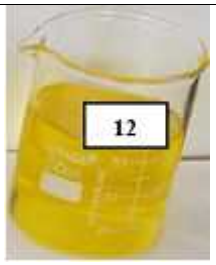
			
Control	**Ajwain leaves**	**Betel leaves**	**Centella**
			
Coriander leaves	**Curry leaves**	**Dill**	**Dodi**
			
Drumstick leaves	**Fenugreek leaves**	**Jequirity leaves**	**Lemon grass**
			
Liquorice	**Mint**	**Shatavari**	**Tulsi**

Figure 4.1: Colour of ghee upon treatment with different herbs

As such study regarding effect of treating ghee with herbs on its colour has been not reported. Therefore, findings obtained in present study could not be compared with that reported in the literature. However, Patel and Rajorhia (1979) noticed that when betel (*Piper betel*) and curry (*Murraya koeniji*) leaves were added to butter during clarification in to ghee, the samples of ghee treated with betel leaves and curry leaves were appreciated for slightly higher intensity of their colour. Therefore, results of the present for colour of ghee on treatment with ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, fenugreek leaves, lemon grass, liquorice, mint, shatavari and tulsi was in general agreement with results reported by these authors.

4.2 EVALUATION OF HERBS FOR THEIR ANTIOXIDANT POTENTIAL

Since use of all the fifteen herbs were found compatible in ghee, these herbs were evaluated for their antioxidant potential. To evaluate for the antioxidant potential, herbs were analysed for their total phenolic content and radical scavenging activity. Moreover, effect of herbs on changes in peroxide value and flavour score of ghee during storage at 80±2^{o}C was also evaluated.

4.2.1 Total Phenolic Content of Herbs

In the present study, the total phenolic content of selected herbs namely ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi were determined. To analyse the phenolic content of the above herbs, extracts (methanol: water = 8:2) were prepared. The total phenolic content was estimated using Folin-Ciocalteau method. The total phenolic content of herbs were expressed in mg of gallic acid equivalents (GAE) per 100 g of dried herb. The data on amount of total phenolics are presented in Table 4.2 and graphically depicted in Figure 4.2.

Table 4.2: Total phenolic content of herbs

Sr. No.	Herb added		Total phenolics content (mg GAE/100 g of dried herbs)
	Common name	Scientific name	
1	Ajwain leaves	*Coleus aromaticus*	0396.67 ± 16.99
2	Betel leaves	*Piper betle*	3653.33 ± 18.86
3	Centella	*Centella asiatica*	1740.00 ± 58.88
4	Coriander leaves	*Coriandrum sativum*	1140.00 ± 32.66
5	Curry leaves	*Murraya koenigii*	1913.33 ± 57.35
6	Dill	*Anethum graveolens*	1613.33 ± 65.99
7	Dodi	*Leptadenia reticulate*	0673.33 ± 24.94
8	Drumstick leaves	*Moringa oleifera*	1673.33 ± 24.94
9	Fenugreek leaves	*Trigonella foenum graecum*	1266.67 ± 57.35
10	Jequirity leaves	*Abrus precatorius*	1846.67 ± 67.99
11	Lemon grass	*Cymbopogon citratus*	0660.00 ± 28.28
12	Liquorice	*Glycyrrhiza glabra*	1030.00 ± 69.76
13	Mint	*Mentha citrata*	1640.00 ± 32.66
14	Shatavari	*Asparagus racemosus*	1106.67 ± 73.64
15	Tulsi	*Ocimum sanctum*	1863.33 ± 49.22

Source of variation	Treatment (herbs)
S. Em.	34.91
CD (0.05)	100.82
CV %	4.08

Data presented as means ± SD (n=3).

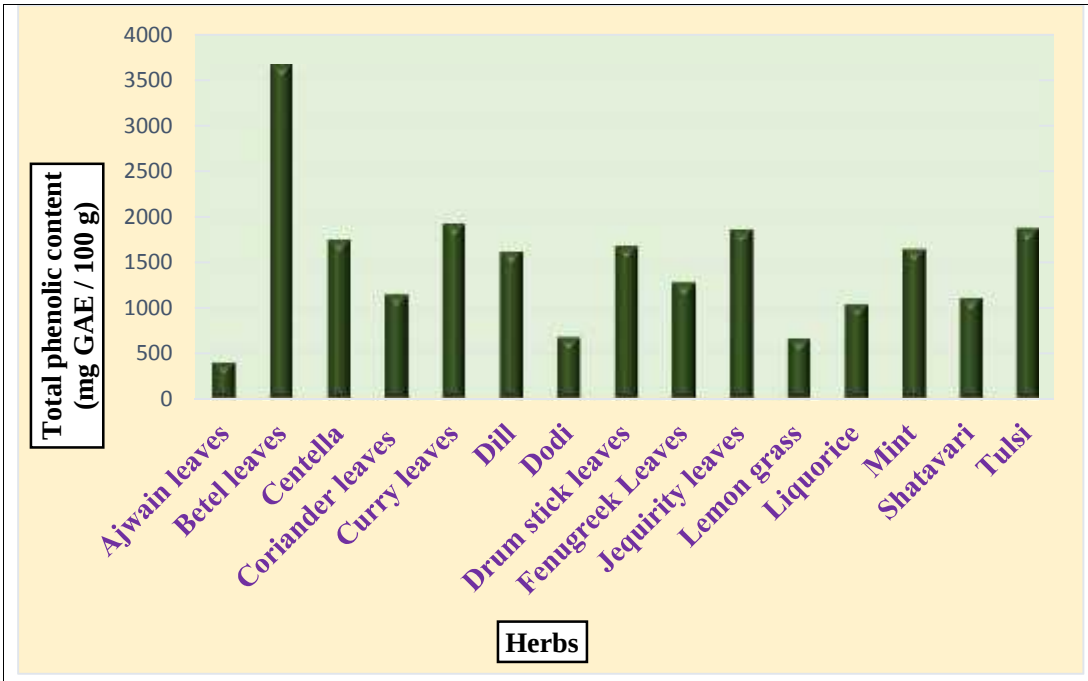


Figure 4.2: Total phenolic content of herbs

The total phenolic content of the fifteen different herbs was in the decreasing order of betel leaves > curry leaves > tulsi > jequirity leaves > centella > drumstick leaves > mint > dill > fenugreek leaves > coriander leaves > shatavari > liquorice > dodi > lemon grass > ajwain leaves respectively. Thus the total phenolic content was highest in betel leaves and the lowest in ajwain leaves.

It was clearly evident from the data that on the basis of total phenolic content the tested herbs could be distributed in three groups. The first group comprised betel leaves which had total phenolic content above 2000 mg GAE per 100 g. The second group had total phenolic content in the range of 1000 to 2000 mg GAE per 100 g and it comprised of centella, coriander leaves, curry leaves, dill, drumstick leaves, fenugreek leaves, jequirity leaves, liquorice, mint, shatavari and tulsi. The third group comprised ajwain leaves, dodi and lemon grass having total phenolic content below 1000 mg GAE per 100 g. The difference in total phenolic content of herbs tested differed significantly.

The total phenolic content of the top ranking herb, the betel leaves (3653.33 mg GAE/100 g) was significantly higher than remaining all other herbs. It was almost 2 to 9 times higher than remaining all other herbs. The second highest total phenolic content was found in curry leaves (1913.33 mg GAE/100 g), which was statistically at par with jequirity leaves and tulsi, but significantly higher than remaining all other herbs, except

betel leaves. The third position in total phenolic content was occupied by tulsi. Its total phenolic content was statistically at par with jequirity leaves, but significantly higher than remaining all other herbs except betel leaves and curry leaves. Jequirity was on fourth position in total phenolic content and it was statistically at par with tulsi, but significantly higher than remaining all other herbs except betel leaves and curry leaves. With respect to total phenolic content, centella (1740 mg GAE/100 g) stood at position five and it was statistically at par with drumstick leaves, but significantly higher than remaining all other herbs except betel leaves, curry leaves, tulsi and jequirity leaves. The total phenolic content of the lowest ranking herb, the ajwain leaves was 396.67 mg GAE 100 g.

Azhar *et al*. (2011) carried out study to elucidate the effect of drought stress on growth, physiology and secondary metabolite production in desi ajwain (*Trachyspermum ammi* L.). Plants were grown in pots and three drought levels (100%, 80% and 60%) of field capacity were created. The total phenolic contents increased significantly with increasing drought stress levels. The highest total phenolic contents (4.44 mg/g) were observed at 60% field capacity, followed by 3.95 mg/g at 80% field capacity and 2.23 mg/g at 100% field capacity. The total content of polyphenols in ajwain samples varied from 1.823 to 4.946 mg/g of dry matter. Oueslati *et al.* (2016) estimated total phenolic content of aqueous and ethanolic extracts (70%) of *Trachyspermum ammi* and found that the total phenolic content in aqueous extract of the *T. ammi*, as estimated by the Folin-Ciocalteu method, had the highest level (162.17 mg GAE per g DW) followed by the ethanolic extract (89.9 mg GAE per g DW).

Jamal *et al.* (2010) studied forty types of Malaysian medicinal plants and found that *Piper betel* L. had phenolic content of 8986.67 mg per liter GAE. Jaiswal *et al.* (2014) collected six different variety of betel leaf [Banarasisafeda (PA), Calcutta (PB), Cuttack (PC), Desibagla (PD), Maharashtra (PE) and Sofia (PF)] from Uttar Pradesh, West Bengal, Odisha, Maharashtra and Bihar and were extracted using five solvents (80% methanol, 80% ethanol, 80% acetone, 80% ethyl acetate and distilled water) to determine total phenol content. The TPC for six variety betel leaf extract in five different solvents were found in the range of 0.29 to 2.62 mg GAE per g dw for PA, 0.08 to 2.59 mg GAE per g dw for PB, 0.09 to 2.38 mg GAE per g dw for PC, 0.07 to 2.87 mg GAE per g dw for PD, 0.16 to 2.07 mg GAE per g dw for PE and 0.04 to 1.16

mg GAE per g dw for PF. Kaur and Mondal (2014) reported the total phenolics content (TPC) of 212±0.50 mg per 100g of fresh weight in *Piper betel*.

Jamal *et al.* (2010) studied forty types of Malaysian medicinal plants and found that *Centella asiatica* had phenolic content of 583.33 mg per liter GAE phenolic content. Mohankumar *et al.* (2017) investigated total phenolic content of selected medicinal plants from Kanyakumari district in which *Centella Asiatica* had highest phenolic content (17.1 mg GAE per g). Rahman *et al.* (2013) investigated the total phenolic content of *Centella asiatica* (Linn.) using different extraction solvent. 100% ethanol, 50% ethanol and water were chosen as extraction solvent. 50% ethanol extract of *C. asiatica* gave highest phenolic content (45.2±0.3 µg PE per mg of extract) followed by water extract (35.6±0.5 µg PE per mg of extract) and 100% ethanol extract (21.6±0.1 µg PE per mg of extract). Leaves of *Centella asiatica* collected from three districts of Assam were analysed for phenolic content by Upadhyaya and Saikia (2012) found total phenol content of the ethanolic extracts between 199.5 and 277 mg per g dry material.

Farah *et al.* (2015) evaluated the total phenolic content of ethanolic extracts coriander (*Coriandrum sativum*) plants grown in Saudi Arabia. They obtained 0.83 ± 0.02 g GAE per 100 g phenolic content from ethanol extract. Paul *et al.* (2013) found total phenolic content of were 63.22±3.59, 62.86±12.81 and 54.17±6.15 mg per g equivalent to gallic acid in coriander using ethanolic extraction, water extraction and petroleum ether extraction respectively. Al-juhaimi and Ghafoor (2011) analysed the total phenols of coriander (*Coriandrum sativum*) leaves in diethyl ether extract. The leaves contained 1.12 mg GAE per 100ml phenolic content. Dutta and singh (2011) studied the aqueous extracts of coriander leaves (CS) for estimation of their total phenol content. They found total phenol content of 0.15 mg GAE per g.

Almey *et al.* (2010) determined the level of total phenolic compounds in curry leaves and found that both methanolic and ethanolic extracts of *Murayya koeniigi* had 20.46±0.20 and 12.31±0.18 mg GAE per g phenolic content respectively. Vijayvargia and Vijayvergia (2016) assessed the total phenolic content of *Murraya Koenigii* Linn leaves. They prepared curry leaves extract to determine the phenolic content using combination of petroleum ether, ethyl acetate and methanol. They found 25.2±0.16 mg GAE per gm DW total phenolic content in curry leaves. 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 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. Devatkal *et al.* (2012) found total phenolic content of 59.2±0.90 mg per g GAE in aqueous extract of curry leaves.

Stankevicius *et al.* (2010) analysed the phenolic content of dill leaves (*Anethum graveolens*). The content of phenolic compounds was found in ethanolic extracts of dill (1399.8±2.4) mg of GAE per 100 g of dry plant material. Abbasi *et al.* (2016) studied total phenolic content of the aqueous extract of *Anethum graveolens* (dill) and showed that dill had 163±3.8 mg GAE per g total phenolic content. Jinesh *et al.* (2010) carried out comparative evaluation of antioxidant properties of leaves of *Anethum graveolens* using ethanol as a solvent. The poly phenol content of green and yellow leaves extracts of *Anethum graveolens* was 10.60% and 14.30% w/w, respectively in terms of gallic acid equivalents.

Hewageegana *et al.* (2014) studied proximate analysis and standardization of leaves: *Leptadenia reticulata* (retz) wight and they found that the mean total polyphenolic content of methanolic extract of *L. reticulata* was 55.6±0.50 mg GAE per g extract. George *et al.* (2015) extracted the dodi leaves in methanol solvent and found that phenolic content of extract was 40 µg GAE per gm. Phytochemical evaluation of *Leptadenia reticulata* (Retz) was carried out by Sonara and Saralaya (2012) for poly phenolic compound. They used methanol and acetone to extract phenolic components. The total phenolic content of the *Leptadenia reticulata* extracts (methanol and acetone) were 1.33 mg% and 2.77 mg% respectively in terms of gallic acid equivalent.

Abdulkadir *et al.* (2015) determined the total phenolic content of drumstic leaves (*Moringa oleifera* Lam.) in methanolic extract was 32.83±1.38 mg GAE per g. Leaves of *Moringa oleifera* (variety: Num Phare, Ang Thong, and PKM1) grown in Thailand were extracted with 95% ethanol and their total phenolic content estimated by Wangcharoen and Gomolmanee (2011). The TPC for three variety *M. oleifera* extract in two different solvents were found 8.43+1.32 mg GAE per g dw for Num Phare, 9.63±1.53 mg GAE per g dw for Ang Thong and 9.69±2.56 mg GAE per g dw for PKM1 respectively in aqueous extract whereas 8.43±1.32 mg GAE per g dw for Num Phare, 9.63±1.53 mg GAE per g dw for Ang Thong and 9.69±2.56 mg GAE per g dw for PKM1 respectively in ethanol extract. Nadeem *et al.* (2013) evaluated the total

phenolic content of drumstick leaves extract (methanolic) and observed that leaves contained 7.6 g per 100 g total phenolic content.

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. Devatkal *et al.* (2012) determined phenolic content of 52.2±0.85 mg per g GAE in aqueous extract of fenugreek leaves.

Gul *et al.* (2013) studied total phenolic content of jequirity leaves. They prepared four different extracts using different solvent such as hexane, ethyl acetate, ethanol and water. The Total phenolic content for four different jequirity leaves extract were found in the range of 1.65±0.22 to 25.48±0.62 GAE mg per g. Jain *et al.* (2015) estimated the polyphenolic content of different parts of *Abrus precatorius.* They found that methanolic extract of *Abrus precatorius* leaves contained 4.80 mg GAE per g polyphenolic content.

Sah *et al*. (2012) determined the total phenolic content of lemongrass leaves extract (40% ethanol). Lemongrass leaves extract had TPC value of 67.28±0.86 mg GAE per g. Hasim *et al.* (2015) prepared extracts of lemongrass leaves (*Cymbopogon citratus*) using 30, 70 and 96% ethanol and determined the total phenolic content of the extracts. The highest total phenol content was in the 30% ethanolic extract of 50.017 GAE mg per g. Gruyal (2014) evaluated total phenolic content of five selected Cantilan herbs. The total phenolic content in methanolic extract of *Cymbopogon citratus* was 44.05±0.147 mg GAE per g.

Tupe *et al.* (2013) found that methanolic extract of liquorice contained 71.29±4.67 mg GAE per g total phenolic. Extraction and identification of natural antioxidants from liquorice (*Glycyrrhiza glabra*) was done by El-sherif *et al.* (2011) and found that liquorice extract (ethanolic) contained 353.93 mg per 100g (gallic acid). Rathanavel and Arasu (2014) analysed the total phenol contents of some selected Indian medicinal plants and found that *Glycyrrhiza glabra* extracts (methanolic) contained 141.80 ± 0.58 mg GAE per 100g total phenol content. Rajurkar and Hande (2011) extracted powdered plant samples with a mixture of methanol and water in the volume ratio 4:1 and found phenolic content of 4.94±0.43 mg/100 g in liquorice.

Leaves and stems of three different herbs from two different families were used to extract phenolic compounds. Diethyl ether was used to make extract. Extract from leaves of mint, which belongs to Lamiaceae family contained 1.24 mg GAE/100 mL of

total phenolic compounds which was significantly higher than those in extracts from coriander and parsley, both of which belong to Apiaceae family (Al-juhaimi and Ghafoor, 2011). Total phenolic content of the ethanolic extracts of *Mentha longifolia* and *Mentha citrata* extracts were 30.327±1.4 and 31.452±1.25 g GAE per 100g, respectively (Al-Okbi *et al.,* 2015). *Mentha arvensis* had total phenolic content of 91.02±5.09 mg GAE per g of dried sample. Total phenolic content of six wild menthe species (*M. aquatica, M. arvensis, M. piperita, M. pulegium, M. rutendifolia and M. villosa*) from Algeria determined by Benabdallah *et al.* (2016). The methanol extract of Algerian mints contained total phenolic content in the range of 14.66±0.30 mg GAE per g dw to 43.21±1.09 mg GAE per g dw.

Pawar (2011) studied the effect of herb extract (*Asparagus racemosus*/shatavari) incorporation on storage stability of ghee and he found the phenolic content of aqueous and ethanolic extract of shatavari was found to be 14.49±0.44 and 24.99±0.74 mg GAE per g extract, respectively.

Basak *et al*. (2014) assessed antioxidant and anti-inflammatory activities of extracts with different polarities (hexane, dichloromethane, ethyl acetate, ethanol and methanol) obtained from *Ocimum sanctum* leaves. Total phenolics content of tulsi leaves extracts was varied from 8.8±0.3 to 127.3±1.6 mg GAE per g dw. Mitra *et al.* (2014) determined the total phenolic content of aqueous tulsi leaf (TLE) (*Ocimum sanctum)* extract. Total phenol content of TLE was 47.1±0.023 mg GAE per g extract. Mohankumar *et al.* (2017) investigated total phenolic content of selected medicinal plants from Kanyakumari district in which *Ocimum sanctum* leaves extract (ethanolic) had 9.4 mg GAE per g phenolic content.

The total phenolics content of different herbs evaluated in the study was more or less within the range reported in the literature. However, some deviations might be attributed to variations variety of the herbs (Benabdallah *et al.*, 2016; Abdulkadir *et al.*, 2015), prevailing agroclimatic conditions of the area in which herb is grown (Jaiswal *et al.,* 2014), agronomic practises followed in the herb farming (Azhar *et al*., 2011; Pakade *et al.,* 2013), maturity of the herb at the stage of harvesting (Jinesh *et al.*, 2010), method followed for post-harvest processing of the herb (Pakade *et al.,* 2013; Al-juhaimi and Ghafoor, 2011; Vijayalakshmi, and Shourie, 2015), the type and concentration of solvent (Hasim *et al.*, 2015; Wangcharoen and Gomolmanee, 2011; Gul *et al.*, 2013) as well as polarity of solvent (Rahman *et al.*, 2013; Basak *et al*., 2014)

used for analysis, the method followed for the estimation of the total phenolics content (Jinesh *et al.,* 2010; Wangcharoen and Gomolmanee, 2011; Rahman *et al.*, 2013), etc.

4.2.2 Radical Scavenging Activity of Herbs by DPPH Assay

DPPH is a stable chromogen radical with a deep purple colour and suitable in study of radical scavenging activity. The DPPH scavenging assay is based on electron donation of antioxidants to neutralize DPPH radical. In this assay, the purple chromogen DPPH$^{\bullet}$ radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 517 nm, which is proportional to concentration of free radical scavenger added to DPPH solution (Boligon *et al.,* 2014).

The DPPH radical scavenging activity of selected herbs ajwain leaves, betel leaves, centella, coriander, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi was determined by following the method as described above. The radical scavenging activity of (methanol: water = 8:2) extract of herbs is given in Table 4.3 and also graphically presented in Figure 4.3.

Table 4.3: Radical scavenging activity of herbs

Sr. No.	Herb added		Radical scavenging activity (% Inhibition)
	Common name	Scientific name	
1	Ajwain leaves	*Coleus aromaticus*	63.52 ± 1.47
2	Betel leaves	*Piper betle*	87.31 ± 0.77
3	Centella	*Centella asiatica*	86.64 ± 0.52
4	Coriander leaves	*Coriandrum sativum*	79.40 ± 0.76
5	Curry leaves	*Murraya koenigii*	84.07 ± 0.93
6	Dill	*Anethum graveolens*	83.62 ± 1.13
7	Dodi	*Leptadenia reticulate*	61.37 ± 0.13
8	Drumstick leaves	*Moringa oleifera*	16.39 ± 0.77
9	Fenugreek leaves	*Trigonella foenum graecum*	79.72 ± 0.82
10	Jequirity leaves	*Abrus precatorius*	65.24 ± 1.44
11	Lemon grass	*Cymbopogon citratus*	37.05 ± 1.21
12	Liquorice	*Glycyrrhiza glabra*	83.20 ± 1.29
13	Mint	*Mentha citrata*	85.76 ± 0.28
14	Shatavari	*Asparagus racemosus*	28.41 ± 0.89
15	Tulsi	*Ocimum sanctum*	39.24 ± 1.46

Source of variation	Treatment (herbs)
SEm	0.71
CD (0.05)	2.06
CV%	1.89

Data are presented as means ± SD (n=3).

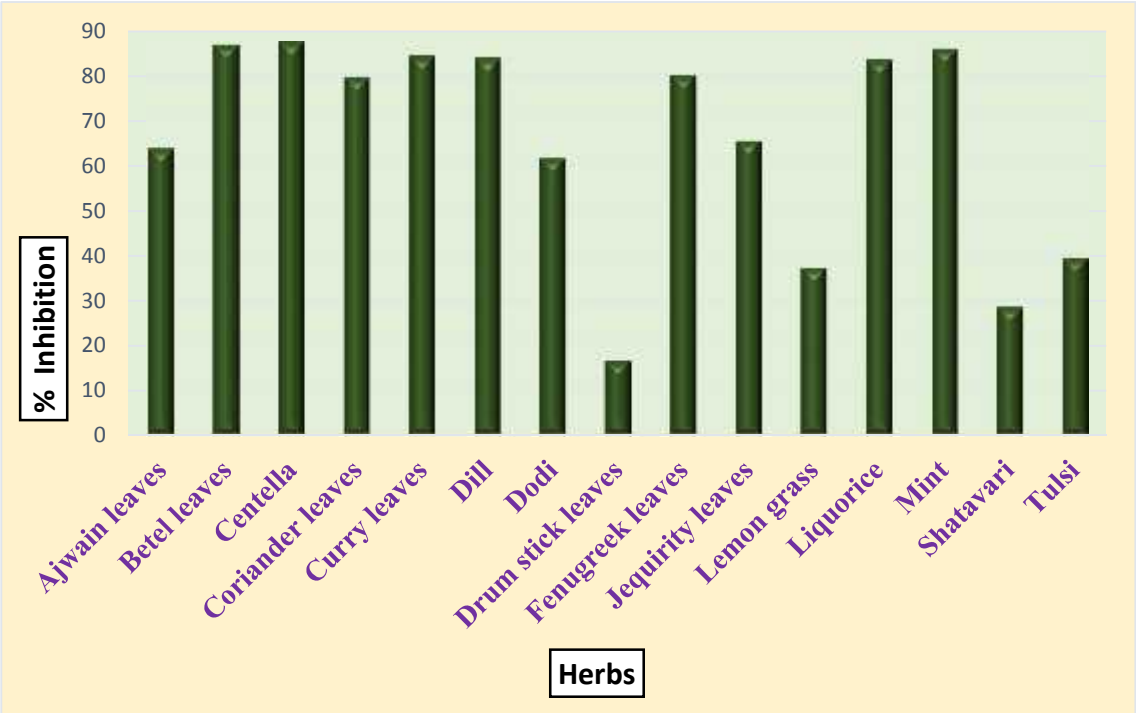


Figure 4.3: Radical scavenging activity of herbs

The radical scavenging activity of the fifteen different herbs was in the decreasing order of betel leaves > centella > mint > curry leaves > dill > liquorice > fenugreek leaves > coriander leaves > jequirity leaves > ajwain leaves > dodi > tulsi > lemon grass > shatavari > drumstick leaves. Thus the radical scavenging activity was highest in betel leaves (87.31% inhibition), which was very closely followed by centella and mint. The lowest radical scavenging activity was found in drumstick leaves (16.39% inhibition).

The difference in radical scavenging activity of herbs tested differed significantly. The radical scavenging activity of the top ranking herb, the betel leaves (87.31% inhibition) was significantly higher than remaining other herbs, except centella and mint. The second highest radical scavenging activity was found in centella (86.64% inhibition), which was statistically at par with mint and significantly higher than remaining other herbs, except betel leaves. The third position in radical scavenging activity was occupied by mint (85.76% inhibition). Its radical scavenging activity was statistically at par with curry leaves, but significantly higher than remaining other herbs, except betel leaves and centella. Curry leaves (84.07% inhibition) was on fourth position in radical scavenging activity and it was statistically at par with dill and liquorice, but significantly higher than remaining other herbs except betel leaves, centella and mint. With respect to the radical scavenging activity dill (83.63% inhibition) stood at position

five and it was statistically at par with liquorice, but significantly higher than remaining all other herbs, except betel leaves, centella, mint and curry leaves.

Oueslati *et al.* (2016) estimated antioxidant activity of aqueous and organic extracts (Ethanol 70%) of *Trachyspermum ammi and* found that the aqueous extract has shown the best antioxidant activity with IC_{50} values of 4.89 µg per ml DPPH assays. Gruyal (2014) evaluated the free radical scavenging capacity of methanolic extract of *Coleus aromaticus* and showed that it scavenged the radicals at 55.62%.

Kaveti *et al.* (2013) carried out In vitro evaluation of antioxidant activity of methanolic extract of piper betel. They found that the concentration of *Piper betel* needed for 50% inhibition was found to be 282.31µg/ml. Jaiswal *et al.* (2014) collected six different variety of betel leaf [Banarasisafeda (PA), Calcutta (PB), Cuttack (PC), Desibagla (PD), Maharashtra (PE) and Sofia (PF)] from Uttar Pradesh, West Bengal, Odisha, Maharashtra and Bihar. These varieties were extracted using five solvents (80% methanol, 80% ethanol, 80% acetone, 80% ethyl acetate and distilled water) in order to determine the effect of solvent and changing variety on total phenol content and antioxidant activity. The DPPH scavenging activity for six variety betel leaf extract in five different solvents were found in the range of 7.20 to 133.5 mg CE per g dw for PF, 127.5±0.25 mg CE/g dw for PE, 77.71±1.62 for PD, 74.96±1.8 for PC, 55.37±1.25 for PB and 2.48 mg CE/g dw for PA. Tamuly *et al.* (2013) obtained IC_{50} value of 36.45 µg per ml for methanol: water (1:1) extract of *P. betel*.

In vitro antioxidant activity of *Centella asiatica* (Linn.) and the impact of extraction solvent polarity on the antioxidant potential were investigated by Rahman *et al.* (2013) in which 100% ethanol, 50% ethanol and water were chosen as extraction solvent. In case of DPPH-scavenging activity, IC_{50} values of 100% ethanol, 50% ethanol and water extracts were 35.6±1.3, 7.1±1.5 and 10.3±1.2 µg per ml respectively. Leaves of *Centella asiatica* collected from three districts of Assam were analysed for phenolic content and antioxidant activity by Upadhyaya and Saikia (2012). DPPH activity was between 73.7% and 88.2% in the ethanolic extract of *C. asiatica*.

Farah *et al.* (2015) evaluated antioxidant activity of ethanolic extracts coriander (*Coriandrum sativum*) plants grown in Saudi Arabia. They found 89.82% DPPH activity in coriander extract. Al-juhaimi and Ghafoor (2011) analysed the antioxidant activities of leaf and stem extracts from coriander and found that DPPH activity of

diethyl ether extract of coriander leaves was 26.82%. Dutta and singh (2011) analysed aqueous extracts of coriander (CG) for scavenging free radical activity. The IC_{50} values of the extracts was found to be 0.53±0.2 mg per ml.

DPPH radical scavenging method was used to determine the antioxidant activity in curry leaves and found that both methanolic and ethanolic extracts of *Murayya koeniigi* had 0.006±0.001 and 0.008±0.001 mg GAE per g phenolic content respectively (Almey *et al.,* 2010). Vijayvargia and Vijayvergia (2016) assessed the antioxidant activity of *Murraya Koenigii* LINN. They prepared curry leaves extract to determine the phenolic content using combination of petroleum ether, ethyl acetate and methanol and found curry leaves extract contained maximum DPPH radical scavenging capacity (IC_{50} 49.86±1.16 mg per ml). In a work carried out by Lalwani *et al.* (2014), curry leaves extract (ethanol) at a concentration of 0.02mg DPPH per ml ethanol exhibited the free radical scavenging activity of 81.13%. Ishtiaque *et al.* (2015) determined the antioxidant activity of methanolic extract of *Murraya koenigii* (curry leaves). Result from their study indicated that curry leaves contained 20-64% DPPH activity. Devatkal *et al.* (2012) determined antioxidant effect of aqueous extracts of curry leaves in raw chicken patties. Curry leaves extract had DPPH free radical scavenging activity of 61.4±1.3%.

Isbilir and Sagiroglu (2011) investigated the antioxidant properties of water, ethanol, and acetone extracts of dill leaves. Among the three extracts, the water extract of dill leaf showed the most potent antioxidative capacity (79.66%) in the DPPH radical scavenging activity. Stankevicius *et al.* (2010) analysed DPPH-free radical scavenging capacity of dill (*Anethum graveolens*) leaves ethanolic extracts. The DPPH activity was 51.7 %. Abbasi *et al.* (2016) assessed the antioxidant properties of aqueous extract of Anethum graveolens (dill) and obtained DPPH radical scavenging activity of 64-86%.

Hewageegana *et al.* (2014) studied proximate analysis and standardization of leaves: *Leptadenia reticulata* and they found that the DPPH radical scavenging activity of methanolic leaf extract was (IC_{50}: 18.56±0.29 µg per ml). Phyto-pharmacological evaluation of *Leptadenia reticulata* was carried out by George *et al.* (2015). They extracted *Leptadenia reticulata* in methanol solvent and found IC_{50} value of *L. reticulata* extract was found to be 220 µg per ml. Sonara and Saralaya (2012) used two solvents (methanol and acetone) for estimation antioxidant activity by DPPH assay. The extracts (methanol and acetone) of *L. Reticulata* had IC_{50} values of 56.66 µg per

ml and 55.55 µg per ml respectively. Tewari *et al.* (2014) studied In vitro antioxidant activity of *Leptadenia pyrotechnica* extract (methanol) and obtained DPPH radical scavenging activity of 38.59-57.18%.

The result obtained from the study of Abdulkadir *et al.* (2015) showed that methanolic leaf extract of drumstick possess mean percentage inhibition (DPPH) of 66.85±1.20%. The antioxidant activity of the leaves of *Moringa oleifera* were investigated by Pakade *et al.* (2013). Fresh *M. oleifera* samples were harvested in April 2011 from two growing areas – Limpopo (LF) and Atteridgeville (AF) – in South Africa. The leaf samples were collected from trees that were planted in different years (2006, 2009 and 2010) and labelled as such, that is, 2006LF, 2009LF, 2010LF and 2010AF. A total of four moringa leaf samples were investigated by extracting their leaves by methanol as a solvent. The DPPH activity for four different moringa samples were found to be in the range of 51.6±15.2 to 59.8±1.7 (percentage of the DPPH concentration left in solution) for 2006LF, 2009LF, 2010LF and 2010AF respectively.

Premanath *et al.* (2011) evaluated the antioxidant activities of fenugreek leaves by DPPH scavenging activity. Among two solvent (methanol and ethanol) extracts tested for antioxidant activity, ethanol extract had the better scavenging activity (59.7±0.46%) followed by methanol (53.2±0.33%). Devatkal *et al.* (2012) determined the antioxidant activity of aqueous extract of fenugreek leaves in raw chicken patties. Fenugreek leaves extract contained 64.2±0.78 % DPPH free radical scavenging activity.

Gul *et al.* (2013) studied antioxidant activity of jequirity leaves extracts (methanol and ethanol) by DPPH method and found that ethanol extract had better ability to scavenge DPPH radical compared to methanol extract. The best IC_{50} values was obtained in ethanol extract of 96.35±2.98 µg per ml compared to methanol (92.63 ±4.63 µg per ml). Jain *et al.* (2015) found that methanolic extract of *Abrus precatorius* leaves had 68% DPPH radical scavenging activity.

Gruyal (2014) showed that methanolic extract of *Cymbopogon citratus* scavenged the radicals by 75.38%. Sah *et al*. (2012) determined the antioxidant activity of lemongrass leaves. The antioxidant activity were evaluated using DPPH radical scavenging ability. Lemongrass leaves extract (40% ethanol) possesses reasonably antioxidant potential with EC_{50} (DPPH) of 192 µg per ml. Hasim *et al.* (2015) prepared extracts of lemongrass leaves (*Cymbopogon citratus*) using 30, 70 and 96% ethanol and

determined the DPPH activity of the extracts. The best IC_{50} value was obtained in the 70% ethanol extract of 79.444 mg per l.

Tupe *et al.* (2013) evaluated the radical-scavenging activity of liquorice extracts using DPPH assay. They found that methanolic extract of liquorice contained 87.64±0.7% DPPH activity. The DPPH-radical-scavenging activity of the methanolic extracts of licorice from different habitats determined by Karahan *et al.* (2016). The IC_{50} values of the extracts were found to be between 588±0.86 µg/ml and 2190±1.73 µg/ml.

Gruyal (2014) evaluated the free radical scavenging capacity of *Mentha arvensis* extract (methanol) which scavenged radicals at 93.48%. Antioxidant activity of six wild menthe species (*M. aquatica, M. arvensis, M. piperita, M. pulegium, M. rutendifolia and M. villosa*) from Algeria determined by Benabdallah *et al.* (2016). The methanol extract of Algerian mints exhibited antioxidant activity (IC_{50}) ranging from 7.5 µg per ml to 44.66 µg per ml. Al-juhaimi and Ghafoor (2011) reported that extract from leaves of mint contained 34.21%.

Kaur and Mondal (2014) evaluated antioxidant activity of alcoholic extracts of *Asparagus racemosus* by DPPH free radical method. *Asparagus racemosus* leaves showed 65%. Pawar (2011) studied the effect of herb extract (*Asparagus racemosus*/shatavari) incorporation on storage stability of ghee and he found the radical-scavenging activity of ethanolic and aqueous of shatavari was 62.89±0.34% and 40.17±0.44% respectively.

Basak *et al*. (2014) assessed antioxidant activities of *Ocimum sanctum* leaves extracts with different polarities (hexane, dichloromethane, ethanol and methanol) obtained from. The antioxidant activity of the methanolic extract was superior to that of all extracts tested with the lowest IC_{50} value of 5.62±0.23 mg per l required to scavenge 50% of DPPH radical, followed by the ethanolic extract (9.25±0.72 mg per l), dichloromethane extract (71.57±3.65 mg per l). However, the extract obtained with hexane exerted the lowest DPPH activity with IC_{50} value of 263.44±12.72 mg per l. Mitra *et al.* (2014) determined the scavenging activity of aqueous tulsi leaf (*Ocimum sanctu*m) extract. The aqueous TLE exhibited over 59.94% scavenging activity of DPPH radical at a concentration of 1 µg per ml. In work carried out by Kaur and Mondal (2014) on antioxidant activity of alcoholic extracts from *Osmium sanctum* leaves showed 74.73% inhibition of DPPH radical.

The quality of natural extracts and their antioxidative performances depends not only on the quality of the original plant, the geographic origin, climatic condition, harvesting date and storage, but also environmental and technological factors affect the activities of antioxidants from residual sources (Moure *et al*., 2001). Carlsen *et al*. (2010) opined that like the content of any food component, antioxidant values will differ for a wide array of reasons, such as growing conditions, seasonal changes and genetically different cultivars, storage conditions and differences in manufacturing procedures and processing. These authors also observed that the differences in unprocessed and processed plant food samples, processed berry products like jam and syrup have approximately half the antioxidant capacity of fresh berries. According to Perez-Jimenez *et al*. (2008) possible interferences is also considered as one of the reasons. There are some aspects that may interfere in the determination of antioxidant capacity and should be taken into account when analysing results. Firstly, the solvent in which the reaction takes place is a key factor in the results, since the polarity of the solvent affects the mechanism of the reaction. Another factor to be considered in the determination of antioxidant capacity is the possible presence in the sample of certain non-antioxidant compounds, which may react in the antioxidant capacity assays, producing over estimations of antioxidant capacity. For instance, it was established that several amino acids may provide a false positive in antioxidant capacity assays. These authors also expressed vies that expression of results is the last step in the determination of antioxidant capacity of a sample, but it is also a key point. Several different ways of expressing the results can be found in works published on antioxidant capacity, even for the same method, making it quite difficult to compare results from similar samples.

The radical scavenging activity of different herbs evaluated in the study was more or less within the range reported in the literature. However, some deviations might be attributed to variations in chemical composition of the different herbs with respect to phenolics and other antioxidants as well as pro-oxidants content (Tupe *et al.,* 2013; Sengul *et al.*, 2009; Kaur and Mondal, 2014; Fukumoto and Mazza, 2000; Bouayed and Bohn, 2010). Some variations observed in antioxidant activities of different herbs in the present study might be attribute one or more of reasons as discussed in section 4.2.1.

4.2.3 Changes in Peroxide Value of Ghee on Storage at 80°±2°C

The oxidative stability of oils and fats with added antioxidants can be determined during storage under normal ambient conditions and packing. However, in general, oxidation can take a long time to occur, e.g. a few days to a few months, which is impractical for routine analysis. For this reason, accelerated oxidation tests or aging tests are conducted.

Tests like Schaal oven test, Sylvester test and Swift test are used for accelerated oxidation test at elevated temperature. Normally, Schaal oven test is used for determination of oxidation of oils but it is also used in dairy industry and oxidative stability of milk fats tested using determination of peroxide value. Schaal oven test involves heating of a convenient quantity of the sample in a thermostatically controlled oven at a specified temperature (either 63°±5° or 70°C) to the time until rancidity starts. Following oxidation, the end point is determined by measuring parameters such as conductivity, peroxide value or diene conjugation. The addition of an antioxidant results in the inhibition of oxidation. Results are quantified by measuring the induction time of a control and sample, with longer induction indicating better antioxidant activity (Bandyopadhyay *et al.*, 2008). Therefore in the present study Schaal oven test was used with a temperature of 80°±2°C, which was selected based on earlier practice established in the laboratory of the Department.

Ghee was prepared as per the procedure described in section 3.3.3 and divided in to sixteen parts. Fifteen different herbs as listed above were added separately at the rate of 0.5 per cent (w/w) to each sample of ghee. The mixtures of ghee and herbs were thoroughly mixed and allowed to stand at 80^{o}C for 30 min for the extraction of phenolic compounds into the ghee. The treated ghee samples were filtered through 6 layers of muslin cloth. The ghee sample without addition of any herb was used as a control sample.

All the samples of ghee were stored at elevated temperature (80°±2°C) to accelerate the oxidation. The samples of ghee were analysed for peroxide value when fresh and also during storage at and interval of every 3 days. The storage study was continued for a period of 12 days where almost all the samples of ghee became unacceptable in sensory evaluation. Total three replications were conducted. The data obtained for changes in peroxide value of the ghee samples are given in Table 4.4 and the trend is presented in Figure 4.4.

Table 4.4: Changes in peroxide value of ghee on storage at 80°±2°C

Sr. No.	Herb used	Peroxide value during storage (meq of O_2 per kg fat)				
		0 day	3rd day	6th day	9th day	12th day
1	- (Control)	0.00	0.98	02.66	04.80	07.88
2	Ajwain leaves	0.00	2.58	04.38	07.23	14.25
3	Betel leaves	0.00	2.11	02.80	04.13	04.99
4	Centella	0.00	2.55	04.63	07.59	20.46
5	Coriander leaves	0.00	1.97	05.39	06.81	13.00
6	Curry leaves	0.00	2.20	03.97	05.77	08.92
7	Dill	0.00	2.82	04.62	07.28	19.77
8	Dodi	0.00	3.18	03.99	08.39	31.49
9	Drumstick leaves	3.24	8.94	18.31	35.80	40.99
10	Fenugreek leaves	0.00	2.59	04.08	05.96	11.66
11	Jequirity leaves	0.00	4.17	08.01	15.81	40.37
12	Lemon grass	1.92	5.39	10.79	28.96	38.02
13	Liquorice	0.00	1.63	02.80	03.47	05.65
14	Mint	0.00	2.68	04.47	07.15	14.81
15	Shatavari	1.47	5.03	10.26	31.22	38.37
16	Tulsi	1.44	4.39	07.35	20.68	33.35

Source of variation	Storage period (P) (days)	Treatment (T) (herbs)	Interaction (P×T)
SEm	0.14	0.24	0.54
CD (0.05)	0.38	0.68	1.51
CV%	10.65		

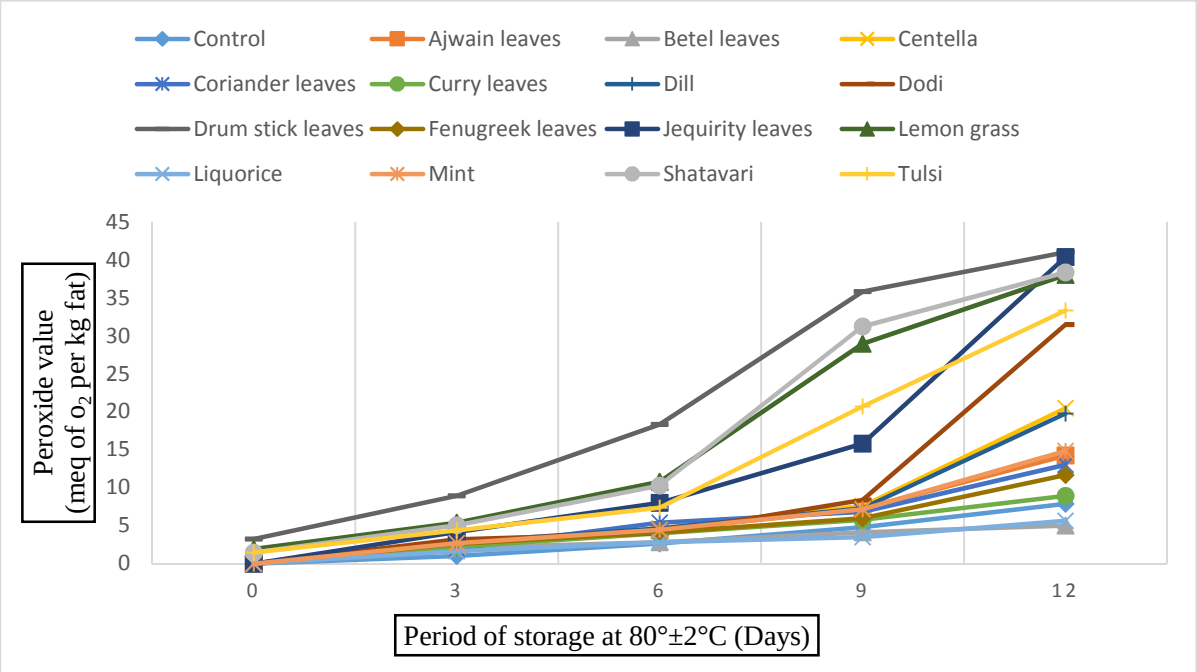


Figure 4.4: Changes in peroxide value of ghee on storage at 80°±2°C

The peroxide value of fresh control ghee sample and that of the different herbs treated ghee samples was in the order of control = ajwain leaves = betel leaves = centella = coriander leaves = curry leaves = dill = dodi = fenugreek leaves = jequirity leaves = liquorice = mint < drumstick leaves < lemon grass < shatavari < tulsi.

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at $80^{o}\pm2°C$ that the treatment (herbs) and period of storage were significant ($P < 0.05$). The interaction between period of storage and herbs was also significant ($P < 0.05$). Therefore, this statistical analysis of the results revealed that different herbs used for in ghee treatment of ghee differed significantly ($P < 0.05$) in their effect on changes in peroxide value of ghee. Similarly, period of storage also differed significantly ($P < 0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the herbs differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of herbs and period of storage were dependent on each other.

The fresh control ghee sample (0 day) (*i.e.* without treatment of any herb) had no peroxide content, as evident from its peroxide value of 0 meq of O_2 per kg fat. Similarly, peroxide value of 0 meq of O_2 per kg fat in ajwain leaves, betel leaves, centella, coriander, curry leaves, dill, dodi, fenugreek leaves, jequirity leaves, liquorice and mint treated ghee samples also suggested that there was peroxide content in these samples. However, ghee samples treated with drumstick leaves, lemon grass, shatavari and tulsi had a peroxide value of 3.24, 1.92, 1.47 and 1.44 meq of O_2 per kg fat. Thus, fresh ghee sample treated with drumstick leaves, lemon grass, shatavari and tulsi had a significantly higher peroxide value compared to that of the control ghee sample as well as other herbs treated ghee samples.

The peroxide value of ghee samples at the end of the storage period of 12 days was in the order of drumstick leaves > jequirity leaves > shatavari > lemon grass > tulsi > dodi > centella > dill > mint > ajwain leaves > coriander leaves > fenugreek leaves > curry leaves > control > liquorice > betel leaves.

On storage, peroxide value of control ghee sample increased gradually during storage and reached to 07.88 meq of O_2 per kg fat on 12^{th} day of storage. Almost similarly trend of gradual rise in peroxide value was also observed in ghee samples treated with betel leaves, curry leaves, dill, dodi, fenugreek leaves and liquorice. In case of ajwain leaves,

centella, dodi and mint treated ghee samples greater rate of rise in peroxide value was observed from 9th day of storage. However, very sharp rise in peroxide value from very beginning was noticed in samples of ghee treated with drumstick leaves, jequirity leaves, lemon grass, shatavari and tulsi. Their peroxide value on 12th day of storage was 40.99, 40.37, 38.02, 38.37 and 33.35 meq of O_2 per kg fat respectively.

Thus, results revealed that the peroxide value of only betel leaves and liquorice treated ghee samples remained lower than the peroxide value of control ghee sample throughout the entire period of storage. The peroxide value of betel leaves and liquorice treated ghee samples was 4.99 and 5.65 meq of O_2 per kg of fat on 12th day of storage. On 12th day of storage the peroxide value of betel leaves and liquorice treated ghee samples was significantly lower than control ghee sample (07.88 meq of O_2 per kg fat). The peroxide value of ghee samples treated with remaining other herbs (ajwain leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, mint, shatavari and tulsi) were significantly higher than the peroxide value of control ghee sample.

The overall results suggested that among fifteen different herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi) evaluated the study, only betel leaves and liquorice were able to control the peroxide formation in ghee during storage. Among the other herbs only curry leaves treated ghee sample was much closer to control ghee sample, with respect to formation of peroxide in ghee. The remaining all other herbs (ajwain leaves, centella, coriander leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, mint, shatavari and tulsi) were had rather prooxidant effect to a varying extent, ranging from moderate to very strong.

In survey of literature it appeared that only one report (Patel and Rajorhia, 1979) is available in which herbs are used as such to increase stability of ghee oxidative deterioration of ghee. In remaining other reports authors have used extracts from herbs in different forms (oleoresin, essential oil, steam distillate, aqueous or organic solvent extract, isolated fractions, etc.). Therefore, results of the present study could not be compared with such reports. Because several factors can induce changes in the antioxidant activity during the distillation of volatile constituents from herbs. Furthermore, hydrolysis and other cleavage processes can release such compounds. Moreover, heat- and water-induced chemical reactions can also change the activity of a

complex extract system consisting of numerous compounds with different chemical and physical properties (Singh *et al.,* 2004). Unfortunately, as with the evaluation of lipid oxidation in general, the published data comparing the antioxidant activities of plant extracts and herbs is difficult to evaluate because of the diverse testing methods used and the questionable conditions of oxidation. Stability tests to evaluate natural antioxidants use a wide variety of oils and food lipid substrates, oxidation conditions varying from room temperature to 180°C, various end points, and a wide range of methods to measure lipid oxidation. Comparison of the antioxidant activities of crude plant extracts is further complicated by the use of varying amounts of test materials with no knowledge of the concentration of active phenolic components present (Frankel, 1993).

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) and curry (*Murraya koeniji*) leaves when added to butter during clarification. The peroxide values of ghee samples treated with curry leaves, betel leaves and antioxidants changed very little up to 30 days of storage. The control (without antioxidants) showed a steep rise in peroxide with value after 60 days of storage. Ghee samples added per cent curry leaves or betel leaves showed relatively smaller increase in peroxide value up to 135 days of storage. Therefore, results found in present study for betel leaves and curry leaves treated ghee samples were in very close agreement with results reported by these authors.

Singh *et al*. (2004) carried out investigation is to compare the chemical composition of volatile oil and its extract as well as to determine the antifungal and antioxidative behaviour on ajwain oil using as additive by various methods. The authors concluded that the ajwain volatile oil and acetone extract have been proven to be alternative sources of natural antioxidants and more efficacious for linseed oil than various synthetic antioxidants like BHA and BHT.

Jaiswal *et al*. (2014) opined that the antioxidant action of betel leaf is very high, due to the presence of phenolic compound hydroxy-chavicol which have been proved to be preservative for vegetable oils up to the concentration of 0.03% without imparting their taste and odour. Use of betel leaf extract as an antioxidant in butter cake retarded its oxidation and extends its shelf life. It was found to be a better source of antioxidant as compared to BHT and BHA.

Patel *et al.* (2013) observed that extracts of coriander (steam distilled and oleoresin) significantly inhibited the peroxide development throughout the 21 days of storage at 80°C as compared to the control. Pawar *et al.* (2014) observed that vidarikand, shatavari and ashwagandha extracts (aqueous and ethanolic) significantly ($P<0.05$) lowered the peroxide value throughout 21 days of storage at 80±1°C as compared to the control.

Nadeem *et al.* (2013) reported that at the end of 90 days storage period, control sample exhibited the highest peroxide value of 16.65 as compared to 5.5 (meq per kg) in butter oil containing 600 ppm methanolic extract of *Moringa olifera* leaves. Siddiq *et al.* (2005) found that the addition of methanolic and acetone extracts of *Moringa oleifera* to sunflower oil significantly inhibited the development of peroxides under accelerated conditions. Perumal and Becker (2003) reported that in Southern Indian villages, people use the fresh leaves of *M. oleifera* during preparation of cow and buffalo ghee to increase its shelf life.

Merai *et al*. (2003) extracted antioxygenic compounds from two varieties of Tulsi (*Ocimum sanctum* L. ('Sri Tulsi' and 'Krishna Tulsi') leaves powder were with methanol. After vacuum drying they were fractionated into water soluble fraction (WSF) and water insoluble fractions (WIF). The WSF carried pro-oxidant principles whereas the WIF exhibited good antioxygenic properties. WIF was further treated with silica gel and charcoal mixture to remove colour-Imparting pigments and was designated as silica gel charcoal treated fraction (SCF). The leaves powder from 'Sri Tulsi' and 'Krishna Tulsi' contained on an average 8.80 and 6.82% moisture, 0.78 and 1.24% volatile oil, 21.78 and 20.34% protein, 12.59 and 11.44% total ash, 9.56 and 10.28% crude fibre, respectively. The SCF fractions from 'Sri' and 'Krishna Tulsi' leaves powder respectively contained on dry matter basis the total phenolics of 107.53 mg/g and 154.13 mg/g with negligible amounts of phospholipids. 'Krishna Tulsi' leaves exhibited slightly higher antioxygenic activity as compared to 'Sri Tulsi' leaves. The addition of SCF pre-extract of 'Krishna Tulsi' leaves powder at a level of 0.6% (w/v) into creamery butter ghee was found almost equally effective as that of BHA at a level of 0.02% in preventing autoxidation for a period of 8 days (192 h) at 80 ± 2°C storage or until the peroxide value of 5 meq of peroxide oxygen was reached. The phenolics present in the 'Tulsi' leaves appeared to be the main contributory factors in extending the oxidative stability of ghee.

Pawar *et al.* (2012) reported that ghee added with ethanolic extract (0.5%) of shatavari developed lower peroxides as compare to control ghee during accelerated storage of

80±1°C. Gandhi *et al.* (2013) evaluated antioxidative properties of vidarikand ethanoic extract in ghee and reported that ethanolic extract of the vidarikand was more effective for preventing the development of the peroxide value and conjugated diene value in ghee during storage.

El-sherif *et al*. (2011) studied the effect of liquorice extract (ethanoic) in refined sunflower oil to give stability against oxidative deterioration and found that the stability was increased with increasing the level of polyphenols ethanoic extracts liquorice addition. They also studied the effect of addition of these polyphenols ethanoic extracts to sweets fortified by full cream milk powder (sesames and folia). They found that liquorice and carob as powder and polyphenols extracts were delayed the rancidity of sesame and peanut significantly.

Damle (2014) reported that high content of phenolic component in ethanolic extract of liquorice (*Glycyrrhiza glabra* L) is responsible for its powerful antioxidant activity by means of significant free radical scavenging, hydrogen-donating, metal ion chelating, anti-lipid peroxidative and reducing abilities. Liquorice flavonoids have exceptionally strong antioxidant activity. Antioxidant activity of liquorice flavonoids was found to be over 100 times stronger than that of antioxidant activity of vitamin E. A dose of 2.58 mg per ml liquorice flavonoids can scavenge more free radicals (20.6% scavenging) than 258 mg per ml of vitamin E (11.2% scavenging). The flavonoids from liquorice are currently the strongest natural antioxidants known. Thus, liquorice extract can be efficiently used to formulate cosmetic products for the protection of skin and hair against oxidative damage.

Hasim *et al.* (2015) evaluated the potential of ethanoic extract of lemon grass as prevention for oil oxidation. Oxidative stability was determined by comparing the time of induction of soybean oil which was added to the sample extract with induction time of soybean oil. The highest antioxidant activity by Rancimat instrument occured in 70% ethanolic extract added soyabean oil (6.18 hrs) compared to soyabean oil without addition of extract (5.18 hrs).

Ramakrishna *et al*. (2015) prepared confectionery jelly with addition of herbal powder enriched with polyphenols and antioxidants. They concluded that indicating the potential of the herb *Coleus aromaticus* extract as a source of natural colour, antioxidants or

nutraceuticals that could be of use in confectionery and traditional Indian confections with a potential application to reduce oxidative stress in living system.

Yanishlieva and Marinova (2001) noticed that the green tea extracts (GTE) exhibited a pro-oxidant effect in menhanden oil and seal blubber oil, perhaps due to the catalytical effect of their chlorophyll constituents. The activity of green tea extracts (GTE) on the oxidation of refined, bleached and deodorized (RBD) seal blubber oil (SBO) and menhaden oil (MHO) was examined under Schaal oven conditions at 65 °C. Progression of oxidation was monitored using weight gain, peroxide value (PV) and 2-thiobarbituric acid-reactive substances (TBARS) data. GTE exhibited a pro-oxidant effect in both oils examined, perhaps due to the catalytic effect of their chlorophyll constituents. Therefore, in follow-up experiments, a column chromatographic technique was employed to remove chlorophyll from GTE. The resultant dechlorophyllized green tea extract (DGTE) was applied to both SBO and MHO at 100, 200, 500 and 1000 ppm levels. The antioxidant activity of DGTE was compared with the effects of the commonly-used antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) at 200 ppm and α-tocopherol at 500 ppm. DGTE at ≥200 ppm exhibited excellent antioxidant activity in both oils and its efficacy was higher than that of BHA, BHT and α-tocopherol, but less than that of TBHQ.

Moure *et al*. (2001) suggested that the potent antioxidants can autoxidize and generate reactive substances and thus also act as prooxidants, depending on the systems, as observed for gallic acid and derivatives, green tea extracts and flavonols in the presence of metal salts. Differences in activity due to differences in the relative partition between phases in different lipid systems can explain why green teas were active antioxidants in corn oil and soybean lecithin liposomes, and prooxidant in oil-in-water emulsions due to greater affinity for the polar surface of the lecithin bilayers. These authors also reported the ability of ginger and garlic to scavenge hydroxyl radicals, but the interaction with iron chelates facilitated OH^{*} generation, and ginger exerted prooxidant action, accelerating damage to DNA in the presence of iron. The prooxidant activity is a result of the ability to reduce metals, such as Fe^{3+} to forms that react with O_2 or H_2O_2 to form initiators of oxidation. As a general rule, the antioxidants extracted from plants can show prooxidant activity at low concentration and antioxidant activity over certain critical values. However, the opposite effect, i.e. antioxidant effect at low concentration and behaved as prooxidant at high concentration is known. Environmental factors, such as

climatic growth conditions, growth, ripening stage, temperature and duration of storage and thermal treatment have been related with antioxidant activity due to inactivation of peroxidases responsible for prooxidant action.

In present study the prooxidant effect observed in ajwain leaves, centella, coriander leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity, lemon grass, mint, shatavari and tulsi treated ghee samples was in line with the results reported in the literature by various research workers and possible reasons for such prooxidant activity might be assigned to reasons mentioned by these research workers.

Mehta *et al*. (2015) study, a comparison of five peroxide analytical methods was performed using oxidized ghee. Six ghee samples were stored at 80°C to accelerate deterioration and sampled periodically (every 48 h) for peroxides. Six ghee samples were stored at 80°C to accelerate deterioration and analysed for peroxides at an interval of every 2 days. The initial peroxide value of fresh ghee samples ranged from 1.79 meq of O_2 per kg ghee. The peroxide value of ghee was 4.32, 4.06, 6.15, 6.38 and 6.38 1.79 meq of O_2 per kg ghee on 2, 4, 6, 8 and 10 days of storage The increase in peroxide value was significantly ($P < 0.05$) on the second day of storage, declined slightly on the fourth day of storage and reached a maximum on the eighth day of storage. The same trend was observed in the present study for the control sample of ghee. Therefore, results obtained for the control sample of ghee were very well in corroboration of the results reported by these authors. No work is reported in the literature regarding changes in peroxide value ghee during storage at 80°± 2°C after addition of herbs. Therefore, results of the present study cannot be compared with published literature.

From the study carried to evaluate selected herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi) for their ability in reducing oxidative reactions in ghee during storage; it was concluded that only betel leaves and liquorice were found promising in retarding flavour deterioration of ghee. Even performance of curry leaves was also worth to take in to account for further study.

4.2.4 Changes in Flavour Score of Ghee on Storage at 80°±2°C

Ghee was prepared as per the procedure described in section 3.3.3 and divided in to sixteen parts. Fifteen different herbs as listed above were added separately at the rate of 0.5 per cent (w/w) to each sample of ghee. The mixtures of ghee and herbs were thoroughly mixed and allowed to stand at 80°C for 30 min for the extraction of phenolic compounds into the ghee. The treated ghee samples were filtered through 6 layers of muslin cloth. The ghee sample without addition of any herb was used as a control sample.

All the samples of ghee were stored at elevated temperature (80°±2°C) to accelerate the oxidation. The samples of ghee were subjected to sensory evaluation for flavour using 9 point hedonic scale when fresh and also during storage at and interval of 2 days. The storage study was continued till flavour score of almost all the sample went below acceptable level (<6). Total three replications were conducted. The data obtained for changes in flavour score of the ghee samples are given in Table 4.5 and the trend is presented in Figure 4.5.

Table 4.5: Changes in flavour score of ghee on storage at 80°±2°C

Sr. No.	Herb used	Flavour score of ghee during storage (out of 9)				
		0 day	3rd day	6th day	9th day	12th day
1	- (Control)	9.00	8.47	7.69	5.75	3.83
2	Ajwain leaves	8.81	7.83	6.94	5.89	4.56
3	Betel leaves	8.86	8.19	7.61	6.94	6.46
4	Centella	9.00	7.83	6.61	5.69	4.47
5	Coriander leaves	9.00	8.03	7.08	5.44	3.92
6	Curry leaves	9.00	8.25	7.31	6.54	6.24
7	Dill	9.00	7.81	6.92	5.81	3.61
8	Dodi	9.00	8.06	7.28	6.42	4.06
9	Drumstick leaves	8.17	7.22	5.69	3.81	2.22
10	Fenugreek leaves	8.22	7.28	6.03	5.31	4.00
11	Jequirity leaves	8.17	7.72	6.61	5.47	3.44
12	Lemon grass	8.28	7.22	4.75	3.36	2.11
13	Liquorice	9.00	8.25	7.19	6.84	6.37
14	Mint	8.22	7.39	6.44	5.53	4.31
15	Shatavari	8.78	7.86	6.22	4.25	1.89
16	Tulsi	8.28	7.61	6.67	4.31	2.89

Source of variation	Storage period (P) (days)	Treatment (T) (herbs)	Interaction (P×T)
SEm	0.07	0.12	0.27
CD (0.05)	0.19	0.34	0.76
CV%	7.21		

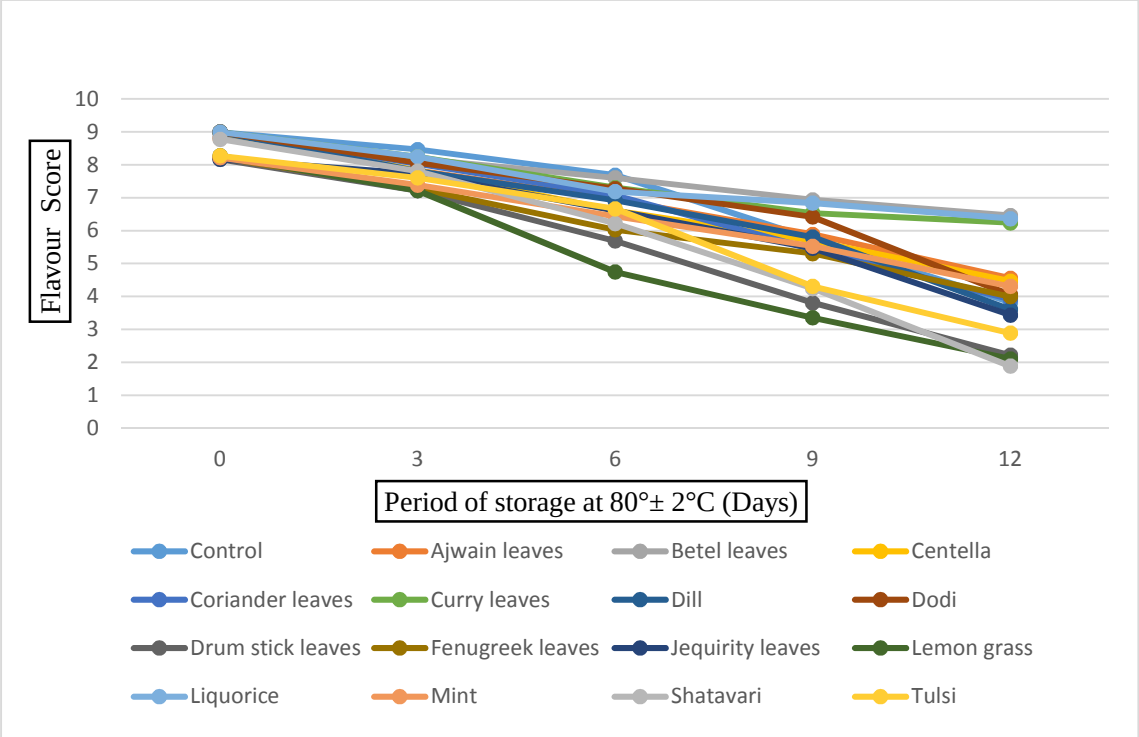


Figure 4.5: Changes in flavour score of ghee on storage at 80°±2°C

The flavour score of fresh control ghee sample and that of the different herbs treated ghee samples was in the order of control = centella = coriander leaves = curry leaves = dill = dodi = liquorice > betel leaves > ajwain leaves > shatavari > tulsi = lemon grass > mint = fenugreek leaves > jequirity leaves = drumstick leaves.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80°±2°C that the treatment (herbs) and period of storage were significant ($P< 0.05$). The interaction between period of storage and herbs was also significant ($P< 0.05$). Therefore, this statistical analysis of the results revealed that different herbs used for in ghee treatment of ghee differed significantly ($P< 0.05$) in their effect on changes in flavour score of ghee. Similarly, period of storage also differed significantly ($P< 0.05$) in their effect on changes in flavour score of ghee. Since, the interaction between period of storage and herbs was found significant it became evident that the effect herbs and period of storage were dependent on each other. In other words the significant interaction effect between period of storage and herbs indicated that the herbs differed significantly from each other in their effect on changes in flavour score of ghee over a period of storage.

The fresh control sample of ghee (*i.e.* without treatment of any herb) (0 day) received full score for flavour, as evident from sore of 9 out of 9. Similarly, full score for flavour

(9 out of 9) in centella, coriander leaves, curry leaves, dill, dodi and liquorice treated fresh ghee samples also suggested that the change in flavour of ghee due to treatment of these herbs had no negative effect the flavour these samples.

On the other hand, fresh ghee samples treated with ajwain leaves, betel leaves, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, mint, shatavari and tulsi acquired lower flavour score than that of the control sample of fresh ghee; indicating that treatment of these herbs had some adverse effect on flavour of ghee. Though the flavour score of fresh ghee treated with ajwain leaves, betel leaves and shatavari was lower than that of the fresh control ghee samples, but the difference was statistically non-significant ($P< 0.05$). Therefore, flavour score of the fresh control ghee sample and that of the ajwain leaves, betel leaves and shatavari treated fresh ghee samples were statistically at par. However, difference flavour score of fresh ghee samples and that of the drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, mint and tulsi was significantly ($P< 0.05$) higher.

The flavour score of fresh control ghee sample and that of the different herbs treated ghee samples at the end of the storage period of 12 days was in the order of betel leaves > liquorice > curry leaves > ajwain leaves > centella > mint > dodi > fenugreek leaves > coriander leaves > **control** > dill > jequirity leaves > tulsi > drumstick leaves > lemon grass > shatavari.

On storage, flavour score of control ghee sample decreased gradually up to 6^{th} day during storage and sharp decrease in flavour score was notices on 9^{th} day of storage. On 9^{th} day of storage flavour score of control ghee sample went below the acceptable level (< 6). Almost similar trend in change of flavour score was observed for ajwain leaves, centella, coriander leaves, dill, fenugreek leaves, jequirity leaves and mint treated ghee samples. The flavour score of shatavari and tulsi treated ghee sample also went below the acceptable level but decline in their flavour score was much larger compared to decline in flavour score of control sample of ghee as well as ajwain leaves, centella, coriander leaves, dill, fenugreek leaves, jequirity leaves and mint treated ghee samples.

In case of drumstick leaves and lemon grass treated ghee samples very sharp declined in their flavour score was observed from beginning of the storage period. The flavour score of these ghee samples went below the acceptable level on 6^{th} day of storage. However, at the same time in betel leaves, curry leaves and liquorice treated ghee samples very

gradual decrease in flavour score was obtained all throughout the storage period. The flavour score of these ghee samples remained above the acceptable level even on 12th day of the storage.

The similar flavour score of fresh control ghee sample and that of the centella, coriander leaves, curry leaves, dill, dodi and liquorice treated fresh ghee samples was attributed pleasing aroma imparted to the ghee by the herbs. On the other hand lower flavour score of ghee sample treated with ajwain leaves, betel leaves, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, mint, shatavari and tulsi as compare to control sample of ghee might be attributed pungent aroma imparted to the ghee by these herbs.

The results of the flavour score on 12th day of storage revealed that among different herbs used in the study dill, drumstick leaves, jequirity leaves, lemon grass, shatavari and tulsi showed negative effect on flavour of ghee during storage, as evident form grater deterioration of flavour on 12th day of storage as compared to control ghee. On the other hand ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dodi, fenugreek leaves, liquorice and mint were able to retard the deterioration in flavour of ghee during the storage. However, from among these beneficial herbs, three top ranking herbs were betel leaves, curry leaves and liquorice giving substantial protection against flavour deterioration of ghee. Only these three herbs were able to retain the flavour score above acceptable level up to termination of the storage study.

In survey of literature it appeared that only one report (Patel and Rajorhia, 1979) is available in which herbs are used as such to increase stability of ghee oxidative deterioration of ghee and reported the changes in flavour score of ghee during storage. In remaining other reports authors have used extracts from herbs in different forms (oleoresin, essential oil, steam distillate, aqueous or organic solvent extract, isolated fractions, etc.) of herbs. Moreover, these authors have not studied the effect of herbs on changes in flavour score of ghee. Therefore, results of the present study could not be compared with such reports.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) and curry (*Murraya koeniji*) leaves when added to butter during clarification. The initial flavour score of control sample, 0.5 per cent curry leaves added samples and 0.5 per cent betel leaves added samples was 8.33, 8.4 and 8.2 respectively.

The flavour score of these ghee samples decreased to 2.16, 5.50 and 5.85 respectively on storage at for a period of 147 days at 30°C. Therefore, results obtained in present study are in general agreement with results reported by these authors. The difference in period of storage to reach the flavour score below acceptable in the reported study and the present study was due to the difference in the temperature used for storage of the samples. In the reported study samples were stored at 30°C whereas, in the present study samples were stored at 80°C.

Mehta *et al*. (2015) study, a comparison of five peroxide analytical methods was performed using oxidized ghee. Six ghee samples were stored at 80°C to accelerate deterioration and sampled periodically (every 48 h) for peroxides. Results were compared using the five methods for analysis as well as a sensory evaluation of ghee for flavour score using 9 point hedonic scale. The initial flavour score of fresh ghee samples was 9.0 which decreased to 7.65, 7.11, 6.39, 5.17 and 4.46 on 2, 4, 6, 8 and 10 days of storage. The flavour score of the ghee declined significantly ($P < 0.05$) after two days of storage, followed by a gradual decrease during the remaining storage period. No attempts were made so far anywhere to compare change in flavour score of ghee with peroxide values (obtained by these five methods) when ghee stored at 80°±2°C. The same trend was observed in the present study for the control sample of ghee. Therefore, results obtained for the control sample of ghee were very well in corroboration of the results reported by these authors. No work is reported in the literature regarding changes in flavour score of ghee during storage at 80°±2°C after addition of herbs. Therefore, results of the present study for changes in flavour score of ghee during storage at 80°±2°C after addition of herbs cannot be compared with published literature.

From the study carried to evaluate selected herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi) for their ability in preserving flavour of ghee during storage; it was concluded that only betel leaves, curry leaves and liquorice were found promising in retarding flavour deterioration of ghee.

4.2.5 Changes in Colour of Different Ghee Samples on Storage at 80°±2°C

The fresh sample of control ghee and fresh of samples various treated ghee were examined for their colour characteristic by visual observation. The samples were stored at 80°±2°C for 12 days and again examined for their colour. The colour characteristic of the fresh ghee samples (before storage) and after storage at 80°±2°C for 12 days is presented in Figure 4.6 (Section 4.1). The comparative appraisal of characteristic of the fresh ghee samples and that of the stored ghee samples is given in Table 4.6.

Table 4.6: Colour of ghee samples before and after storage at 80°±2°C for 12 days

Sr. No.	Herb used	Colour of ghee	
		Before storage	After storage
1	- (Control)	Golden yellow	Dark golden yellow
2	Ajwain leaves	Golden yellow	Light golden yellow
3	Betel leaves	Golden yellow	Golden yellow
4	Centella leaves	Golden yellow	Faint golden yellow
5	Coriander leaves	Golden yellow	Light golden yellow
6	Curry leaves	Golden yellow	Golden yellow
7	Dill	Golden yellow	Faint golden yellow
8	Dodi	Golden yellow	Faint golden yellow
9	Drumstick leaves	Dark olive green	Olive green
10	Fenugreek leaves	Golden yellow	Golden yellow
11	Jequirity leaves	Greenish yellow	Faint olive green
12	Lemon grass	Golden yellow	Faint golden yellow
13	Liquorice	Golden yellow	Golden yellow
14	Mint	Golden yellow	Faint golden yellow
15	Shatavari	Golden yellow	Faint golden yellow
16	Tulsi	Golden yellow	Faint golden yellow

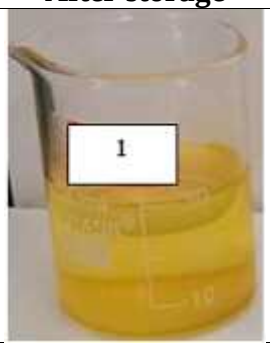
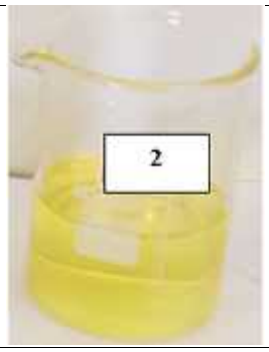
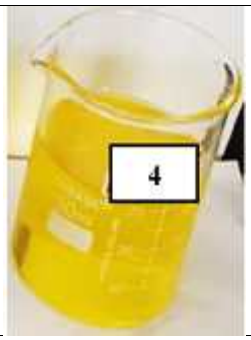
Before storage	After storage	Before storage	After storage
			
Control		**Coriander leaves**	
			
Ajwain leaves		**Curry leaves**	
			
Betel leaves		**Dill**	
			
Centella		**Dodi**	

Figure 4.6: Colour of ghee samples before and after storage at 80°±2°C for 12 days (Cont. …….)

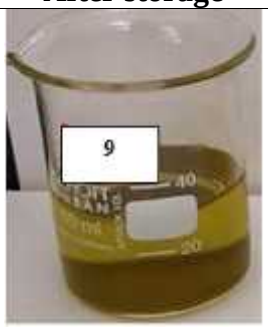
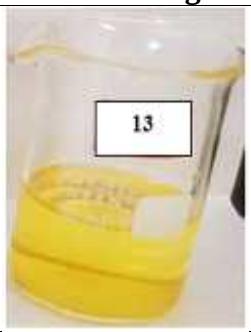
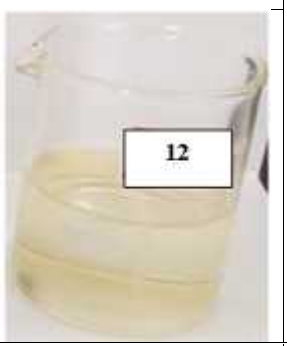
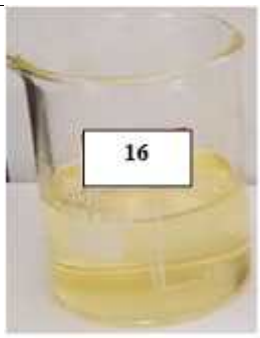
Before storage	After storage	Before storage	After storage
			
Drumstick leaves		**Liquorice**	
			
Fenugreek leaves		**Mint**	
			
Jequirity leaves		**Shatavari**	
			
Lemon grass		**Tulsi**	

Figure 4.6: Colour of ghee samples before and after storage at 80°±2°C for 12 days

Initial golden yellow colour of the control sample of ghee changed to dark golden yellow with slight brownish tinge after the storage. Among fifteen herbs used in the study to treat the ghee, only the samples of ghee treated with betel leaves and liquorice were able to retain the original colour without any change in its shade and intensity. The samples of ghee treated with coriander leaves, curry leaves and fenugreek leaves also retained their golden yellow colour, with slight change in shade and intensity in of the colour, up to the end of the storage study.

However, in case of ajwain leaves, centella, dill, dodi, jequirity leaves, lemon grass, mint, shatavari and tulsi treated ghee samples substantial difference in intensity of colour between colour of freshly prepared ghee samples and colour of the same samples of ghee after twelve storage at 80°±2°C. The golden yellow colour of ajwain leaves and coriander leaves treated fresh ghee sample changed to light golden yellow colour after the storage.

In centella, dill, dodi, lemon grass, mint, shatavari and tulsi treated ghee samples; golden yellow colour of their fresh sample changed to faint yellow colour after the storage. The drumstick leaves and jequirity leaves treated ghee sample also followed the same trend. The dark olive green colour of drumstick leaves treated ghee sample decreased to olive green and greenish yellow colour of jequirity treated ghee sample decreased to faint olive greenish.

The darkening of golden yellow colour of control ghee sample with slight tinge towards brownish side on storage as observed in the present study might be attributed to formation of caramelization and maillard reactions products of yellow to brown colour. The ability of betel leaves, coriander, curry leaves, fenugreek leaves and liquorice treated samples of ghee to retain their golden yellow colour on storage at 80°±2°C for 12 days might be attributed to protective effect provided by antioxidant activity of these herbs. At the same time fading of the golden yellow colour in samples of ghee treated ajwain leaves, centella, dill, dodi, lemon grass, mint, shatavari and tulsi on storage at 80°±2°C for 12 days might be attributed to destructive effect exerted by pro-oxidants activity of these herbs. The pro-oxidants activity of these herbs had been established form changes in peroxide values of these ghee samples (Section 4.2.3). Changing of colour in samples of ghee treated drum stick leaves and jequirity leaves on storage also might be due to the pro-oxidant activity of herbs.

Visual colour in processed foods is largely due to coloured products of non-enzymic browning reactions such as caramelization and maillard. Both caramelization and maillard reaction has long been recognized and quantitated as the source of colour in processed foods. Maillard (1912, 1916) from his earliest observations from amino acid-sugar reactions reported the progressive change in formation of colour ranging from colourless, yellow, orange, wine-red and finally brown. Similarly, caramel colour ranges from the yellow to dark brown (Kamuf *et al*., 2003). Therefore, views and findings of these authors supported the observations made in the present on darkening of colour in controlled sample of ghee on storage.

The colour of ghee is typical golden yellow. The yellow colour of ghee is attributed to its β-carotene content, which in turn originates from grass fed to the animals. It undergoes oxidative degradation during storage resulting in an alteration of major quality parameters such as colour, aroma and the nutritive value (Pawar *et al.,* 2012). The β-carotene bleaching causes discoloration of yellow colour of a β-carotene in solution due to the breaking of π-conjugation by addition reaction of lipid or lipid peroxyl radical (L^* or LOO^*) to a C=C double bond of β-carotene. The radical species is generated from the autoxidation of lipid by heating under air atmosphere (Ueno *et al*., 2014). Therefore, views and findings of these authors supported the observations made in the present on fading of golden yellow colour in of ghee treated ajwain leaves, centella, dill, dodi, jequirity leaves, lemon grass, mint, shatavari and tulsi on storage.

4.3 SELECTION OF STAGE TO ADD HERBS IN PREPARATION OF GHEE

When anybody thinks of food processing, heat treatment is often the first process that comes to mind. Antioxidative activity of a given compound may increase, decrease or remain unchanged as a function of temperature. Stability of an antioxidant to heat is advantageous in the food industry, since many fat and oil-containing foods are heated during processing and since heat is often the initiator of lipid oxidation (Brever, 2011).

It is well known that many food antioxidants can be significantly lost as a consequence of food processing such as sterilisation, pasteurisation 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 antioxidant activity. The antioxidant activity is diminished owing to inactivation of antioxidant compounds caused by different chemical reactions enhanced by the effect of heat. The positive effects of food processing include in some cases transformation of antioxidants into more active compounds, such as the deglycosylation of onion quercetin, as well as an increase in the antioxidant activity owing to inhibition of enzymes. An important primary effect of food browning caused by the maillard reactions may be the formation of antioxidants in a thermal treatment conditions as well (Horvathova *et al*., 2007).

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.

As it is very well known that ghee is almost anhydrous milk fat and 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 antioxidant components present in the herbs. Even possibility also exist about interaction of antioxidants present in herbs with ghee residue, leading to decrease in effectiveness of the antioxidants. At the same there is possibility of improvement in extraction of these antioxidants 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 into ghee, as listed below.

(1) Initial stage of heat clarification of butter in to ghee (in melted butter)

(2) Final stage of heat clarification of butter in to ghee (near 105°C temperature)

(3) After the heat clarification and separation from ghee residue (in hot ghee at 80°C)

An adoption of third possibility for treatment of ghee with herb (in hot ghee after the heat clarification and separation from ghee residue) avoids possibilities of losing antioxidants due destruction by heat or interaction with ghee residue. Moreover, adopting the practice of adding herb to 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.

During first phase of the study (15 herb treated + 1 control) samples of ghee were involved while assessment of herbs for their compatibility in ghee (Section 4.1) and also in second phase of the study evaluating the herbs for their antioxidant potential in ghee (Section 4.2). Therefore, use of the third stage for addition of herbs (in hot ghee at 80°C) was followed during first and second stage of the study. Since, work with the third stage for addition of herbs (in hot ghee at 80°C) was already carried out, in the present phase of the remaining two stages (initial stage of heat clarification of butter in to ghee, *i.e.* in melted butter and final stage of heat clarification of butter in to ghee, *i.e.* near 105°C temperature) were studied to evaluate their performance in treatment ghee with selected herbs.

From the work carried to evaluate antioxidant potential of 15 different herbs betel leaves, curry leaves and liquorice were selected for further study. These herbs were used to evaluate performance of two different stages in preparation of ghee for addition of the herbs. The herbs were added the rate 0.5 per cent of the expected yield of ghee.

For evaluating two different stages in preparation of ghee (initial stage of clarification and final stage of clarification) for treatment with the selected herbs, the sample of butter (120 g) was taken in to each of seven of 500 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 centre of the sand bath. The sand bath was heated by gas fired

burner with flame at the centre. When butter was melted completely betel leaves, curry leaves and liquorice was added separately in the melted butter. In another 3 samples of melted butter betel leaves, curry leaves and liquorice was added separately when their temperature reached to 105°C. Out of 7 samples of the butter kept for heat clarification to prepared ghee, one sample was not treated with any herb to serve as a control. The heating was continued till temperature reached to 120°C.

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 cloth, ghee was collected in 150 ml glass beakers and stored in incubator at 80°±2°C for 12 days. To prepare 7 samples of ghee simultaneously under similar and uniform heating conditions sand bath was used.

All the 7 samples of fresh ghee were analysed for peroxide value by titration against standard sodium thiosulfate solution using starch indicator and expressed as meq of O_2 per kg fat. The samples of ghee were also evaluated for flavour score by sensory evaluation using 9 point hedonic scale. Similarly, during storage all the samples of ghee were analysed for peroxide value and evaluated for flavour score at a regular interval of every 2 days. Total four replications were conducted and average results obtained from the four replications are presented below.

4.3.1 Performance of Betel Leaves Added at Two Different Stages in Ghee Preparation

Effect of two different stages of addition on performance of betel leaves for retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.3.1.1 Peroxide value of betel leaves treated ghee during storage at 80°±2°C

The changes in peroxide value of different ghee samples (control and treated with betel leaves) during storage at 80°±2°C are presented in Table 4.7 and graphically presented in Figure 4.7.

Table 4.7: Changes in peroxide value of betel leaves treated ghee during storage

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)		
	Control	Betel leaves added at initial stage of clarification	Betel leaves added at final stage of clarification
0	0.19	0.63	0.60
2	0.53	1.50	1.28
4	1.44	2.31	1.62
6	1.93	3.07	2.46
8	4.04	4.23	2.81
10	6.19	5.43	3.72
12	9.33	7.09	5.05

Source of Variation	Storage period (P) (days)	Treatment (T) (Stage of addition)	Interaction (P×T)
SEm	0.18	0.12	0.31
CD (0.05)	0.51	0.34	0.89
CV%	20.19		

Data are presented as means (n=4).

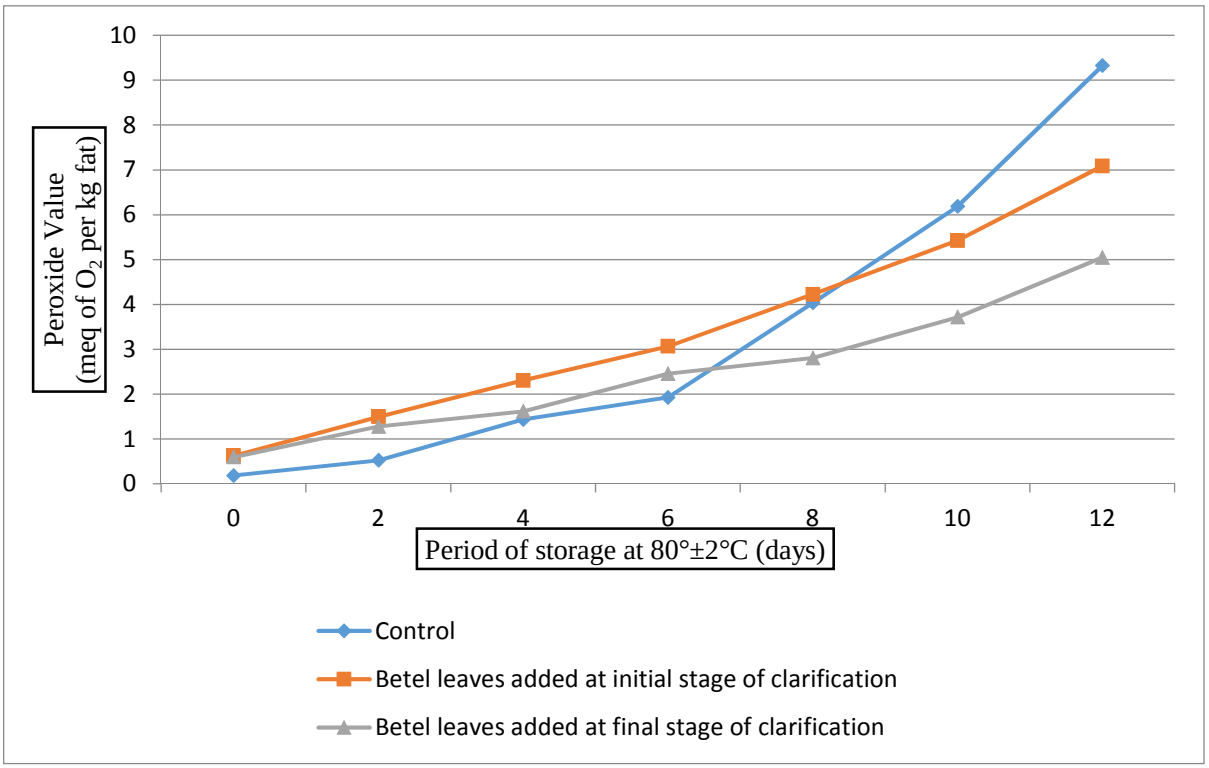


Figure 4.7: Changes in peroxide value of betel leaves treated ghee on storage

The peroxide value of different fresh ghee samples was in the order of control ghee < ghee treated with betel leaves at final stage of clarification < ghee treated with betel leaves at initial stage of clarification. However, the order of peroxide value of different ghee samples was just reversed at the end of the 12 days at 80°±2°C. The order of peroxide value of different samples was just reversed at the end of the storage was ghee treated with betel leaves at final stage of clarification < ghee treated with betel leaves at initial stage of clarification < control ghee.

The peroxide value of all the three types of ghee samples increased at a steady rate up 6^{th} day of the storage. The rate of rise in peroxide value became steep from 6^{th} day onwards in case of control ghee and that in ghee treated with betel leaves at initial stage of clarification. On the other hand in ghee treated with betel leaves at final stage of clarification, no steep rate of rise in peroxide value was noticed at any stage during the entire storage period.

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at 80°±2°C that the treatments (stage of betel leaves addition in ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and stage of betel leaves addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stages in ghee preparation used for treatment of ghee with the betel leaves differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with betel leaves and period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with betel leaves and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) control sample of ghee had significantly ($P<0.05$) lower peroxide value compared to samples of ghee treated with betel leaves, irrespective of their stage of addition in preparation of ghee. Moreover, up to 2 days peroxide value of control ghee sample also remained significantly ($P<0.05$) lower than that of the samples of ghee treated with betel leaves, irrespective of their stage of addition. However on 4^{th} day of storage peroxide value of control ghee sample was

statistically at par with sample of ghee treated with betel leaves at final stage of clarification. Moreover, on 6th day of storage peroxide value of control ghee sample remained significantly ($P<0.05$) lower than that of the samples of ghee treated with betel leaves, irrespective of their stage of addition. However, peroxide value of control ghee sample became significantly ($P<0.05$) higher than that of sample of ghee treated with betel leaves at final stage of clarification on 8th and 10th day respectively. Whereas sample of ghee treated with betel leaves at initial stage of clarification was statistically at par with control ghee on 8th day of storage. On 12th day of storage, peroxide value of control ghee sample remained significantly ($P<0.05$) higher than both the ghee samples treated with betel leaves at initial and final stage of clarification.

Among the sample of ghee treated with betel leaves at two different stages of clarification (initial and final) in preparation of ghee the peroxide values of ghee sample treated with betel leaves at initial stage of clarification remained higher all throughout the storage period than that of sample of ghee treated with betel leaves at final stage of clarification. However, peroxide values of both ghee samples were statistically at par up to 2 day of the storage. Thereafter in remaining storage period peroxide values of ghee sample treated with betel leaves at initial stage of clarification were significantly ($P<0.05$) higher than that of sample of ghee treated with betel leaves at final stage of clarification.

From the forgoing resume it became evident that treating the ghee with betel leaves at final stage of clarification reduced the peroxide formation more effectively compared to the ghee with betel leaves at initial stage of clarification.

No report is available in the literature for evaluating effect of treatment of ghee with betel leaves at different stages in preparation of ghee on changes in peroxide value of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature. Though Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves. These authors added the betel leaves to only melted butter (*i.e.* during initial stage of clarification, but they have not carried the study about final stage of clarification.

4.3.1.2 Flavour score of betel leaves treated ghee during storage at 80°±2°C

The changes in flavour score of different ghee samples (control and treated with betel leaves) during storage at 80°±2°C are presented in Table 4.8 and graphically presented in Figure 4.8.

Table 4.8: Changes in flavour score of betel leaves treated ghee during storage

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)		
	Control	Betel leaves added at initial stage of clarification	Betel leaves added at final stage of clarification
0	9.00	8.80	8.83
2	8.58	8.30	8.33
4	7.75	7.88	7.90
6	6.60	7.48	7.53
8	5.60	7.05	7.15
10	4.10	6.68	6.70
12	2.40	6.20	6.35

Source of variation	Storage period (P) (days)	Treatment (T) (Stage of addition)	Interaction (P×T)
SEm	0.04	0.03	0.07
CD (0.05)	0.12	0.08	0.21
CV%	2.11		

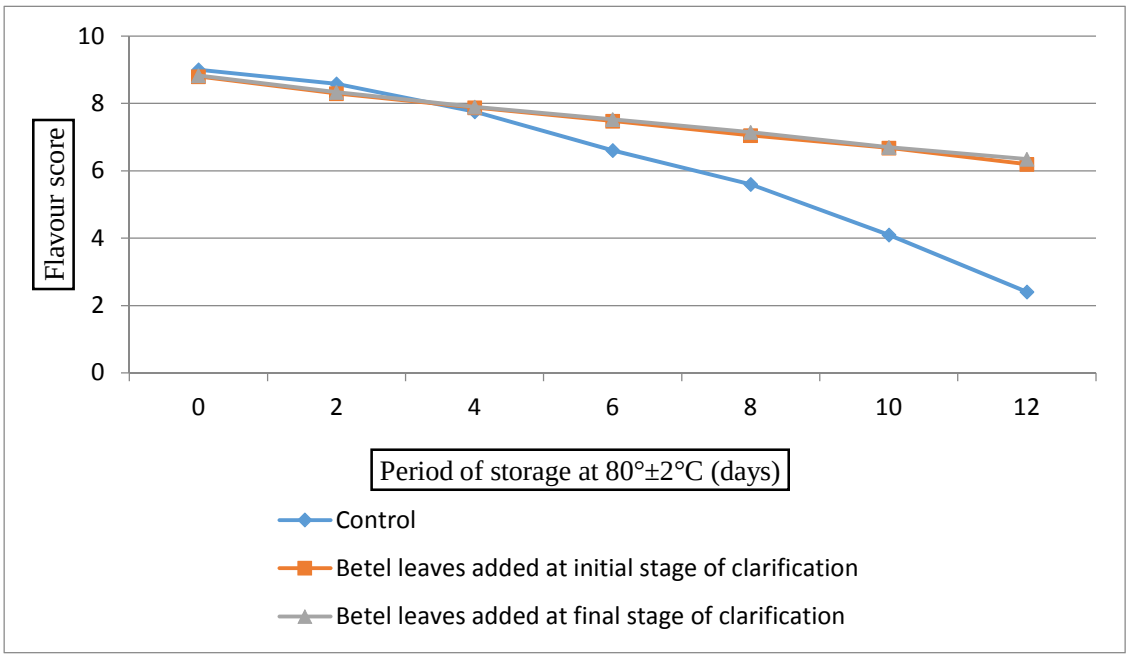


Figure 4.8: Changes in flavour score of betel leaves treated ghee during storage

The flavour score of different fresh ghee was in the order of control ghee > ghee treated with betel leaves at final stage of clarification > ghee treated with betel leaves at initial stage of clarification. However, the order of flavour score of different ghee samples was just reversed at the end of the 12 days at 80°±2°C. The order of flavour score of different ghee samples was just reversed at the end of the storage was ghee treated with betel leaves at final stage of clarification > ghee treated with betel leaves at initial stage of clarification > control ghee.

The flavour score of all the three types of ghee samples decreased at a steady rate up fourth day of the storage. The rate of decline in flavour score of control ghee sample became steep from 6^{th} day onwards. However, in case of ghee treated with betel leaves at initial stage of clarification as well as at final stage of clarification no steep rate of decline in flavour score was noticed at any stage during the entire storage period. The flavour score of control ghee sample went below the acceptable level (<6) on 8^{th} day of the storage. However, flavour score of ghee samples treated with betel leaves at initial stage or final stage of clarification remained acceptable even on 12^{th} day of the storage.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80°±2°C that the treatments (stage of betel leaves addition in ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and stage of betel leaves addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stage in ghee preparation used for treatment of ghee with the betel leaves differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with betel leaves and period of the storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with betel leaves and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) control sample of ghee had significantly ($P<0.05$) higher flavour score compared to samples of ghee treated with betel leaves, irrespective of their stage of addition in preparation of ghee. Even on 2^{nd} day of storage flavour score of control sample of ghee had significantly ($P<0.05$) higher flavour score

compared to samples of ghee treated with betel leaves, irrespective of their stage of addition in preparation of ghee. However, from 4th day of the storage flavour score of control ghee sample became significantly ($P<0.05$) lower than that of sample of ghee treated with betel leaves at initial stage of clarification and sample of ghee treated with betel leaves at final stage of clarification.

Among the sample of ghee treated with betel leaves at two different stages of clarification (initial and final) in preparation of ghee the flavour score of ghee sample treated with betel leaves at initial stage of clarification remained higher all throughout the storage period than that of sample of ghee treated with betel leaves at final stage of clarification. Moreover only on 8th and 12th day of storage flavour score of ghee sample treated with betel leaves at initial stage of clarification was significantly ($P<0.05$) higher than that of sample of ghee treated with betel leaves at final stage of clarification.

From the forgoing resume it became evident that treating the ghee with betel leaves at final stage of clarification reduced the peroxide formation more effectively compared to the ghee with betel leaves at initial stage of clarification.

No report is available in the literature for evaluating effect of treatment of ghee with betel leaves at different stages in preparation of ghee on changes in flavour score of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature. Though Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves. These authors added the betel leaves to only melted butter (*i.e.* during initial stage of clarification, but they have not carried the study about final stage of clarification.

4.3.2 Performance of Curry Leaves Added at Two Different Stages in Ghee Preparation

Effect of two different stages of addition on performance of curry leaves for retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.3.3.1 Peroxide value of curry leaves treated ghee during storage

The changes in peroxide value of different ghee samples (control and treated with curry leaves) during storage at 80°±2°C are presented in Table 4.9 and graphically presented in Figure 4.9.

Table 4.9: Changes in peroxide value of curry leaves treated ghee during storage

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)		
	Control	Curry leaves added at initial stage of clarification	Curry leaves added at final stage of clarification
0	0.19	0.62	0.49
2	0.53	2.30	1.37
4	1.44	4.02	2.66
6	1.93	5.86	4.23
8	4.04	8.08	5.49
10	6.19	11.44	6.47
12	9.33	14.76	8.13

Source of variation	Storage period (P) (days)	Treatment (T) (Stage of addition)	Interaction (P×T)
SEm	0.42	0.27	0.73
CD (0.05)	1.18	0.77	2.05
CV%	30.59		

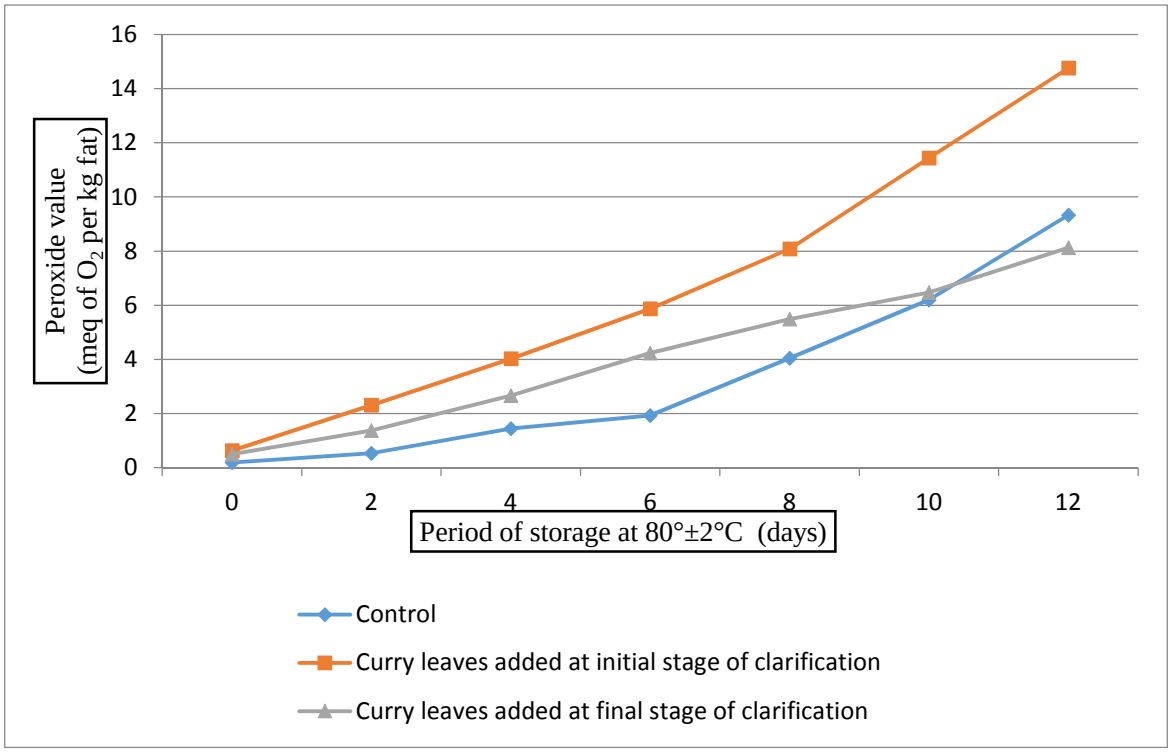


Figure 4.9: Changes in peroxide value of curry leaves treated ghee during storage

The peroxide value of different fresh samples of ghee was in the order of control ghee < ghee treated with curry leaves at final stage of clarification < ghee treated with curry leaves at initial stage of clarification. However, the order of peroxide value of different ghee samples was just reversed at the end of the 12 days storage at 80°±2°C. The order of peroxide value of different ghee samples was just reversed at the end of the storage was ghee treated with curry leaves at final stage of clarification < control ghee < ghee treated with curry leaves at initial stage of clarification.

The peroxide value of all the three types of ghee samples increased at a steady rate up sixth day of the storage. The rate of rise in peroxide value became steep from 6^{th} day onwards in case of control ghee and that in ghee treated with curry leaves at initial stage of clarification. On the other hand in ghee treated with curry leaves at final stage of clarification, no steep rate of rise in peroxide value was noticed at any stage during the entire storage period.

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at 80°±2°C that the treatment (stage of curry leaves addition in ghee) and period of storage were significant ($P<0.05$). The interaction between period of storage and stage of curry leaves addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stage in preparation of ghee used for treatment of ghee with the curry leaves differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with curry leaves and period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with curry leaves and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) peroxide value of control ghee sample was statistically at par with samples of ghee treated with curry leaves, irrespective of their stage of addition in preparation of ghee. Moreover, peroxide value of control ghee sample remained significantly ($P<0.05$) lower than that of the samples of ghee treated with curry leaves at initial stage of clarification during entire period of storage.

Similarly, up to 10th day of storage peroxide value of control ghee sample also remained lower than that of the samples of ghee treated with curry leaves at final stage of clarification, but their difference was statistically at par on 10th day of storage. However, peroxide value of control ghee sample became significantly ($P<0.05$) higher than that of sample of ghee treated with curry leaves at final stage of clarification on 12th day of the storage.

Among the sample of ghee treated with curry leaves at two different stages of clarification (initial and final) in preparation of ghee the peroxide values of ghee sample treated with curry leaves at initial stage of clarification remained higher all throughout the storage period than that of sample of ghee treated with curry leaves at final stage of clarification. Of course, peroxide values of both ghee samples were statistically at par only on 0 day (when both the samples were fresh). Thereafter peroxide values of ghee sample treated with curry leaves at initial stage of clarification were significantly ($P<0.05$) higher than that of sample of ghee treated with curry leaves at final stage of clarification throughout the storage period.

From the forgoing resume it became evident that treating the ghee with curry leaves at final stage of clarification reduced the peroxide formation more effectively compared to the ghee with curry leaves at initial stage of clarification. The examination of the results clearly suggested that the treatment of ghee with curry leaves at initial stage of clarification adversely affected the stability of the ghee against oxidative deterioration, due to strong prooxidant effect, when compared with the results of the control ghee sample. The careful comparison of the data also suggested that even treatment of ghee with curry leaves at final stage of clarification also had adverse effect the stability of the ghee against oxidative deterioration of the ghee up to 8th day of storage, when compared with the results of the control ghee sample. On 12th of storage ghee with curry leaves at final stage of clarification showed slightly lower peroxide value compared to that of the control ghee sample.

No report is available in the literature for evaluating effect of treatment of ghee with curry leaves at different stages in preparation of ghee. Therefore, results obtained in present study could not be compared as such with the reports in the literature.

No report is available in the literature for evaluating effect of treatment of ghee with curry leaves at different stages in preparation of ghee on changes in peroxide value of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature. Though Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of curry (*Murraya koeniji*) *leaves*. These authors added the curry leaves to only melted butter (*i.e.* during initial stage of clarification, but they have not carried the study about final stage of clarification.

4.3.2.2 Flavour score of curry leaves treated ghee during storage at 80°±2°C

The changes in flavour score of different ghee samples (control and treated with curry leaves) during storage at 80°±2°C are presented in Table 4.10 and graphically presented in Figure 4.10.

Table 4.10: Changes in flavour score of curry leaves treated ghee during storage

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)		
	Control	Curry leaves added at initial stage of clarification	Curry leaves added at final stage of clarification
0	9.00	8.03	8.80
2	8.58	7.45	8.30
4	7.75	6.85	7.88
6	6.60	6.10	7.35
8	5.60	5.38	6.93
10	4.10	4.15	6.57
12	2.40	2.10	6.18

Source of variation	Storage period (P) (days)	Treatment (T) (Stage of addition)	Interaction (P×T)
SEm	0.06	0.04	0.10
CD (0.05)	0.17	0.11	0.29
CV%	3.12		

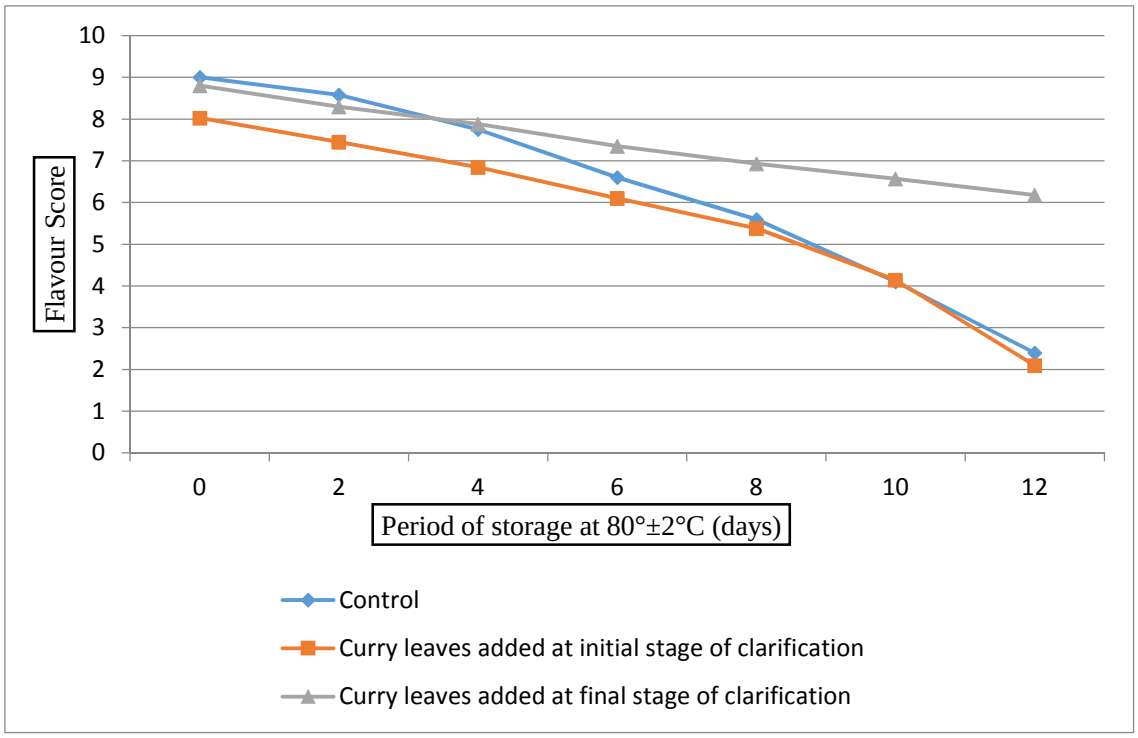


Figure 4.10: Changes in flavour score of curry leaves treated ghee during storage

The flavour score of different fresh ghee was in the order of control ghee > ghee treated with curry leaves at final stage of clarification > ghee treated with curry leaves at initial stage of clarification. However, the order of flavour score of different ghee samples was just reversed at the end of the 12 days at 80°±2°C. The order of flavour score of different ghee samples was just reversed at the end of the storage was ghee treated with curry leaves at final stage of clarification > control ghee > ghee treated with curry leaves at initial stage of clarification.

The flavour score of all the three types of ghee samples decreased at a steady rate up 4th day of the storage. The rate of decline in flavour score of control ghee sample became steep from 6th day onwards. However, in case of ghee treated with curry leaves at initial stage of clarification as well as at final stage of clarification no steep rate of decline in flavour score was noticed at any stage during the entire storage period. The flavour score of control ghee sample went below the acceptable level (<6) on 8th day of the storage. Similarly, the flavour score of ghee sample treated with curry leaves at initial stage of clarification also went below the acceptable level on 8th day of the storage. However, flavour score of ghee samples treated with curry leaves at final stage of clarification remained acceptable even on 12th day of the storage.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80°±2°C that the treatments (stage of curry leaves addition in ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and stage of curry leaves addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stage in ghee preparation used for treatment of ghee with the curry leaves differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with curry leaves and period of the storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with curry leaves and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) control sample of ghee had significantly ($P<0.05$) higher flavour score compared to samples of ghee treated with curry leaves, irrespective of their stage of addition in preparation of ghee. Even on 2nd day of storage flavour score of control sample of ghee had significantly ($P<0.05$) higher flavour score compared to samples of ghee treated with curry leaves, irrespective of their stage of addition in preparation of ghee. Moreover, control ghee had statistically higher ($P<0.05$) flavour score compared to sample of ghee treated with curry leaves at initial stage of clarification during the entire storage period. However only on 10th day of storage flavour score of control ghee sample was statistically at par with sample of ghee treated with curry leaves at initial stage of clarification. However, from 6th day of the storage flavour score of control ghee sample became significantly ($P<0.05$) lower than that of sample of ghee treated with curry leaves at final stage of clarification.

Among the sample of ghee treated with curry leaves at two different stages of clarification (initial and final) in preparation of ghee the flavour score of ghee sample treated with curry leaves at final stage of clarification remained higher all throughout the storage period than that of sample of ghee treated with curry leaves at initial stage of clarification.

From the forgoing resume it became evident that treating the ghee with curry leaves at final stage of clarification reduced the peroxide formation more effectively compared to the ghee with curry leaves at initial stage of clarification.

No report is available in the literature for evaluating effect of treatment of ghee with curry leaves at different stages in preparation of ghee on changes in flavour score of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature. Though Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of curry (*Piper curry*) leaves. These authors added the curry leaves to only melted butter (*i.e.* during initial stage of clarification, but they have not carried the study about final stage of clarification.

4.3.3 Performance of Liquorice Added at Two Different Stages in Ghee Preparation

Effect of two different stages of addition on performance of liquorice for retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.3.3.1 Peroxide value of liquorice treated ghee during storage

The changes in peroxide value of different ghee samples (control and treated with liquorice) during storage at 80°±2°C are presented in Table 4.11 and graphically presented in Figure 4.11.

Table 4.11: Changes in peroxide value of liquorice treated ghee during storage

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)		
	Control	Liquorice leaves added at initial stage of clarification	Liquorice leaves added at final stage of clarification
0	0.19	0.15	0.44
2	0.53	0.64	0.67
4	1.44	0.95	1.11
6	1.93	1.50	1.59
8	4.04	1.88	2.13
10	6.19	2.65	2.98
12	9.33	4.04	4.93

Source of Variation	Storage period (days)	Treatment (stage of addition)	Interaction (P×T)
SEm	0.12	0.08	0.21
CD (0.05)	0.34	0.22	0.58
CV%	17.62		

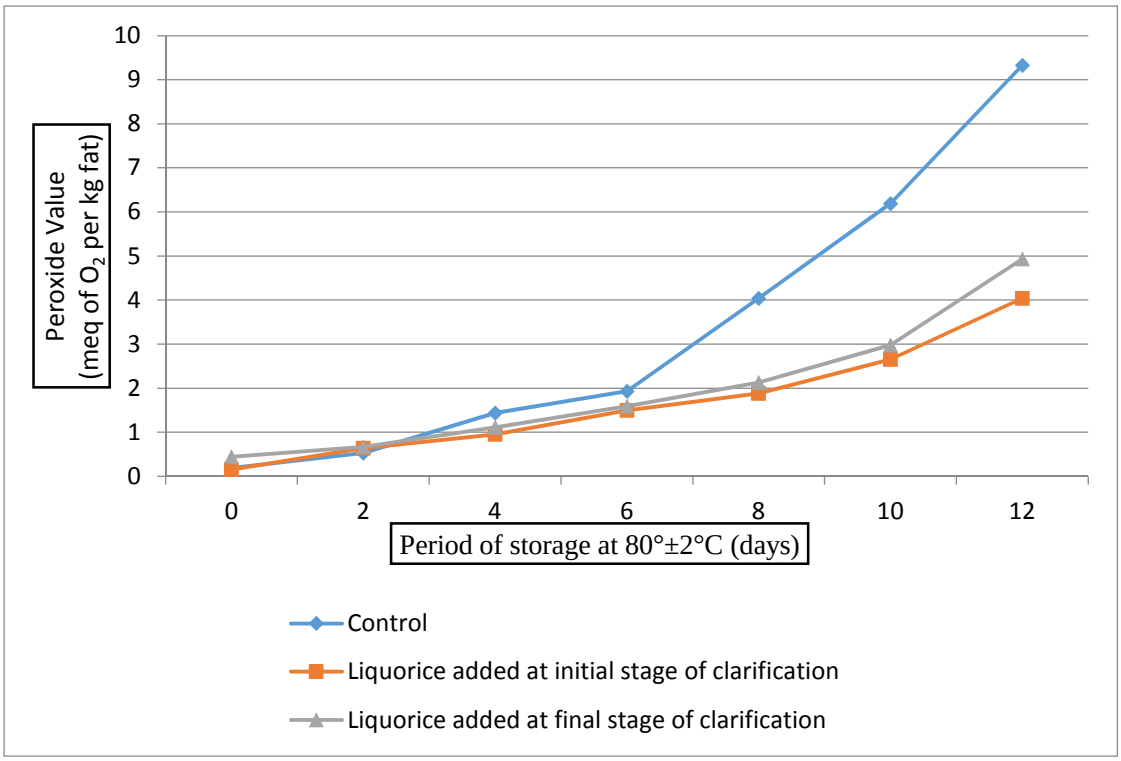


Figure 4.11: Changes in peroxide value of liquorice treated ghee during storage

The peroxide value of different fresh ghee was in the order of ghee treated with liquorice at initial stage of clarification < control ghee < ghee treated with liquorice at final stage of clarification. However, the order of peroxide value of different ghee samples changed at the end of the 12 days storage at 80°±2°C. The order of peroxide value of different ghee samples was just reversed at the end of the storage was ghee treated with liquorice at initial stage of clarification < ghee treated with liquorice at final stage of clarification < control ghee.

The peroxide value of all the three types of ghee samples increased at almost steady rate up 6^{th} day of the storage. The rate of rise in peroxide value became steep from 6^{th} day onwards in case of control ghee sample. However, in ghee treated with liquorice at final stage of clarification slightly steep rise was noticed from 10^{th} day of the storage. On the other hand in ghee treated with liquorice at initial stage of clarification, no noticeable steep rate of rise in peroxide value was noticed at any stage during the entire storage period.

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at 80°±2°C that the treatment (stage of liquorice addition in ghee) and period

of storage were significant ($P<0.05$). The interaction between period of storage and stage of liquorice addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stage in ghee preparation used for treatment of ghee with the liquorice differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with liquorice and period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with liquorice and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) peroxide value of control ghee sample was statistically at par with sample of ghee treated with liquorice at initial stage of clarification whereas it was significantly ($P<0.05$) lower than sample of ghee treated with liquorice at final stage of clarification. Only on 2^{nd} day of the storage peroxide value of all three types of ghee samples became statistically at par. Moreover, after 2^{nd} day of the storage period peroxide value of control ghee sample remained higher than samples of ghee treated with liquorice, irrespective of their stage of addition in preparation of ghee. From 4^{th} day of storage, control ghee sample was significantly ($P<0.05$) higher than both ghee samples treated with liquorice at initial and final stage of clarification.

Among the sample of ghee treated with liquorice at two different stages of clarification (initial and final) in preparation of ghee the peroxide values of ghee sample treated with liquorice at initial stage of clarification remained lower all throughout the storage period than that of sample of ghee treated with liquorice at final stage of clarification. However, peroxide values of both these ghee samples were statistically at par up to 6^{th} day of the storage except on 0 day. On subsequent storage period peroxide value of ghee sample treated with liquorice at initial stage of clarification was significantly ($P<0.05$) lower than that of sample of ghee treated with liquorice at final stage of clarification.

From the forgoing resume it became evident that treating the ghee with liquorice at initial stage of clarification reduced the peroxide formation more effectively compared to the ghee with liquorice at final stage of clarification.

No report is available in the literature for evaluating effect of treatment of ghee with liquorice at different stages in preparation of ghee on changes in peroxide value of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature.

4.3.3.2 Flavour score of liquorice treated ghee during storage

The changes in flavour of different ghee samples (control and treated with liquorice) during storage at 80°±2°C are presented in Table 4.12 and graphically presented in Figure 4.12.

Table 4.12: Changes in flavour score of liquorice treated ghee during storage

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)		
	Control	Liquorice leaves added at initial stage of clarification	Liquorice leaves added at final stage of clarification
0	9.00	8.88	8.85
2	8.58	8.60	8.55
4	7.75	8.20	8.23
6	6.60	7.93	7.83
8	5.60	7.55	7.48
10	4.10	7.18	7.08
12	2.40	6.90	6.65

Source of variation	Storage period (P) (days)	Treatment (T) (stage of addition)	Interaction (P×T)
SEm	0.05	0.03	0.08
CD (0.05)	0.13	0.09	0.23
CV%	2.25		

Data are presented as means (n=4).

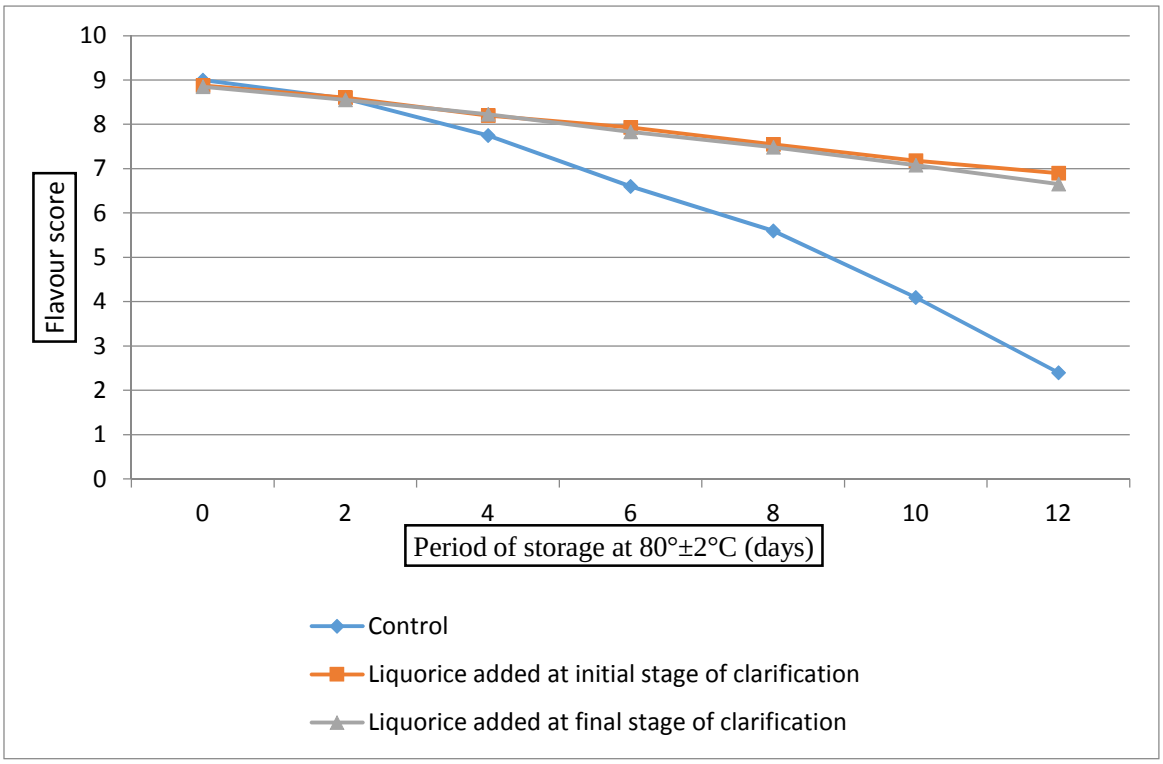


Figure 4.12: Changes in flavour score of liquorice treated ghee during storage

The flavour score of different fresh ghee was in the order of control ghee > ghee treated with liquorice at initial stage of clarification > ghee treated with liquorice at final stage of clarification. However, the order of flavour score of different ghee samples changed at the end of the 12 days storage at 80°±2°C. The order of flavour score of different ghee samples was in the order of ghee treated with liquorice at initial stage of clarification > ghee treated with liquorice at final stage of clarification > control ghee.

The flavour score of all the three types of ghee samples decreased at almost equal rate on 2nd day of the storage. The rate of decline in flavour score of control ghee sample became steep from 4th day onwards. However, in case of ghee treated with liquorice at initial stage of clarification as well as at final stage of clarification no steep rate of decline in flavour score was noticed at any stage during the entire storage period. The flavour score of control ghee sample went below the acceptable level (<6) on 8th day of the storage. However, flavour score of ghee samples treated with liquorice at initial stage and final stage of clarification remained acceptable even on 12th day of the storage.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80°±2°C that the treatments (stage of liquorice addition in ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and stage of liquorice addition in ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different stage in ghee preparation used for treatment of ghee with the liquorice differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the stage in preparation of ghee used for treatment with liquorice and period of the storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of stage in preparation of ghee used for treatment with liquorice and period of storage were dependent on each other.

Among the fresh ghee samples (0 day) control sample of ghee had significantly ($P<0.05$) higher flavour score compared to samples of ghee treated with liquorice, irrespective of their stage of addition in preparation of ghee. Moreover, differences between flavour scores all three types of ghee were statistically at par on 2^{nd} day of the storage. However, from 4^{th} day of the storage flavour score of control ghee sample became significantly ($P<0.05$) lower than both ghee samples treated with liquorice at initial and final stage of clarification.

Among the sample of ghee treated with liquorice at two different stages of clarification (initial and final) in preparation of ghee the flavour score of ghee sample treated with liquorice at initial stage of clarification remained higher all throughout the storage period than that of sample of ghee treated with liquorice at final stage of clarification. Moreover, flavour score of both ghee samples were statistically at par up to 10^{th} day of the storage. However on 6^{th} and 12^{th} day of storage flavour score of ghee sample treated with liquorice at initial stage of clarification was significantly ($P<0.05$) higher than that of sample of ghee treated with liquorice at final stage of clarification.

From the forgoing resume it became evident that treating the ghee with liquorice at initial stage of clarification reduced the peroxide formation more effectively compared to the ghee with liquorice at final stage of clarification.

No report is available in the literature for evaluating effect of treatment of ghee with liquorice at different stages in preparation of ghee on changes in flavour score of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature.

Conflicting views are reported in the literature about effect of heat on performance antioxidants from natural sources. Some of the reports suggested that heat treatment adversely affected the performance of the antioxidants. However, according to other reports heat improves the performance of the antioxidants from natural sources. The quality of natural extracts and their antioxidative performances depends not only on the quality of the original plant, the geographic origin, climatic condition, harvesting date and storage but also environmental and technological factors affect the activities of antioxidants from residual sources (Moure *et al*., 2001). Moreover, in extract from natural materials content of substances with an antioxidant activity could be rather low. The content of active substances does not depend only on the raw material, but also on processing conditions which should be optimised in each case (Pokorny and Korczak, 2001).

According to Lal and Narayanan (1980) as well as Sree and Lal (1990) stability of antioxidants decreased with increase in storage period of ghee. Sree and Lal (1990) stated that the losses of antioxidants observed during storage might be due to the scavenging of antioxidants by the free radicals formed during autoxidation of ghee. As per the views expressed by Nicoliy *et al*. (1999) in the majority of cases, food processing carried out industrially or even during home meal preparation, may be responsible for a significant loss of natural antioxidants. This is due to the fact that most of the compounds are relatively unstable. Pradhan *et al.* (2014) found that percentage content of hydroxyl chavicol in aqueous extract of *Piper betle* L. reduced upon duration with heating. Nwaichi and Anyanwu (2013) studied phytoscreening and the effect of heat treatment on the antioxidant activity in three medicinal plant parts *Tetrapleura tetraptera*, *Piper guineense* and *Xylopia ethiopica*. The recorded results clearly indicate total phenolic content and antioxidant activities decreased due to thermal treatment.

Rahim *et al*. (2010) examined the effects of variations in boiling temperature and boiling time on the antioxidant activity of the boiling extract of *Plecranthus amboinicus* Lour. Fresh plants were boiled at 45, 60, 100 and 120°C for 1, 2 and 3 h respectively. Results of study indicated that the antioxidant activity increased with the rise of

temperature from 45-100°C but dropped when the extraction temperature was raised to 120°C. The authors opined that knowing the best boiling temperature and time will guide the future research in preserving antioxidant content of the plant when processing by boiling. The author also opined that thermal treatments are the main cause of the depletion in natural antioxidants. Horvathova *et al*. (2007) studied effect of heat treatment and storage on antioxidant activity of black pepper, allspice and oregano. Thermal treatment at 130°C for 5 minutes caused significant decrease of all antioxidant activity parameters with the exception of increased content of phenolic substances in black pepper. Except for shallot and cabbage, the antioxidant activities of kale, spinach and swamp cabbage were significantly decreased ($p < 0.05$) after thermal treatment.

Horvathova *et al*. (2007) studied effect of heat treatment and storage on antioxidant activity of black pepper, allspice and oregano by determination of antiradical activity, reducing power, thiobarbituric acid number and content of total phenolic substances. Thermal treatment at 130°C for 5 minutes caused significant decrease of all antioxidant activity parameters with the exception of increased content of phenolic substances in black pepper. The initial reduction in the overall antioxidant activity was due to the thermal degradation of naturally occurring antioxidants. Horvathova *et al*. (2007) reported that the when powders and oils were analysed in case of ginger this antioxidant activity was reduced on heating (120°C for 1 hour).

Therefore, in present study reduction in performance of curry leaves and betel leaves used in treatment of ghee at initial stage of clarification during preparation of ghee compared to final stage of the clarification might be attributed to heat induced destruction of antioxidants. Thus, observations of present study were in corroboration with findings reported and views expressed by various authors as presented above.

Some of the studies indicated that heat increases the antioxidant capacity of herbs (Chohan *et al.,* 2008; Halvorensen *et al.,* 2006). Kim *et al.* (2006) studied the effect of heat on the antioxidant capacity of grape seed extracts and came to the conclusion that the antioxidant capacity of these extracts increased through the liberation of phenolic compounds by heat. Hence the additive effects are shown well by partitioning easily and spreading evenly well in favourable medium provided by heating. Jeong *et al*. (2004) evaluated effect of heat treatment on the antioxidant activity of extracts from *Citrus unshiu* peels (CP). Their results indicated that the antioxidant activity of CP extracts was significantly affected by heating temperature and duration of treatment on

CP. This means that phenolic compounds with antioxidant activity in plants present several kinds of bound states, and a simple heating process can be used as a tool for increasing the antioxidant activity of CP. The TPC in extracts of CP significantly increased by heat treatments. Horvathova *et al*. (2007) suggested that that the thermal processing of tomatoes enhances their nutritional value by increasing the bioaccessible lycopene content as well as the total antioxidant activity. The antioxidant activity of green beans was reduced by 20%. Horvathova *et al*. (2007) reported that the antioxidant activity of spice extracts were retained even after boiling for 30 min at 100 °C, indicating that the spice constituents were resistant to thermal denaturation and turmeric showed an increase in antioxidant activity on heating (120°C for 1 hour).

Brever (2011) reported that ginger extract has good thermal stability and inhibits more than 85% of linoleic acid peroxidation when heated at 185°C for 120 min. Heating (120°C) dry ginger and turmeric essential oils results in different degrees of antioxidant activity retention. The antioxidant activity of turmeric oil is higher after heating (120°C). As per the views expressed by Nicoliy *et al*. (1999) polyphenols with an intermediate oxidation state can exhibit higher radical scavenging efficiency than the non-oxidized ones. The higher antioxidant properties of the partially oxidized polyphenols could be attributed to their increased ability to donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or to the capacity of their aromatic structures to support the unpaired electron through delocalization around the π-electron system. Processing and/or prolonged storage times can promote or enhance the progressive enzymatic or chemical oxidation of phenolic compounds; these reactions proceed at different rates depending on some intrinsic food variables as well as on processing conditions (a_w, pH, time, temperature, oxygen availability, etc.). Thus, the increase or the decrease in the overall antioxidant properties of polyphenol-containing products are consequences of the same oxidation reactions.

Therefore, in present study better performance of liquorice used in treatment of ghee at initial stage of clarification during preparation of ghee compared to final stage of the clarification might be attributed to increased liberation of phenolic compounds, easy partitioning, spreading evenly and formation of polyphenols with an intermediate oxidation state of antioxidants by heat. Thus, findings of present study were in corroboration with findings reported and views expressed by various authors as presented above.

4.4 OPTIMIZATION OF RATE FOR ADDITION OF HERB 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 herbs in turn adversely affect the organoleptic property of the products like ghee, because people are not accustomed with it. Moreover, it has been reported that the effect of antioxidant depends on its concentration, both at low and high concentration they may become pro-oxidant (Gordon, 1990). Therefore, it was essential to optimize rate of addition of the selected herb for use in treatment of ghee.

In studies carried for compatibility assessment of different herbs for use in ghee (Section 4.1), evaluation of herbs for their antioxidant potential (Section 4.2) and selection of stage for treatment of ghee with herbs (Section 4.3); the herbs were used at the rate of 0.5 per cent of the expected yield of the ghee. In these studies strong aroma of the respective herbs was experienced in the ghee samples. Therefore, to optimize rate of selected herbs (betel leaves, curry leaves and liquorice) for treatment of ghee, a concentration range of 0.1 to 0.4 per cent was chosen for the study.

From the work carried to evaluate antioxidant potential of fifteen different herbs betel leaves, curry leaves and liquorice were selected for further study. These herbs were used to evaluate performance of two different stages in preparation of ghee for addition of the herbs. For betel leaves and curry leaves final stage of clarification was used for the treatment of ghee, whereas, for liquorice initial stage of clarification was used for the treatment of ghee.

For evaluating different rates of the selected herbs (betel leaves, curry leaves and liquorice) for treatment of ghee, the sample of butter (120 g) was taken in to each of thirteen 500 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 centre of the sand bath. The sand bath was heated by gas fired burner with flame at the centre. When butter was melted completely liquorice was added in the melted butter at the rate of 0.1, 0.2, 0.3 and 0.4 per cent of the expected yield of ghee. Similarly, in remaining eight beakers betel leaves and curry leaves were separately added 0.1, 0.2, 0.3 and 0.4 per cent of the expected yield of ghee when temperature of the content

reached to 105°±2°C. Out of thirteen samples of the butter kept for heat clarification to prepared ghee, one sample was not treated with any herb to serve as a control. The heating was continued till temperature reached to 120°C.

In the entire process (from beginning to end) for preparation of ghee each sample was mixed gently with stainless steel spatula turn by turn. The content of each beaker was then filtered through 6 folded muslin cloth, ghee was collected in 150 ml glass beakers and stored in incubator at 80°±2°C. To prepare thirteen (13) samples of ghee simultaneously under similar and uniform heating conditions sand bath was used.

All the thirteen samples of fresh ghee were analysed for peroxide value by titration against standard sodium thiosulfate solution using starch indicator and expressed as meq of O_2 per kg fat. The samples of ghee were also evaluated for flavour score by sensory evaluation using 9 point hedonic scale. Similarly, during storage all the samples of ghee were analysed for peroxide value and evaluated for flavour score at a regular interval of every 2 days for 12 days and then at an interval of 1 day. The storage of the samples and their analysis was continued till flavour score of all most all the samples went below acceptable level (*i.e.* 22 days). Total three replications were conducted and average results obtained from the three replications are presented below.

4.4.1 Effect of Rate of Betel Leaves Used in Treatment of Ghee

Effect of different rates of addition of betel leaves on its performance in retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.4.1.1 Changes in peroxide value of betel leaves treated ghee during storage at 80°±2°C

The results obtained for rate of betel leaves addition on changes in peroxide value of ghee during storage are presented in Table 4.13 and the trend is presented in Figure 4.13.

Table 4.13: Changes in peroxide value of ghee during storage after treating with betel leaves at different rates

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)				
	Rate of betel leaves used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	0.19	0.39	0.32	0.19	0.26
2	0.77	0.90	0.78	0.58	0.64
4	1.67	1.16	1.17	1.24	0.92
6	3.68	1.48	1.85	1.80	1.20
8	4.73	3.61	2.41	2.18	1.69
10	6.71	4.39	2.76	2.35	2.18
12	8.71	4.76	3.19	2.53	2.54
13	10.88	5.01	3.56	2.66	2.82
14	12.79	5.41	4.17	2.71	3.28
15	14.72	5.91	5.34	2.75	3.65
16	17.41	7.41	6.64	2.81	4.03
17	20.35	8.91	7.09	2.87	4.33
18	24.71	**10.44**	7.57	2.92	5.38
19	27.76	11.71	**11.31**	2.97	6.39
20	31.02	13.03	13.32	3.12	**7.57**
21	32.97	15.56	14.89	3.31	9.48
22	35.80	18.95	17.16	3.51	11.91

Source of Variation	Storage period (P) (days)	Treatment (T) (Rate of addition)	Interaction (P× T)
SEm	0.25	0.13	0.55
CD (0.05)	0.69	0.37	1.53
CV%	13.79		

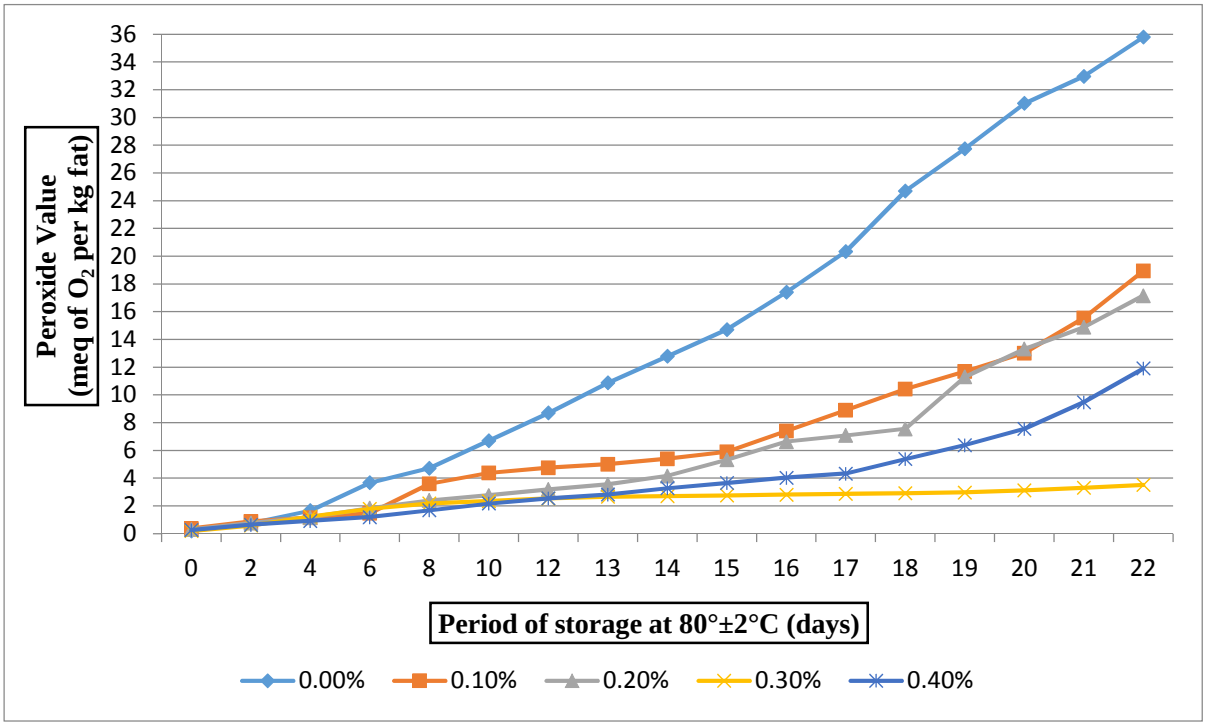


Figure 4.13: Changes in peroxide value of ghee during storage after treating with betel leaves at different rates

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at $80^{o}\pm2°C$ that the treatments (rate of betel leaves used in treatment of ghee) and storage period both were significant ($P<0.05$). The interaction between storage period and rate of betel leaves used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different rate of betel leaves used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, storage period also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the rate of betel leaves used in treatment of ghee and period of storage differed significantly from each other in their effect on peroxide value of ghee over a storage period. Thus, it became evident that the effect of rate of betel leaves in treatment of ghee and storage period were dependent on each other.

The peroxide value of different fresh ghee sample was in the order of control ghee = ghee treated with 0.3% betel leaves < ghee treated with 0.4% betel leaves < ghee treated with 0.2% betel leaves < ghee treated with 0.1% betel leaves. However, position of control ghee sample changed in the order of peroxide value of different ghee samples at the end of the 22 days storage at $80^{o}\pm2°C$. At the end of the storage the order of peroxide value of different ghee samples was ghee treated with 0.3% betel leaves < ghee treated with 0.4% betel leaves < ghee treated with 0.2% betel leaves < ghee treated with 0.1% betel leaves < control ghee.

The control ghee sample showed steeped rise in peroxide value on 16^{th} day of the storage. Whereas, samples of ghee treated with betel leaves at 0.1, 0.2, and 0.4 per cent rate showed steep rise in peroxide value on 18^{th}, 19^{th} and 20^{th} day of the storage. Interestingly, sample of ghee treated with 0.3 per cent betel leaves peroxide value of ghee increased at a steady rate, no sharp rise in peroxide value was observed in entire period of storage.

Among the fresh ghee samples (0 day) control sample of ghee had lower peroxide value compared to samples of ghee treated with betel leaves at different rate of addition except 0.3 per cent treated ghee sample. However, while remaining entire period of storage peroxide value of control ghee sample also remained higher compared peroxide value of all the betel leaves treated ghee samples. The peroxide value of control ghee became significantly ($P<0.05$) higher than that of samples of ghee treated with betel

leaves from 4th day of storage and on subsequent storage up to end it remained significantly (P<0.05) higher than that of samples of ghee treated with betel leaves.

Among the sample of ghee treated with betel leaves at different rate of addition (0.1, 0.2, 0.3 and 0.4 per cent), the peroxide value of ghee treated with 0.1, 0.2 and 0.4 per cent betel leaves remained higher than that of the treated with 0.3 per cent betel leaves when fresh and also during entire period of their storage. Though, peroxide values of all the betel leaves treated ghee samples was statistically at par up to 4th day of the storage, thereafter in remaining storage period peroxide values of ghee sample treated with 0.3 per cent betel leaves was significantly (P<0.05) lower than samples of ghee treated with 0.1 and 0.2 per cent betel leaves. From 14th day of storage period peroxide value of ghee sample treated with 0.3 per cent betel leaves was significantly (P<0.05) lower than 0.4 per cent betel leaves treated ghee sample. It can be sum-up that treatment of ghee with betel leaves at four rate used in the treatment of ghee, all the four rates were able to reduce the peroxide formation in ghee during storage 80°±2°C, compared to formation of peroxide in control sample of ghee. However, best control in formation of peroxide in ghee during storage was given by 0.3 per cent betel leaves. Any increase or decrease in the rate of betel leaves from 0.3 per cent resulted into reduction in effectiveness of the treatment in controlling the peroxide formation in ghee during storage 80°±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent betel leaves was most effective for reducing the peroxide formation in ghee during storage 80°±2°C, compared to the ghee with betel leaves at 0.1, 0.2 and 0.4 per cent rate. Any deviation from 0.3 per cent resulted in to decrease in performance of the treatment. Therefore, in the present study 0.3 per cent betel leaves was considered as optimum for treatment of ghee for better retention of flavour during storage.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves when added to melted butter during clarification. Fresh betel leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of betel leaves 0.2, 0.5 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C The sample were examined for Peroxide value. (meq of 0.002N $Na_2S_2O_3$/g fat). The peroxide values of ghee samples treated with betel leaves and antioxidants changed

very little up to 30 days of storage. The control (without antioxidants) showed a steep rise in peroxide with value after 60 days of storage. Ghee samples added 1.0 per cent betel leaves showed least increase in peroxide value up to 135 days of storage. However, ghee samples treated with betel leaves 1 per cent concentration proved to be most stable even up to 147 days of storage at 30°C.

The above cited report (Patel and Rajorhia, 1979) is the only record available in the literature for application of betel leaves in treatment of ghee at different rates to evaluate its antioxidative effects in ghee. In that study addition of betel leaves at the rate of 1 per cent rate was found most effective, whereas, in present study 0.3 per cent was found most effective. Such variations might be attributed to number of reasons. There were several differences between work carried in present study and the study carried out by the authors to optimize rate of betel leaves: (1) form of betel leaves used (dried leaves or fresh leaves respectively), (2) stage in ghee preparation at which betel leaves were added (final and initial respectively) and (3) temperature at which ghee was stored (80° and 30°C respectively). Even difference in variety might also be responsible for the difference, however, the authors have not reported the variety of the betel leaves used in their study.

4.4.1.2 Changes in flavour score of betel leaves treated ghee during storage at 80°±2°C

The results obtained for rate of betel leaves addition on changes in flavour score of ghee during storage are presented in Table 4.14 and the trend is presented in Figure 4.14.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80^{o}±2°C that the treatments (rate of betel leaves used in treatment of ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and rate of betel leaves used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different rate of betel leaves used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the rate of betel leaves used in treatment of ghee and period of the storage significantly differed from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of rate of betel leaves used for treatment of ghee and period of storage were dependent on each other.

Table 4.14: Changes in flavour score of ghee during storage after treating with betel leaves at different rates

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)				
	Rate of betel leaves used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	9.00	9.00	9.00	9.00	8.73
2	8.70	8.67	8.73	8.83	8.50
4	7.97	8.07	8.23	8.73	8.23
6	6.77	7.33	8.50	7.63	8.00
8	**5.57**	6.27	8.23	7.40	7.70
10	4.53	**5.40**	6.93	7.47	7.23
12	3.27	4.80	6.27	7.27	6.93
13	2.67	4.23	6.00	7.00	6.77
14	2.00	3.73	**5.43**	6.80	6.63
15	1.53	3.53	5.17	6.67	6.23
16	1.37	2.97	4.93	6.47	**5.93**
17	1.23	2.33	4.47	6.20	5.73
18	1.00	1.93	4.17	6.03	5.47
19	1.00	1.20	3.73	6.00	5.27
20	1.00	1.00	2.93	6.00	4.53
21	1.00	1.00	2.53	6.00	3.83
22	1.00	1.00	2.00	**6.00**	3.00

Source of variation	Storage period (P) (days)	Treatment (T) (Rate of addition)	Interaction (P×T)
S. Em	0.08	0.05	0.19
CD (0.05)	0.24	0.13	0.53
CV %	6.09		

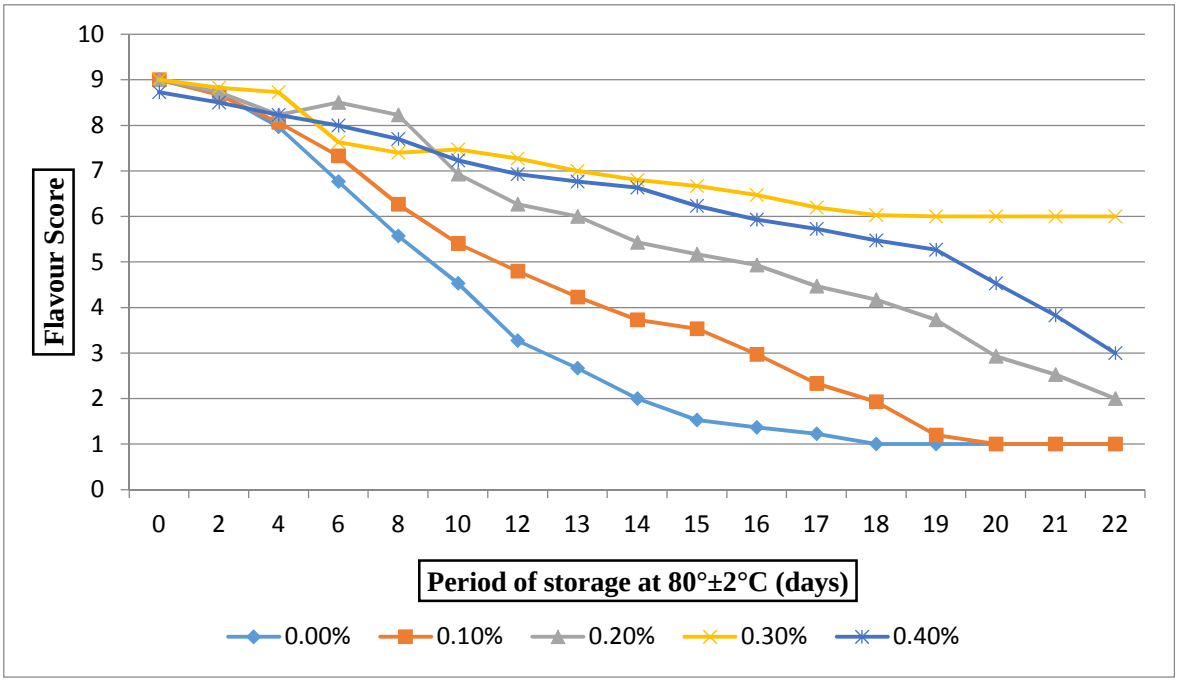


Figure 4.14: Changes in flavour score of ghee during storage after treating with betel leaves at different rates

The flavour score of different fresh ghee sample was in the order of control ghee = ghee treated with 0.1% betel leaves = ghee treated with 0.2% betel leaves = ghee treated with 0.3% betel leaves > ghee treated with 0.4% betel leaves. However, order of flavour score of ghee samples was reserved at the end of the 22 days storage at 80°±2°C. At the end of the storage the order of flavour score of different ghee samples was ghee treated with 0.3% betel leaves > ghee treated with 0.4% betel leaves > ghee treated with 0.2% betel leaves > ghee treated with 0.1% betel leaves = control ghee.

The flavour score of control ghee sample decreased at a rapid rate from beginning of the storage and showed steeped descale on 17th day of the storage. The sample of ghee treated with 0.1 per cent betel leaves also showed almost similar trend in changes in flavour score of ghee on storage. Whereas, samples of ghee treated with betel leaves at 0.2 per cent rate showed gradual declined in flavour score up to 10th day of the storage ant thereafter it started declining at a rapid rate. Similarly samples of ghee treated with betel leaves at 0.4 per cent rate showed gradual declined in flavour score up to 19th day of the storage ant thereafter it started declining at a rapid rate. Interestingly, sample of ghee treated with 0.3 per cent betel leaves flavour score of ghee increase at a steady rate, no sharp rise in flavour score was observed in entire period of storage.

Among the fresh ghee samples (0 day) control ghee sample as well as samples of ghee treated with betel leaves at the rate of 0.1, 0.2 and 0.3 acquired full flavour score (9 out of 9). Only the samples of ghee treated with betel leaves at different rate of addition except 0.4 per cent got slightly lower flavour score compared other samples in the group. On 2nd day of the storage flavour score of all the samples of ghee decreased, but relative trend between the samples was almost similar as noticed in case of the fresh samples. On 4th of the storage flavour score of control ghee lowest among all the sample of ghee. The flavour score of control ghee became significantly lower than all the betel leaves treated ghee samples.

Among the samples of ghee treated with betel leaves at different rate of addition (0.1, 0.2, 0.3 and 0.4 per cent), the flavour score of ghee treated with 0.1 per cent betel leaves followed trend almost parallel to control ghee and with its values slightly lower than that of the control ghee sample. The flavour score of ghee treated with 0.2 and 0.4 per cent betel leaves followed trend almost parallel to each other and the values of sample of ghee treated with 0.2 per cent betel leaves slightly lower than that of the ghee treated with 0.4 per cent betel leaves. The flavour score of ghee treated with 0.1 per cent betel

leaves remained lowest and 0.3 per cent betel leaves remained highest all throughout the storage.

Among all the samples of ghee, the flavour score of control ghee went below the acceptable level on 8th day of the storage. On the other hand flavour score of ghee treated with 0.1, 0.2 and 0.4 per cent betel leaves went below the acceptable level on 10th, 14th and 16th day of the storage. However, flavour score of ghee treated with 0.3 per cent betel leaves remained acceptable even at the end of 22 days storage. Thus, best control in retention of flavour score of ghee during storage was achieved by treatment of ghee with 0.3 per cent betel leaves. Any increase or decrease in the rate of betel leaves from 0.3 per cent resulted in to reduction in effectiveness of the treatment in controlling the deterioration of ghee during storage 80^{o}±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent betel leaves was most effective for reducing the deterioration of flavour of ghee during storage 80^{o}±2°C, compared to the ghee with betel leaves at 0.1, 0.2 and 0.4 per cent rate.

The chief constituent of the leaves is a volatile oil known as betel oil varying in chemical composition in betel varieties growing in different countries. The flavour of the leaf is due to the presence of essential oil. The leaves are aromatic with a varied taste ranging from sweet to pungent due to presence of essential oil. The essential oil composing of safrole, allyl pyrocatechol monoacetate, eugenol, terpinen-4-ol, eugenyl acetate, etc. as the major components. Hydroxychavicol was the major component of essential oil (Preethy *et al.,* 2016). The specific pungent aromatic flavour is due to the presence of phenol and terpene-like compounds. The major terpenoids and phenols include 1,8-cineole, cadinene, camphene, caryophyllene, limonene, pinene, chavicol, allyl pyrocatechol, catechol, carvacrol, safrole, eugenol and chevibetel (Uddin *et al.,* 2015; Nirmala and Kumari, 2015). Therefore, decrease in flavour score of ghee upon addition of betel leaves observed in the present study, up to 0.4 per cent may be attributed to above mentioned constituents of betel leaves.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves when added to melted butter during clarification. Fresh betel leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of betel

leaves 0.2, 0.5 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C The sample were examined for flavour score. All the samples of ghee were rated excellent at the beginning of the experiment. The judges preferred ghee samples treated with betel leaves as indicated by their highest scores for flavour. The flavour scores of ghee samples treated with betel leaves and antioxidants changed very little up to 30 days of storage. Initial flavour score of control ghee was 8.33 whereas, ghee treated with 0.2, 0.5 and 1.0 per cent betel leaves received flavour score of 8.3, 8.2 and 8.4 respectively. After storage for 147 days flavour score of control ghee was 2.16 whereas, ghee treated with 0.2, 0.5 and 1.0 per cent betel leaves received flavour score of 2.20, 5.85 and 6.50 respectively.

The above cited repot (Patel and Rajorhia, 1979) is the only record available in the literature for application of betel leaves in treatment of ghee at different rates to evaluate its antioxidative effects in ghee. In that study addition of betel leaves at the rate of 1 per cent rate was found most effective, whereas, in present study 0.3 per cent was found most effective. Such variations might be attributed to number of reasons. There were several differences between work carried in present study and the study carried out by the authors to optimize rate of betel leaves: (1) form of betel leaves used (vacuum dried powder and fresh respectively), (2) stage in ghee preparation at which betel leaves were added (final and initial respectively) and (3) temperature at which ghee was stored (80° and 30°C respectively). Even difference in variety might also be responsible for the difference, however, the authors did not reported the variety of the betel leaves used in their study.

4.4.2 Effect of Rate of Curry Leaves Used in Treatment of Ghee

Effect of different rates of addition of curry leaves on its performance in retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.4.2.1 Changes in Peroxide value of curry leaves treated ghee during storage at 80°±2°C

The results obtained for rate of curry leaves addition on changes in peroxide value of ghee during storage are presented in Table 4.15 and the trend is presented in Figure 4.15.

Table 4.15: Changes in peroxide value of ghee curry leaves during storage after treating with at different rates

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)				
	Rate of curry leaves used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	0.19	0.57	0.31	0.19	0.39
2	0.77	0.90	0.85	0.88	0.97
4	1.67	1.46	1.23	1.15	1.42
6	3.68	2.56	1.96	1.88	1.87
8	4.73	3.51	2.11	2.17	2.48
10	6.71	3.72	2.54	2.50	3.32
12	8.71	4.14	3.08	3.54	4.02
13	10.88	4.46	3.68	3.77	5.66
14	12.79	7.06	4.53	4.78	7.82
15	14.72	8.50	6.53	6.33	9.64
16	17.41	8.67	7.85	6.63	9.59
17	20.35	**13.04**	8.00	7.03	9.53
18	24.71	15.36	8.30	7.73	**11.14**
19	27.76	15.64	8.70	8.07	11.43
20	31.02	18.10	**11.70**	8.43	12.12
21	32.97	21.70	15.70	8.91	13.87
22	35.80	27.56	17.67	9.44	15.83

Source of variation	Storage period (P) (days)	Treatment (T) (Rate of addition)	Interaction (P×T)
SEm	0.37	0.20	0.82
CD (0.05)	1.03	0.56	2.30
CV%	16.79		

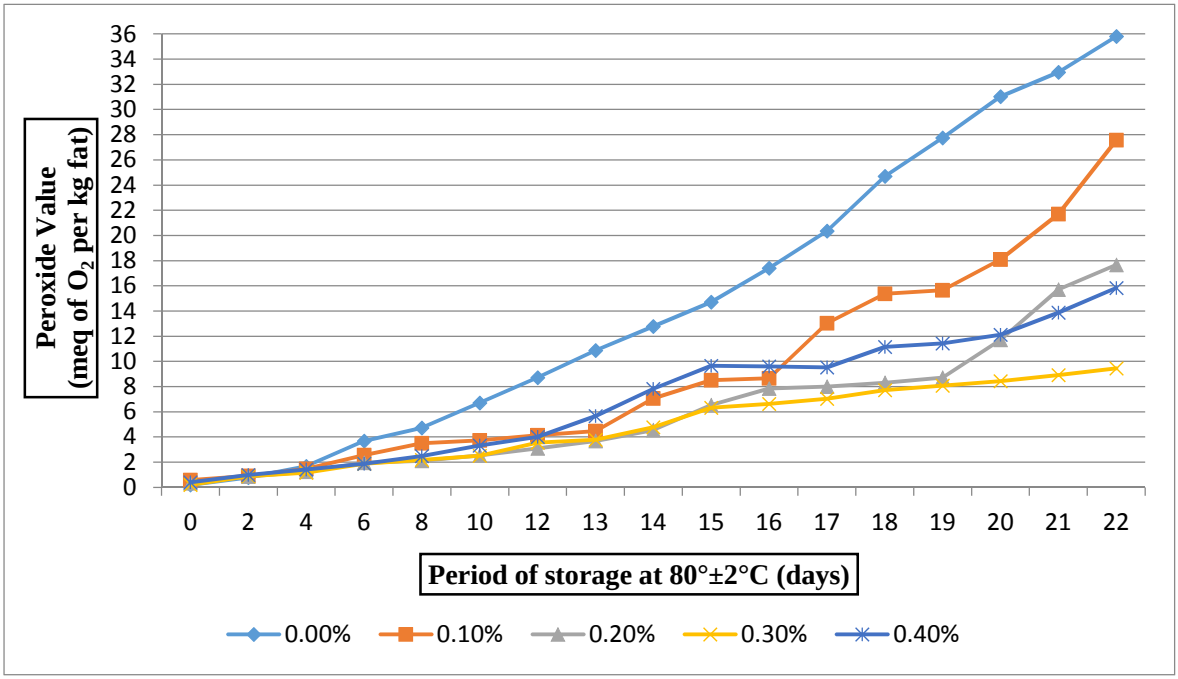


Figure 4.15: Changes in peroxide value of ghee during storage after treating with curry leaves at different rates

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at $80^{o}\pm2°C$ that the treatments (rate of curry leaves used in treatment of ghee) and storage period both were significant ($P<0.05$). The interaction between storage period and rate of curry leaves used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different rate of curry leaves used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, storage period also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the rate of curry leaves used in treatment of ghee and period of storage differed significantly from each other in their effect on peroxide value of ghee over a storage period. Thus, it became evident that the effect of rate of curry leaves used in treatment of ghee and storage period were dependent on each other.

The peroxide value of different fresh ghee sample was in the order of control ghee = ghee treated with 0.3% curry leaves < ghee treated with 0.2% curry leaves < ghee treated with 0.4% curry leaves < ghee treated with 0.1% curry leaves. However, position of control ghee sample changed in the order of peroxide value of different ghee samples at the end of the 22 days storage at $80^{o}\pm2°C$. At the end of the storage the order of peroxide value of different ghee samples was ghee treated with 0.3% curry leaves < ghee treated with 0.4% curry leaves < ghee treated with 0.2% curry leaves < ghee treated with 0.1% curry leaves < control ghee.

The control ghee sample showed steeped rise in peroxide value on 16^{th} day of the storage. Whereas, samples of ghee treated with curry leaves at 0.1, 0.2, and 0.4 per cent rate showed steeped rise in peroxide value on 17^{th}, 20^{th} and 18^{th} day of the storage. Interestingly, sample of ghee treated with 0.3 per cent curry leaves peroxide value of ghee increased at a steady rate, no sharp rise in peroxide value was observed in entire period of storage.

Among the fresh ghee samples (0 day) control ghee sample had lower peroxide value compared to samples of ghee treated with curry leaves at different rate of addition except 0.3 per cent treated ghee sample. However, while remaining entire period of storage peroxide value of control ghee sample also remained higher compared peroxide value of all the curry leaves treated ghee samples. The peroxide value of control ghee became significantly ($P<0.05$) higher compared peroxide value of all the curry leaves

treated ghee samples (at the rate of 0.1, 0.2, 0.3 and 0.4 per cent) from 6th day of storage and on subsequent storage up to end.

Among the sample of ghee treated with curry leaves at different rate of addition (0.1, 0.2, 0.3 and 0.4 per cent), the peroxide value of ghee treated with 0.1, 0.2 and 0.4 per cent curry leaves remained higher than that of the treated with 0.3 per cent curry leaves when fresh and also during almost entire period of their storage. Though, peroxide values of curry leaves treated ghee samples was statistically at par up to 4th day of the storage, then after in remaining storage period peroxide values of ghee sample treated with 0.3 per cent curry leaves was significantly ($P<0.05$) lower than that of sample of ghee treated with 0.1 and 0.4 per cent curry leaves. Sample of ghee treated with 0.2 per cent curry leaves was statistically at par with 0.3 per cent curry leaves added ghee sample up to 15th day of the storage. It can be sum-up that treatment of ghee with curry leaves at four different rates used in the treatment of ghee, all the 4 rates were able to reduce the peroxide formation in ghee during storage 80^{o}±2°C, compared to formation of peroxide in control sample of ghee. However, best control in formation of peroxide in ghee during storage was given by 0.3 per cent curry leaves. Any increase or decrease in the rate of curry leaves from 0.3 per cent resulted in to reduction in effectiveness of the treatment in controlling the peroxide formation in ghee during storage 80^{o}±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent curry leaves was most effective for reducing the peroxide formation in ghee during storage 80^{o}±2°C, compared to the ghee with curry leaves at 0.1, 0.2 and 0.4 per cent rate. Any deviation from 0.3 per cent resulted in to decrease in performance of the treatment. Therefore, in the present study 0.3 per cent curry leaves was considered as optimum for treatment of ghee for better retention of flavour during storage.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of curry leaves (*Murraya koenigi*) when added to melted butter during clarification. Fresh curry leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of curry leaves 0.5, 0.8 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C The sample were examined for Peroxide value. (meq of 0.002N $Na_2S_2O_3$/g fat). The peroxide values of ghee samples treated with curry leaves changed very little up to 30 days of storage. The control (without antioxidants) showed a steep rise in peroxide with

value after 60 days of storage. Ghee samples added 1.0 per cent curry leaves showed least increase in peroxide value up to 135 days of storage. However, ghee samples treated with curry leaves 1 per cent concentration proved to be most stable even up to 147 days of storage at 30°C.

The above cited repot (Patel and Rajorhia, 1979) is the only record available in the literature for application of curry leaves in treatment of ghee at different rates to evaluate its antioxidative effects in ghee. In that study addition of curry leaves at the rate of 1 per cent rate was found most effective, whereas, in present study 0.3 per cent was found most effective. Such variations might be attributed to number of reasons. There were several differences between work carried in present study and the study carried out by the authors to optimize rate of curry leaves: (1) form of curry leaves used (vacuum dried powder and fresh respectively), (2) stage in ghee preparation at which curry leaves were added (final and initial respectively) and (3) temperature at which ghee was stored (80° and 30°C respectively). Even difference in variety might also be responsible for the difference, however, the authors did not reported the variety of the curry leaves used in their study.

4.4.2.2 Changes in Flavour score of curry leaves treated ghee during storage at 80°±2°C

The results obtained for rate of curry leaves addition on changes in flavour score of ghee during storage are presented in Table 4.16 and the trend is presented in Figure 4.16.

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at 80°±2°C that the treatments (rate of curry leaves used in treatment of ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and rate of curry leaves used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different rate of curry leaves used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the rate of curry leaves used in treatment of ghee and period of the storage significantly differed from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of rate of

curry leaves used for treatment of ghee and period of storage were dependent on each other.

Table 4.16: Changes in flavour score of ghee during storage after treating with curry leaves at different rates

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)				
	Rate of curry leaves used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	9.00	8.90	8.77	8.77	8.70
2	8.70	8.47	8.40	8.50	8.50
4	7.97	7.63	8.03	8.20	8.13
6	6.77	6.93	7.40	8.00	7.53
8	**5.57**	**5.63**	6.87	7.50	7.13
10	4.53	4.90	6.00	6.97	6.70
12	3.27	4.43	**5.30**	6.50	6.17
13	2.67	3.70	4.73	6.37	**5.70**
14	2.00	2.33	3.40	**5.63**	5.40
15	1.53	1.53	2.07	4.23	4.50
16	1.37	1.00	1.53	3.10	3.20
17	1.23	1.00	1.00	2.33	2.13
18	1.00	1.00	1.00	1.67	1.00
19	1.00	1.00	1.00	1.00	1.00
20	1.00	1.00	1.00	1.00	1.00
21	1.00	1.00	1.00	1.00	1.00
22	1.00	1.00	1.00	1.00	1.00

Source of Variation	Storage period (P)(days)	Treatment (T) (Rate of addition)	Interaction (P×T)
SEm	0.14	0.07	0.30
CD (0.05)	0.38	0.21	0.85
CV%	12.55		

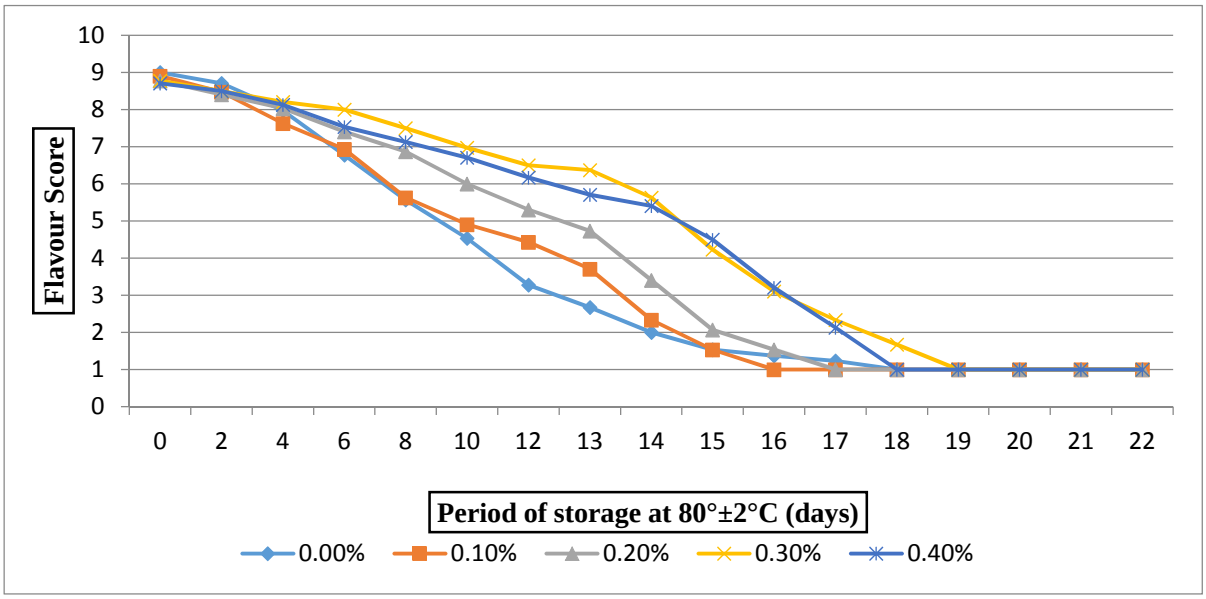


Figure 4.16: Changes in flavour score of ghee during storage after treating with curry leaves at different rates

The flavour score of different fresh ghee sample was in the order of control ghee > ghee treated with 0.1% curry leaves > ghee treated with 0.2% curry leaves = ghee treated with 0.3% curry leaves > ghee treated with 0.4% curry leaves. On 13th day of the storage the order of flavour score of different ghee samples was ghee treated with 0.3% curry leaves > ghee treated with 0.4% curry leaves > ghee treated with 0.2% curry leaves > ghee treated with 0.1% curry leaves > control ghee. However, at the end of the 22 days storage at 80^{o}±2°C, flavour score of all the ghee samples were equal.

The flavour score of control ghee sample decreased at a rapid rate from beginning of the storage and showed steeped descale on 17th day of the storage. The sample of ghee treated with 0.1 per cent curry leaves also showed almost similar trend in changes in flavour score of ghee on storage as that of the control ghee sample. Interestingly, sample of ghee treated with 0.3 per cent curry leaves flavour score of ghee increased at a steady rate, no sharp rise in flavour score was observed in entire period of storage. The sample of ghee treated with 0.4 per cent curry leaves also showed almost similar trend in changes in flavour score of ghee on storage as that of the sample treated with 0.3 per cent curry leaves. However, trend in changes in flavour score of ghee treated with 0.2 per cent curry leaves was intermediate to that of the control ghee and the ghee treated with 0.1 per cent curry leaves.

Among the fresh ghee samples (0 day) only control sample of ghee acquired full flavour score (9 out of 9). All the samples of ghee treated with curry leaves at different rate of addition got lower flavour score compared control ghee sample and the decreased in the flavour score was proportional to the rate of curry leaves used for treatment of ghee. Thus, it was evident from the data that flavour score of the fresh ghee samples was decreasing with increasing rate of curry leaves used for treatment of ghee.

Among the samples of ghee treated with curry leaves at different rate of addition (0.1, 0.2, 0.3 and 0.4 per cent), the flavour score of ghee treated with 0.1 per cent curry leaves followed trend almost parallel to control ghee and with its values sometimes slightly lower and sometimes slightly higher than that of the control ghee sample. The flavour score of ghee treated with 0.2 and 0.4 per cent curry leaves followed trend almost parallel to the sample of ghee treated with 0.3 per cent curry leaves and with their values slightly lower than that of the ghee treated with 0.3 per cent curry leaves. The flavour

score of ghee treated with 0.3 per cent curry leaves remained highest all throughout the storage.

Among all the samples of ghee, the flavour score of control ghee and ghee treated with 0.1 curry leaves went below the acceptable level on 8^{th} day of the storage. On the other hand flavour score of ghee treated with 0.2, 0.3 and 0.4 per cent curry leaves went below the acceptable level on 12^{th}, 14^{th} and 13^{th} day of the storage. Thus, among different levels of curry used in treatment of ghee, best control for retention of flavour score of ghee during storage was achieved by 0.3 per cent curry leaves. Any increase or decrease in the rate of curry leaves from 0.3 per cent resulted in to reduction in effectiveness of the treatment in controlling the deterioration ghee flavour during storage 80^{o}±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent curry leaves was most effective for reducing the deterioration of flavour of ghee during storage 80^{o}±2°C, compared to the ghee with curry leaves at 0.1, 0.2 and 0.4 per cent rate.

Curry leaves contains phytochemical such as flavonoid, phenols, saponins, alkaloids and tannins. Flavonoids are the major constituent of curry leaves. The major constituent responsible for the aroma and flavor has been reported as pinene, sabinene, caryophyllene, cadinol and cadinene (Singh *et al.*, 2014; Igara *et al.*, 2016). Therefore, decrease in flavour score of ghee upon addition of curry leaves observed in the present study, up to 0.4 per cent may be attributed to above mentioned constituents.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of curry (*Murraya koenigi*) leaves when added to melted butter during clarification. Fresh curry leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of curry leaves 0.2, 0.5 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C The sample were examined for flavour score. All the samples of ghee were rated excellent at the beginning of the experiment. The judges preferred ghee samples treated with curry leaves as indicated by their highest scores for flavour. The flavour scores of ghee samples treated with curry leaves and antioxidants changed very little up to 30

days of storage. Initial flavour score of control ghee was 8.33 whereas, ghee treated with 0.5, 0.8 and 1.0 per cent curry leaves received flavour score of 8.5, 8.4 and 8.4 respectively. After storage for 147 days flavour score of control ghee was 2.16 whereas, ghee treated with 0.2, 0.5 and 1.0 per cent curry leaves received flavour score of 5.5, 6.1 and 6.5 respectively.

The above cited repot (Patel and Rajorhia, 1979) is the only record available in the literature for application of curry leaves in treatment of ghee at different rates to evaluate its antioxidative effects in ghee. In that study addition of curry leaves at the rate of 1 per cent rate was found most effective, whereas, in present study 0.3 per cent was found most effective. Such variations might be attributed to number of reasons. There were several differences between work carried in present study and the study carried out by the authors to optimize rate of curry leaves: (1) form of curry leaves used (vacuum dried powder and fresh respectively), (2) stage in ghee preparation at which curry leaves were added (final and initial respectively) and (3) temperature at which ghee was stored (80° and 30°C respectively). Even difference in variety might also be responsible for the difference, however, the authors did not reported the variety of the curry leaves used in their study.

4.4.3 Effect of Rate of Liquorice Used in Treatment of Ghee

Effect of different rates of addition of liquorice on its performance in retarding oxidative deterioration of ghee was measured in terms of changes in peroxide value and flavour score of ghee during storage at 80°±2°C.

4.4.3.1 Changes in peroxide value of liquorice treated ghee during storage at 80°±2°C

The results obtained for rate of liquorice addition on changes in peroxide value of ghee during storage are presented in Table 4.17 and the trend is presented in Figure 4.17.

Table 4.17: Changes in peroxide value of ghee during storage after treating with liquorice at different rates

Storage period (days)	Peroxide value of ghee during storage at 80°±2°C (meq of O_2 per kg fat)				
	Rate of liquorice used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	0.19	0.26	0.31	0.38	0.57
2	0.77	0.65	0.72	0.79	0.90
4	1.67	0.88	0.96	1.04	1.16
6	3.68	1.23	1.26	1.33	1.56
8	4.73	1.83	2.28	1.72	1.80
10	6.71	2.36	2.38	2.16	2.20
12	8.71	3.27	2.78	2.48	2.54
13	10.88	3.65	3.54	2.66	2.76
14	12.79	4.53	3.84	2.87	3.24
15	14.72	4.89	4.28	3.18	3.67
16	**17.41**	6.50	4.55	3.64	3.80
17	20.35	**6.95**	7.01	4.18	4.09
18	24.71	7.53	**8.81**	4.55	4.59
19	27.76	8.30	10.54	4.76	4.90
20	31.02	10.95	11.37	5.16	**6.36**
21	32.97	13.23	12.71	5.57	8.17
22	35.80	15.81	15.08	5.95	11.10

Source of variation	Storage period (P) (days)	Treatment (T) (rate)	Interaction (P×T)
S. Em	0.23	0.13	0.52
CD (0.05)	0.65	0.35	1.45
CV %	13.75		

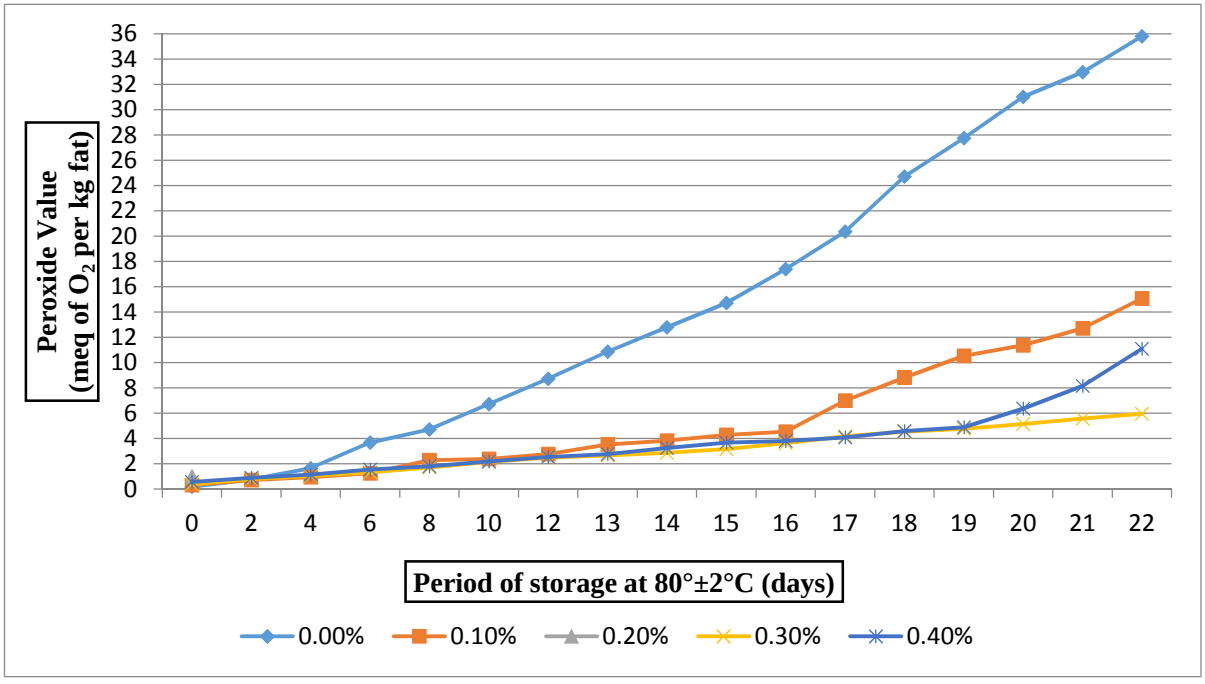


Figure 4.17: Changes in peroxide value of ghee during storage after treating with liquorice at different rates

It was revealed from statistical analysis of data on changes in peroxide value of ghee on storage at 80°±2°C that the treatments (rate of liquorice used in treatment of ghee) and period of storage both were significant (P<0.05). The interaction between period of storage and rate of liquorice used in treatment of ghee was also significant (P<0.05). Therefore, this statistical analysis of the results suggested that different rate of liquorice used in treatment of ghee differed significantly (P<0.05) in their effect on changes in peroxide value of ghee during storage. Similarly, storage period also differed significantly (P<0.05) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the rate of liquorice used in treatment of ghee and storage period differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of rate of liquorice used for treatment of ghee and period of storage were dependent on each other.

The peroxide value of different fresh ghee sample was in the order of control ghee < ghee treated with 0.1% liquorice < ghee treated with 0.2% liquorice < ghee treated with 0.3% liquorice < ghee treated with 0.4% liquorice. However, the order of peroxide value of different ghee samples changed at the end of the 22 days storage at 80°±2°C. At the end of the storage the order of peroxide value of different ghee samples was ghee treated with 0.3% liquorice < ghee treated with 0.4% liquorice < ghee treated with 0.2% liquorice < ghee treated with 0.1% liquorice < control ghee.

The control ghee sample showed steeped rise in peroxide value on 16th day of the storage. Whereas, samples of ghee treated with liquorice at 0.1, 0.2, and 0.4 per cent rate showed steeped rise in peroxide value on 17th, 18th and 20th day of the storage. Interestingly, sample of ghee treated with 0.3 per cent liquorice peroxide value of ghee increased at a steady rate, no sharp rise in peroxide value was observed in entire period of storage.

Among the fresh ghee samples (0 day), control sample of ghee had the lowest peroxide value compared to samples of ghee treated with liquorice at different rates of addition. It was noticed that among fresh ghee samples peroxide value increased with increase in rate of liquorice addition. Even up to 6th day of the storage same trend continued among liquorice treated ghee samples. However, from 4th of the storage peroxide value of control ghee sample became significantly higher (P<0.9) compared to all the ghee

samples treated with liquorice and remained significantly higher during subsequent storage up to end.

Among the sample of ghee treated with liquorice at different rate of addition (0.1, 0.2, 0.3 and 0.4%), on 8th of the storage the peroxide value of ghee treated with 0.3 per cent liquorice was lowest and also remained lowest during remaining period of the storage. Though, peroxide values of liquorice treated ghee samples was statistically at par up to 6th day of the storage, thereafter from 14th day of storage period peroxide values of ghee sample treated with 0.3 per cent liquorice was significantly ($P<0.05$) lower than all the liquorice treated ghee samples. Thus, it can be sum-up that treatment of ghee with liquorice at four different rate used in the treatment of ghee, all the four rates were able to reduce the peroxide formation in ghee during storage 80^{o}±2°C, compared to formation of peroxide in control ghee sample. However, best control in formation of peroxide in ghee during storage was given by 0.3 per cent liquorice. Any increase or decrease in the rate of liquorice from 0.3 per cent resulted in to reduction in effectiveness of the treatment in controlling the peroxide formation in ghee during storage 80^{o}±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent liquorice was most effective for reducing the peroxide formation in ghee during storage 80^{o}±2°C, compared to the ghee with liquorice at 0.1, 0.2 and 0.4 per cent rate. Any deviation from 0.3 per cent resulted in to decrease in performance of the treatment. Therefore, in the present study 0.3 per cent liquorice was considered as optimum in treatment of ghee for better control of peroxide formation during storage.

No report is available in the literature for evaluating effect of treatment of ghee with liquorice at different rates in preparation of ghee on changes in peroxide value of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature.

4.4.3.2 Changes in flavour score of liquorice treated ghee during storage at 80^{o}±2°C

The results obtained for rate of liquorice addition on changes in flavour score of ghee during storage are presented in Table 4.18 and the trend is presented in Figure 4.18.

Table 4.18: Changes in flavour score of ghee during storage after treating with liquorice at different rates

Storage period (days)	Flavour score of ghee during storage at 80°±2°C (Out of 9)				
	Rate of liquorice used in treatment of ghee				
	0.0%	**0.1%**	**0.2%**	**0.3%**	**0.4%**
0	9.00	9.00	9.00	9.00	8.53
2	8.70	8.60	8.67	8.73	8.37
4	7.97	8.03	8.03	8.40	8.27
6	6.77	7.30	7.50	8.13	7.87
8	**5.57**	6.20	7.13	7.70	7.40
10	4.53	**5.10**	6.47	7.23	6.77
12	3.27	4.23	6.00	6.87	6.30
13	2.67	4.00	6.00	6.70	6.17
14	2.00	3.70	**5.67**	6.40	6.03
15	1.53	3.27	5.17	6.13	**5.80**
16	1.37	3.03	4.90	6.00	4.70
17	1.23	2.67	4.30	6.00	4.07
18	1.00	1.93	3.67	**5.40**	3.53
19	1.00	1.20	3.27	5.07	3.07
20	1.00	1.00	2.20	4.20	2.40
21	1.00	1.00	1.00	3.57	1.33
22	1.00	1.00	1.00	2.87	1.00

Source of variation	Storage period (P)(days)	Treatment (T) (Rate of addition)	Interaction (P×T)
SEm	0.09	0.05	0.21
CD (0.05)	0.26	0.14	0.58
CV%	7.26		

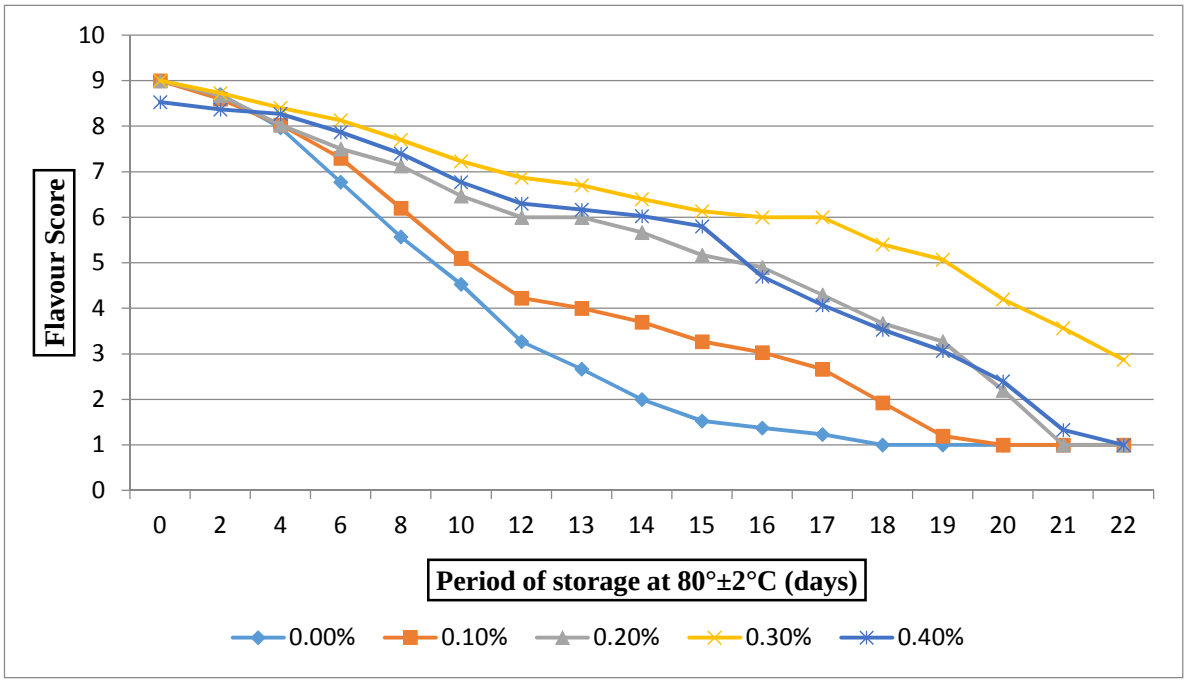


Figure 4.18: Changes in flavour score of ghee during storage after treating with liquorice at different rates

It was revealed from statistical analysis of data on changes in flavour score of ghee on storage at $80^{o}\pm2°C$ that the treatments (rate of liquorice used in treatment of ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage and rate of liquorice used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different rate of liquorice used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the rate of liquorice used in treatment of ghee and period of the storage significantly differed from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of rate of liquorice used for treatment of ghee and period of storage were dependent on each other.

The flavour score of different fresh ghee sample was in the order of control ghee = ghee treated with 0.1% liquorice = ghee treated with 0.2% liquorice = ghee treated with 0.3% liquorice > ghee treated with 0.4% liquorice. On 13^{th} day of the storage the order of flavour score of different ghee samples was ghee treated with 0.3% liquorice > ghee treated with 0.4% liquorice > ghee treated with 0.2% liquorice > ghee treated with 0.1% liquorice > control ghee.

Among the fresh ghee samples (0 day) control ghee sample as well as ghee samples treated with liquorice at the rate of 0.1, 0.2 and 0.3 acquired full flavour score (9 out of 9). Only the ghee samples treated with liquorice at 0.4 per cent got slightly lower flavour score compared other samples in the group. On 2^{nd} day of the storage flavour score of all the ghee samples decreased, but relative trend between the samples was almost similar as noticed in case of the fresh ghee samples. On 4^{th} of the storage flavour score of control ghee was lowest among all the ghee samples. The flavour score of control ghee became significantly lower than all the liquorice treated ghee samples.

The flavour score of control ghee decreased at a rapid rate from beginning of the storage and showed steeped descale on 17^{th} day of the storage. Flavour score of the control sample reached to a significantly lower level compared to liquorice treated ghee on 4^{th} day of the storage.

Among the samples of ghee treated with liquorice at different rate of addition (0.1, 0.2, 0.3 and 0.4 per cent), the sample of ghee treated with 0.1 per cent liquorice also showed

almost similar trend in changes in flavour score of ghee on storage as that of the control ghee sample, except slight variation between 13th to 19th days of the storage. Interestingly, sample of ghee treated with 0.3 per cent liquorice flavour score of ghee increased at a steady rate, no sharp rise in flavour score was observed up to 17th day of the storage. The samples of ghee treated with 0.2 and 0.4 per cent liquorice also followed almost similar trend in changes in flavour score of ghee as that of the 0.3 per cent treated ghee sample up to 13th day of the storage. However, their trend change towards the control and 0.1 per cent treated sample of ghee after on storage up to 15th day of the storage, however, their trend changed thereafter and tended toward fatter rate of decline. The flavour score of ghee treated with 0.3 per cent liquorice remained highest all throughout the storage.

Among all the samples of ghee, the flavour score of control ghee went below the acceptable level on 8th day of the storage. On the other hand flavour score of ghee treated with 0.1, 0.2, 0.3 and 0.4 per cent liquorice went below the acceptable level on 10th, 14th, 18th and 15th day of the storage. Thus, among different levels of liquorice used in treatment of ghee, best control for retention of flavour score of ghee during storage was achieved by 0.3 per cent liquorice. Any increase or decrease in the rate of liquorice from 0.3 per cent resulted in to reduction in effectiveness of the treatment in controlling the deterioration ghee flavour during storage 80^{o}±2°C.

From the forgoing resume it became very clearly evident that treatment of ghee with 0.3 per cent liquorice was most effective for restricting flavour deterioration of ghee during storage 80^{o}±2°C, compared to the ghee with liquorice at 0.1, 0.2 and 0.4 per cent rate. Any deviation from 0.3 per cent resulted in to decrease in performance of the treatment. Therefore, in the present study 0.3 per cent liquorice was considered as optimum in treatment of ghee for better retention of flavour during storage.

From the entire study carried to optimize rate of betel leaves, curry leaves and liquorice for treatment of ghee it was found that for all three herbs a rate of 0.3 was optimum. The variation in rate of the herbs on any side (towards lower or higher) resulted in to reduction in the effectiveness of the treatment. The reduction in the effectiveness of the treatment on lowering the amount of herb in treatment of ghee might be attributed insufficient availability of antioxidants for the action and/or possible pro-oxidant effect of the antioxidant due to their presence in low concentration. On the other hand decrease in the effectiveness of the treatment on increasing the amount of herb in treatment of

ghee might be attributed possible pro-oxidant effect of the antioxidant due to their presence in higher concentration. From careful examination of the data obtained the study order of relative pro-oxidant effect of three herbs used in the present could be work out as curry leaves > liquorice > betel leaves.

Liquorice contains phytochemicals such as alkaloids, flavonoids, phenols and tannins. The flavour of liquorice is due the presence of volatile components. The volatile components of liquorice includes pentanol, hexanol, linalool oxide A and B, tetramethyl pyrazine, terpinen-4-ol, α-terpineol, geraniol and others (Roshan *et al.,* 2012). Therefore, decrease in flavour score of ghee upon addition of liquorice observed in the present study, up to 0.4 per cent may be attributed to above mentioned constituents of liquorice.

No report is available in the literature for evaluating effect of treatment of ghee with liquorice at different rates in preparation of ghee on changes in flavour score of ghee during storage. Therefore, results obtained in present study could not be compared as such with the reports in the literature.

Duga (1976) pointed out that some antioxidants provide increased protection with increasing concentration, while others have optimal levels after which higher levels exert pro-oxidant effects. According to Fukumoto and Mazza (2000) most phenolic compounds had pro-oxidant activity at low concentrations. Bouayed and Bohn (2010) opined that high concentrations of antioxidants including BHT and BHA in food items, can also increase spoilage of food items due to pro-oxidant activities. Similar views were expressed by Ling *et al*. (2010). These authors stated that high concentrations of antioxidants may have pro-oxidant activity. Moure *et al*. (2001) suggested that potent antioxidants can autoxidize and generate reactive substances and thus also act as pro-oxidants, depending on the systems, According to Gordon (1990) as well as Cao and Cutler (1993) at high concentrations of antioxidant, their pro-oxidant effects could arise due to the involvement of the phenolic compounds in initiation reactions (i.e., formation of radicals). As per Cillard *et al*. (1980) hydroxyl radical absorbance capacity of antioxidants decreases at high concentrations due to their involvement in initiation reactions such as at high concentrations.

4.5 COMBINATIONS OF THE SELECTED HERBS AS ANTIOXIDANTS IN GHEE

Many plant-based foods are good sources of unique phytochemical antioxidant. Structurally diverse phytochemicals may possess similar, overlapping, or different but complementary effects in their antioxidant activities. A combination of different plant-based foods may exhibit additive, synergistic or antagonistic interactions among their different phytochemicals. An additive effect refers to a food combination that provides the sum of the effects of the individual components; a synergistic effect occurs when the effect is greater than the sum of individual components, and antagonism occurs when the sum of the effects is less than the mathematical sum that would be predicted from individual components (Wang *et al*., 2011). Therefore, different herbs have different bioactive constituents with different antioxidant activities. When they are taken together, the mixtures shows higher or lower antioxidant capacity than their individual extracts. It may be due to synergistic, additive or antagonistic interaction among different compounds (Gupta *et al*., 2014).

In view of the above resume, attempt was made in the present study to evaluate the selected herbs *i.e.* betel leaves (B), curry leaves (C) and liquorice (L) for their action in combination for application in ghee as an antioxidant to retard oxidative deterioration of ghee during storage. To study the effect of these three herbs in combination, individual herbs were mixed to prepare binary and ternary mixtures as: betel leaves and curry leaves (B+C), betel leaves and liquorice (B+L), curry leaves and liquorice (C+L) as well as betel leaves, curry leaves and liquorice (B+C+L). Each of these herbs were used at the rate optimized in the previous phase of this study (*i.e.* @ 0.3% of the expected yield of ghee) (Section 4.4). Moreover, stage in preparation of ghee at which addition of the herbs to be added was decided on the basis of work out in the previous phase of this study (Section 4.3). Accordingly betel leaves and curry leaves were added at the final stage of clarification and liquorice was added at the initial stage of clarification. Along with the combinations of herbs, individual herbs were also tested simultaneously under identical condition to decide whether action of the combination of herbs was synergistic, additive or antagonistic.

For evaluating the action of selected herbs (betel leaves, curry leaves and liquorice) in combination for treatment of ghee, the sample of butter (120 g) was taken in to each of the 8 glass beakers of 500 ml. The beakers containing butter were arranged in round

shaped sand bath in such a way that each beaker remained at equal distance from the centre of the sand bath. In 1st beaker no herb was added to serve as control. Betel leaves (0.3%), curry leaves (0.3%) and liquorice (0.3%) were added in 2nd, 3rd and 4th beaker respectively. The combination of betel leaves (0.3%) and curry leaves (0.3%), betel leaves (0.3%) and liquorice (0.3%), curry leaves (0.3%) and liquorice (0.3%) as well as betel leaves (0.3%), curry leaves (0.3%) and liquorice (0.3%) were added to 5th, 6th, 7th and 8th beaker. The rate of addition was on the basis of expected yield of ghee.

Liquorice was added at initial stage of clarification (*i.e.* to melted butter) and betel leaves and curry leaves were added at final stage of clarification (*i.e.* when temperature of the content reached to 105°C). The sand bath was heated by gas fired burner with flame at the centre. The heating was continued till temperature reached to 120°C. In the entire process (from beginning to end) for preparation of ghee each sample was mixed gently with stainless steel spatula turn by turn. The content of each beaker was then filtered through 6 folded muslin cloth, ghee was collected in 150 ml glass beakers and stored in incubator at 80°±2°C.

All the 8 samples of fresh ghee were analysed for peroxide value by titration against standard 0.002N sodium thiosulfate solution using starch indicator and expressed as meq of O_2 per kg fat. The samples of ghee were also evaluated for flavour score by sensory evaluation using 9 point hedonic scale. Similarly, during storage all the samples of ghee were analysed for peroxide value and evaluated for flavour score at a regular interval of every 2 days up to 12 days and then every day. The storage of the samples and their analysis was continued till flavour score of almost all the ghee samples went below acceptable level. Total three replications were conducted and average results obtained from the three replications are presented below.

4.5.1 Effect of Herbs and their Combinations on Peroxide Value of Ghee during Storage

Effect of selected herbs and their combination on reducing peroxide formation in ghee was measured in terms of changes in peroxide value of the ghee during storage at 80°±2°C. The results obtained for changes in peroxide value of ghee during storage are presented in Table 4.19 and the trend is presented in Figure 4.19.

Table 4.19: Changes in peroxide value of ghee during storage at 80°±2°C after treating with selected herbs and their combinations

Storage period (days)	Peroxide value of ghee (meq of O_2 per kg fat) Herbs and their combinations used in treatment of ghee							
	Control	**B**	**C**	**L**	**B+C**	**B+L**	**C+L**	**B+C+L**
0	0.26	0.19	0.39	0.39	0.39	0.39	0.19	0.19
2	1.15	0.39	0.96	0.58	0.92	0.39	0.57	0.39
4	1.90	1.08	1.65	1.56	1.18	0.57	0.96	0.76
6	2.19	1.38	2.33	1.77	1.37	0.78	1.17	1.59
8	3.85	1.62	2.78	2.12	1.70	1.37	1.52	2.08
10	5.66	1.79	3.04	2.35	1.87	1.75	1.75	2.46
12	6.68	2.07	4.36	2.65	2.09	1.93	2.15	2.83
13	7.63	2.77	5.97	2.92	2.55	2.14	2.67	3.22
14	9.81	3.14	7.72	3.15	2.79	2.32	2.97	3.54
15	11.24	3.22	9.49	3.63	3.19	2.54	3.38	3.85
16	12.87	3.30	11.06	3.82	3.30	2.68	3.55	4.07
17	18.91	3.42	9.62	4.25	3.62	2.91	3.69	4.29
18	22.74	3.52	8.79	4.51	3.70	3.27	3.79	4.56
19	33.30	3.68	11.83	4.71	3.94	3.48	4.06	4.75
20	38.03	3.94	14.50	5.43	4.17	3.71	4.32	4.93
21	35.80	4.38	21.33	5.91	4.76	3.82	6.23	5.25
22	33.68	4.62	30.05	6.24	5.01	4.21	6.69	5.37
23	30.21	5.15	38.37	6.83	5.34	4.52	6.95	5.56
24	28.33	5.28	**40.11**	8.36	5.48	4.65	7.28	5.67
25	21.62	5.39	34.92	9.28	5.93	4.89	8.52	5.72
26	20.67	5.57	25.25	9.81	6.44	5.12	8.73	5.89
27	23.87	5.71	18.18	13.14	6.66	5.39	9.01	6.00
28	26.07	5.90	13.89	19.09	8.28	5.53	**12.93**	6.43
29	27.75	6.08	10.75	27.13	11.28	5.74	9.64	6.93

Source of variation	Storage period (P) (days)	Treatment (T) (Herb/combination)	Interaction (P× T)
SEm	0.16	0.09	0.44
CD (0.05)	0.43	0.25	1.22
CV%	10.74		

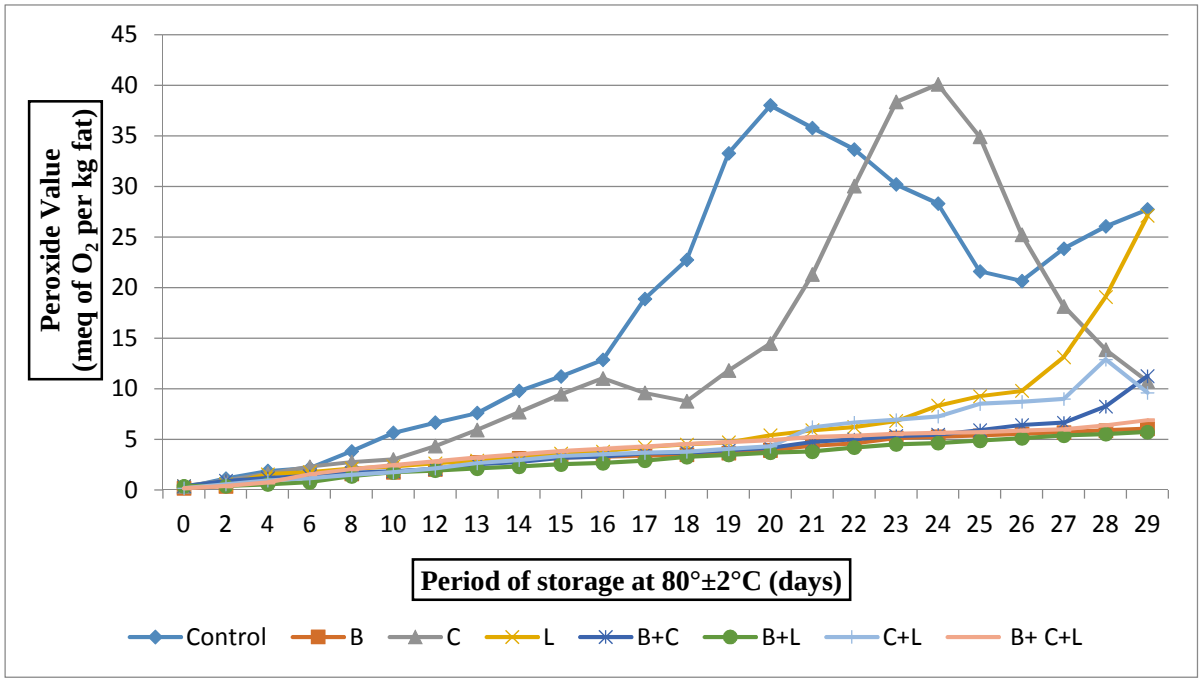


Figure 4.19: Changes in peroxide value of ghee during storage at 80°±2°C after treating with selected herbs and their combinations

It was revealed from statistical analysis of data on changes in peroxide value of ghee during storage at $80^{o}\pm2°C$ that the treatments (herbs and their combinations used in treatment of ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as herbs and their combinations used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different herbs and their combinations used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that the herbs and their combinations used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of herbs and their combinations used in treatment of ghee and period of storage were dependent on each other.

The peroxide value of different fresh ghee sample was in the order of curry leaves = liquorice = betel leaves and curry leaves = betel leaves and liquorice > control > betel leaves = curry leaves and liquorice = betel leaves, curry leaves and liquorice. However, the order of maximum level of peroxide value to which peroxide value reached during the storage was curry leaves > control > liquorice > betel leaves and curry leaves > curry leaves and liquorice > betel leaves, curry leaves and liquorice > betel leaves > betel leaves and liquorice. The maximum level of peroxide value peroxide values of these ghee samples were 40.11, 38.03, 27.13, 11.28, 12.93, 6.93, 6.08 and 5.74 meq of O_2 per kg fat respectively.

In control sample of peroxide value rise at very rapid from beginning, showed steeped rise in peroxide value on 17^{th} day of the storage, reached to the maximum level (38.03 meq of O_2 per kg fat) on 20^{th} day of the storage and then started falling down on further storage. The sample of ghee treated with curry leaves alone also followed almost similar trend of rise peroxide value up to 6^{th} day of the storage as that of the control sample of ghee. There was slight destine in peroxide value of this ghee sample ghee for a brief period of 2 days, then again it regain its moment of rise in the peroxide value, reached to the maximum level (40.11 meq of O_2 per kg fat) to surpass the peroxide value of control ghee on 24^{th} day of the storage and the started declining on further storage.

In sample of ghee treated with liquorice alone, peroxide value rise very gradually up to 23rd day of the storage, on further storage its rate of rise in peroxide value was accelerated, much steeped rise in peroxide value was noticed from 26th day of the storage and peroxide value reached to 27.13 meq of O_2 per kg fat on 29th day of the storage. The sample of ghee treated with combination of curry leaves and liquorice also followed almost similar trend of rise in peroxide value up to 23rd day of the storage as that of the sample of ghee treated with only liquorice, with peroxide values below the liquorice treated ghee and reached to 12.93 meq of O_2 per kg fat on 28th day of the storage. The sample of ghee treated with combination of betel leaves and curry leaves followed somewhat similar trend of rise in peroxide value as that of the ghee treated with combination of curry leaves and liquorice. In ghee treated with combination of betel leaves and curry leaves, peroxide value rise very gradually up to 27th day of the storage, on further storage its rate of rise in peroxide value was accelerated and it reached to 11.28 meq of O_2 per kg fat on 29th day of the storage.

In samples of ghee treated with betel leaves alone and combination of betel leaves and liquorice peroxide value rise very gradually all throughout the storage. There was no sign of rapid rise of peroxide value was observed in these ghee samples at any stage of the entire storage. On 29th of the storage peroxide value of ghee treated with betel leaves alone and combination of betel leaves and liquorice was 6.08 and 5.74 meq of O_2 per kg fat respectively. Betel leaves treated ghee had significantly ($P<0.05$) higher peroxide value than sample treated with combination of betel leaves and liquorice. The data indicated that there was a significant increase in effectiveness to control peroxide formation in ghee when betel leaves were blended with liquorice. Neither synergistic nor additive effect was noticed. The probable reason for such lack of synergistic or additive action between betel leaves and liquorice might be attributed to strong pro-oxidant effect of liquorice at later stage of the storage.

In ghee treated with ternary blend of the selected herbs comprising betel leaves peroxide value rise very gradually almost entire the storage, except towards the end of the storage. In this sample of ghee treated with combination of betel leaves, curry leaves and liquorice gradual rise in peroxide value was noticed till the end of the storage. On 29th day of the storage peroxide value of this ghee sample reached to 6.93. It was significantly higher ($P <0.05$) that the peroxide values of ghee treated with betel leaves alone or combination of betel leaves and liquorice.

Combination of betel leaves and liquorice treated ghee sample had significantly ($P<0.05$) lower peroxide value than the sample treated with betel leaves alone at the end of storage.

The effectiveness of selected herbs and their different combinations in controlling formation of peroxide in ghee during the storage can be arranged in the order of betel leaves and liquorice > betel leaves > betel leaves, curry leaves and liquorice > betel leaves and curry leaves > curry leaves and liquorice > liquorice > curry leaves.

From the results obtained on the effectiveness of selected herbs and their different combinations, they can be grouped in to three categories: high, medium and low. The herbs and/or their combination falling under category of low effectiveness was curry leaves. Similarly, herbs and/or their combination falling under category of medium effectiveness were combination of betel leaves and curry leaves, combination of curry leaves and liquorice as well as liquorice. Whereas, herbs and/or their combination falling under category of high effectiveness were of combination of betel leaves and liquorice, betel leaves as well as combination of betel leaves, curry leaves and liquorice.

The examination of the data on changes in peroxide values of different samples of ghee it was evident that curry leaves gave a marginal control on development of peroxide in during in initial phase of the storage (*i.e.* up to ~12 days). Thereafter, on further storage it started behaving as a pro-oxidant. The pro-oxidant effect of curry leaves was evident from the highest peroxide value (40.11 meq of O_2 per kg fat) attained by this sample amongst all the samples of ghee. Its peroxide value was higher that of the even control ghee (38.03 meq of O_2 per kg fat).

The pro-oxidant action of curry leaves had caused antagonistic effect when it was used in combination with betel leaves as well as along with blend betel leaves and liquorice. This fact was proved from the peroxide value of the sample on 29^{th} day of the storage. The peroxide value of ghee treated with betel leaves alone was 6.08 meq of O_2 per kg fat on 29^{th} day of the storage, at the same time peroxide value of ghee treated with combination of betel leaves and curry leaves was 11.28 meq of O_2 per kg fat. Similarly, peroxide value of ghee treated with combination of betel leaves and liquorice was 5.74 meq of O_2 per kg fat on 29^{th} day of the storage, whereas, at the same time peroxide value of ghee treated with combination of betel leaves, curry leaves and liquorice was 6.93 meq of O_2 per kg fat.

On the other hand careful examination of the data suggested some synergistic effect between curry leaves and liquorice. Their synergistic action was very clearly evident from the maximum extent to which peroxide value of each ghee sample treated with curry leaves, liquorice and their combination reached during entire storage period. As stated earlier, when ghee was treated with curry leaves, the maximum peroxide value of this sample was 40.11 meq of O_2 per kg fat. Similarly, when ghee was treated with liquorice, the maximum peroxide value of this sample was 27.13 meq of O_2 per kg fat. When both of these herbs were applied together for treatment of ghee, theoretically the maximum peroxide value of ghee was expected to be much higher than their individual ghee samples. However, the peroxide value of this ghee sample treated with blend curry leaves and liquorice reached to maximum of only 12.93 meq of O_2 per kg fat on 28^{th} day of the storage and then started declining on further storage.

The synergistic action of curry leaves and liquorice was further supported by comparing maximum level of peroxide value of ghee samples treated with blend of betel leaves plus curry leaves and maximum level of peroxide value of ghee samples treated with blend of betel leaves, curry leaves plus liquorice. The maximum level of peroxide value of ghee samples treated with blend of betel leaves plus curry leaves was 11.28 meq of O_2 per kg fat on 29^{th} day of the storage. When liquorice was also incorporated in blend along with betel leaves and curry leaves for treatment of ghee, the maximum level of peroxide value of this ghee sample was 6.93 meq of O_2 per kg fat on 29^{th} day of the storage. Thus, formation of peroxide was restricted due to possible synergistic action of curry leaves and liquorice.

It was evident from the work carried out to evaluate effectiveness of selected herbs and their different combinations to control formation of peroxide in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of various herbs and their different combinations in controlling formation of peroxides in ghee during the storage. Therefore, results obtained in the present study could not be compared with the reports in the literature.

4.5.2 Effect of Herbs and their Combinations on Flavour Score of Ghee during Storage

Effect of selected herbs and their combinations on flavour of ghee was measured in terms of changes in flavour score of the ghee during storage at 80°±2°C. The results obtained for changes in flavour score of ghee during storage are presented in Table 4.20(a) and the trend is presented in Figure 4.20.

Table 4.20(a): Changes in flavour score of ghee during storage at 80°±2°C after treating with selected herbs and their combinations

Storage period (days)	Flavour score of ghee (out of 9)							
	Herbs and their combinations used in treatment of ghee							
	Control	B	C	L	B+C	B+L	C+L	B+C+L
0	9.00	8.72	8.57	8.20	8.63	8.90	8.57	8.77
2	8.77	8.63	8.10	7.83	9.00	9.00	9.00	9.00
4	8.27	8.49	7.63	7.70	8.50	8.88	8.37	8.53
6	7.30	8.38	7.40	7.60	8.23	8.79	8.03	8.27
8	6.60	8.27	7.00	7.50	8.07	8.68	7.83	8.03
10	**5.77**	8.00	6.73	7.33	8.00	8.53	7.73	7.67
12	4.90	7.73	6.40	7.27	7.77	8.41	7.57	7.43
13	4.00	7.53	**5.43**	6.97	7.57	8.32	7.37	7.13
14	2.70	7.47	4.80	6.87	7.33	8.27	7.07	7.00
15	2.03	7.30	3.43	6.77	7.13	8.03	6.90	6.73
16	1.33	7.20	2.90	6.60	6.90	7.83	6.83	6.67
17	1.00	7.13	2.33	6.53	6.83	7.63	6.70	6.57
18	1.00	7.00	2.07	6.50	6.63	7.57	6.40	6.57
19	1.00	6.87	2.00	6.27	6.43	7.43	6.23	6.50
20	1.00	6.77	1.93	**4.57**	6.30	7.17	6.13	6.50
21	1.00	6.77	1.83	4.17	6.13	7.03	6.03	6.50
22	1.00	6.73	1.53	4.17	**5.80**	6.90	6.00	6.43
23	1.00	6.47	1.13	3.90	5.53	6.77	**5.90**	6.27
24	1.00	6.37	1.00	3.60	5.33	6.70	5.77	6.03
25	1.00	6.30	1.00	3.33	4.93	6.67	5.60	6.03
26	1.00	6.23	1.00	3.27	4.53	6.57	5.27	6.00
27	1.00	6.17	1.00	3.00	4.00	6.50	4.37	6.00
28	1.00	6.10	1.00	2.87	3.73	6.33	3.87	**5.43**
29	1.00	**6.03**	1.00	2.70	3.27	**6.20**	3.63	4.39

Source of variation	Storage period (P) (days)	Treatment (T) (Herb/combination)	Interaction (P× T)
SEm	0.10	0.06	0.27
CD (0.05)	0.27	0.15	0.75
CV%	8.11		

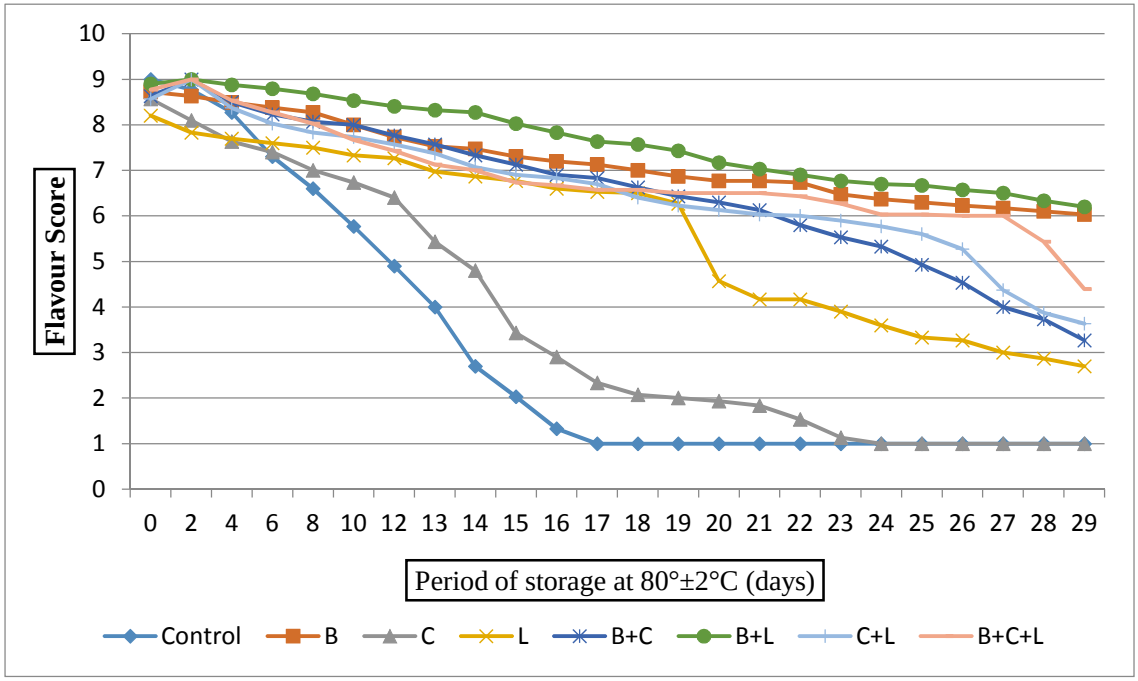


Figure 4.20: Changes in flavour score of ghee during storage at 80°±2°C after treating with selected herbs and their combinations

It was revealed from statistical analysis of data on changes in flavour score of ghee during storage at 80°±2°C that the treatments (herbs and their combinations used in treatment of ghee) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as herbs and their combinations used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different herbs and their combinations used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, period of storage also had significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that the herbs and their combinations used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of herbs and their combinations used in treatment of ghee and period of storage were dependent on each other.

The flavour score of different fresh ghee sample was in the order of control > betel leaves and liquorice > betel leaves, curry leaves and liquorice > betel leaves > betel leaves and curry leaves > curry leaves = curry leaves and liquorice > liquorice. On 2nd of the storage slight improvement in flavour score of the ghee samples treated different blend of the herb was experienced. The possible reason for the improvement in flavour

might be attributed little harsh aroma imparted by the herbs in fresh ghee, which might have subsided on storage. However, the order of flavour score changed significantly during the storage and at the end of the 29 days storage the order of flavour score was betel leaves and liquorice > betel leaves > betel leaves, curry leaves and liquorice > curry leaves and liquorice > betel leaves and curry leaves > liquorice > control = curry leaves. On 29th day of the storage, the flavour score of these ghee samples (out of 9) were 6.03, 6.20, 4.39, 3.63, 3.27, 2.70, 1.00 and 1.00, respectively.

In control sample flavour score decreased at very rapid from beginning, decline in flavour score was steeped from 4th day of the storage, it went below the acceptable level on 10th day of the storage and then went down on further storage. The sample of ghee treated with curry leaves alone also followed almost similar trend of decline in flavour score during the storage, as that of the control sample of ghee, but comparatively at slightly lower rate than the control. The flavour score of curry leaves treated ghee sample went below the acceptable level on 13th day of the storage and continued to decline on further storage.

In ghee treated with liquorice alone, flavour score decreased very gradually up to 19th day of the storage and the flavour score reached below the acceptable level on the 20th day of the storage and then gradually decline till the end of the storage. The sample of ghee treated with combination of curry leaves and liquorice also followed almost similar trend in decrease of flavour score on further storage and its flavour score went below the acceptable level on 23rd day of the storage. The sample of ghee treated with combination of betel leaves and curry leaves followed somewhat similar trend of decrease in flavour score as that of the ghee treated with combination of curry leaves and liquorice and became unacceptable on 22nd of the storage.

In ghee treated with betel leaves alone, combination of betel leaves and liquorice as well as combination of betel leaves, curry leaves and liquorice; flavour score decreased very gradually all throughout the storage period. Especially, in samples of ghee treated with betel leaves alone or combination of betel leaves there was no sign of rapid decline in flavour score was observed at any stage of the entire storage. However, in samples of ghee treated with combination of betel leaves, curry leaves and liquorice rapid decline in flavour score was noticed on 28th day of the storage and its flavour score also went below the acceptable level on 28th day of the storage. At the end of the study (on 29th of the storage) flavour score of ghee treated with betel leaves alone and

combination of betel leaves of liquorice was 6.03 and 6.20 respectively. Flavour score of combination of betel leaves and liquorice treated ghee sample was significantly ($P<0.05$) higher than the sample treated with betel leaves alone.

The effectiveness of selected herbs and their different combinations in retaining the flavour of ghee during the storage can be arranged in the order of betel leaves and liquorice > betel leaves > betel leaves, curry leaves and liquorice > curry leaves and liquorice > betel leaves and curry leaves > liquorice > curry leaves.

From the results obtained on the effectiveness of selected herbs and their different combinations, they can be grouped in to three categories: high, medium and low. The herbs and/or their combination falling under category of low effectiveness was curry leaves. Similarly, herbs and/or their combination falling under category of medium effectiveness were combination of betel leaves and curry leaves, combination of curry leaves and liquorice as well as liquorice. Whereas, herbs and/or their combination falling under category of high effectiveness were of combination of betel leaves and liquorice, betel leaves as well as combination of betel leaves, curry leaves and liquorice.

It was evident from the work carried out to evaluate effectiveness of selected herbs and their different combinations to control deterioration of flavour in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of various herbs and their different combinations in controlling deterioration of flavour in ghee during the storage. Therefore, results obtained in the present study could not be compared with the reports in the literature.

Moure *et al*. (2001) suggested that reported since there is no single antioxidant that can scavenge all kinds of radicals or that performs optimally for all lipid products, mixtures of antioxidants resulting in a synergistic effect are preferred for preventing free radical-induced diseases. The use of synergistic mixtures of antioxidants allows a reduction in the concentration of each and also increases the antioxidant effectiveness with respect to the activity of the separate components although, even in widely used and commercialized extracts, such as rosemary, the antioxidative behaviour and synergistic actions of most of the compounds remain unknown. Sherwin (1990) summarized the beneficial effects of using mixtures of antioxidants as: (1) advantages of their different

effectiveness; (2) minimalisation of solubility or colour problems presented by individual compounds; (3) better control and accuracy of application; (4) complete distribution or solution of antioxidants and chelating agents. Moure *et al*. (2001) reported synergistic effects of phenols from grape seeds and pomace polyphenols have been reported. Mixtures of tocopherol and carotene, as well as mixtures with other substances (ascorbic acid, lecithin). An antagonist effects were observed between ellagic acid and catechin. The authors suggested the possible existence of hydrogen-bonding between carbonyls in ellagic acid and o-dihydroxyl groups in catechin. Chu and Hsu (1999) observed a two or three times higher oxidative stability index for peanut oil when mixtures of antioxidants were used. Gupta *et al*. (2014) evaluated the synergistic antioxidant activity of tea with ginger, black pepper and tulsi and found that all the combinations (tea and ginger; tea and black pepper; tea and tulsi; tea, ginger and black pepper; tea, black pepper and tulsi; tea, ginger and tulsi; tea, ginger, black pepper and tulsi) exhibited radical scavenging activity for aqueous and methanolic extract.

Among all the mixtures, the mixture of methanolic extract of different herbs, the best result was obtained in the synergistic combination; a (tea: ginger). This clearly shows that the amount of antioxidant compounds present in methanolic extract were more than the aqueous extracts of above herbs. The results showed that selected polyherbal combinations of all the extracts with tea were found to produce best antioxidant activity in comparison to their individual extracts. Therefore, findings of the present study on synergistic and antagonistic effect of selected herbs and their combinations in reducing oxidative deterioration of ghee were in general agreement with views and observations reported by the various worker in the literature.

4.6 COMPARISON OF HERBS WITH SYNTHETIC ANTIOXIDANT (BHA)

After selecting the potential herbs, stage in preparation of ghee for treatment with herbs and optimum rate of herbs for use as well as evaluating the combination effect of the selected herbs; in last phase of the study work was carried out to compare the performance of the herbs with butylated hydroxyl anisole (BHA), a synthetic antioxidant permitted in ghee under FSSAI rules for reducing the oxidative deterioration of ghee during storage. The fresh samples of ghee were also analysed for quality standards as prescribed under FSSAI.

Based on the performance in reducing the oxidative deterioration of ghee during the storage, betel leaves (B) as well as combination of betel leaves and liquorice (B+L) were selected for the comparison of their performance with BHA in reducing the oxidative deterioration of ghee. To compare the performance of the betel leaves and blend of betel leaves plus liquorice with BHA, the sample of butter (120 g) was taken in to each of the 4 glass beakers of 500 ml. The beakers containing butter were arranged in round shaped sand bath in such a way that each beaker remained at equal distance from the centre of the sand bath. In 1st and 2nd beakers nothing was added. 1st beaker was used as a control sample. In betel leaves (0.3%) and betel leaves (0.3%) plus liquorice (0.3%) were added in 3rd and 4th beaker respectively. Liquorice was added at initial stage of clarification (*i.e.* to melted butter) and betel leaves and curry leaves were added at final stage of clarification (*i.e.* when temperature of the content reached to 105°C). The sand bath was heated by gas fired burner with flame at the centre. The heating was continued till temperature reached to 120°C. In the entire process (from beginning to end) for preparation of ghee each sample was mixed gently with stainless steel spatula turn by turn. The content of each beaker was then filtered through 6 folded muslin cloth, ghee was collected in 150 ml glass beakers and then in 2nd beaker BHA (0.02%) was added. All the ghee samples were divided in to 2 parts. One part was stored in incubator at 35°±2°C and another part was stored at 80°±2°C. Total four replications were conducted.

All the samples of fresh ghee were analysed for quality standards and evaluated for sensory attributes. All the samples of ghee stored at elevated temperature (80°±2°C) as well as at normal room temperature (35°±2°C) and regularly monitored for effect of antioxidants on oxidative changes taking place in the ghee.

4.6.1 Quality Parameters of Ghee

All the fresh samples of ghee were analysed for various quality parameters as specified under FSSAI standards including moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and Baudouin test. Their results are presented in Table 4.20(b).

Table 4.20(b): Quality parameters of ghee treated with different antioxidants

Antioxidant used	Quality parameter of ghee					
	Moisture (%)	BR reading at 40°C	RM value	Polenske value	FFA (% oleic acid)	Baudouin test
Control	0.15	42.16	33.52	1.38	0.25	Negative
Betel leaves	0.14	41.05	33.91	1.45	0.23	Negative
Betel leaves + Liquorice	0.17	40.61	32.31	1.50	0.21	Negative
BHA	0.18	41.57	34.07	1.53	0.24	Negative
Source of variation						
SEm	0.01	0.26	0.30	0.12	0.01	-
CD (0.05)	NS	0.80	0.93	NS	NS	-
CV%	14.79	1.25	1.81	16.34	10.86	-

4.6.1.1 Moisture content

It is observed that the moisture content in different ghee samples varied on an average from 0.14 to 0.18 %. 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% (FSSAI, 2011). As per Agmark standards moisture content of ghee should not be more than 0.3% (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.

4.6.1.2 BR reading

BR reading of different ghee samples at 40°C varied on an average from 40.61 to 42.16. The difference in BR reading of different samples of ghee was statistically significant ($P< 0.05$). All the samples of ghee treated with both the samples (betel leaves and blend of betel leaves plus liquorice) showed significantly lower BR reading compared to BR reading of the control ghee. The results revealed that the treatment of ghee with antioxidants (betel leaves and blend of betel leaves plus liquorice) significantly lowered their BR reading.

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 different samples of ghee 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).

4.6.1.3 RM value

RM value of different ghee samples varied on an average from 32.31 to 34.07. The difference in RM value of different ghee samples was statistically significant ($P< 0.05$). The results revealed that the treatment of ghee with antioxidants (BHA, betel leaves and blend of betel leaves plus liquorice) had significant effect on RM value of ghee. However, no definite trend or relationship between control ghee sample and samples treated with antioxidant or between the different antioxidant was observed.

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 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).

4.6.1.4 Polenske value

The Polenske value of ghee samples varied on an average from 1.38 to 1.53 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 standards as well as Agmark standards, Polenske value of ghee should be 1 to 2. Thus, Polenske value 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).

4.6.1.5 FFA content

The FFA content of ghee samples varied on an average from 0.21 to 0.25 per cent 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 (as per cent oleic acid) 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).

4.6.1.6 Baudouin test

According to FSSAI and Agmark standards for ghee Baudouin test should be negative. In the present Baudouin test was found negative for all ghee samples including control ghee and ghee treated with BHA, betel leaves as well blend of betel leaves plus liquorice. Thus, all the ghee samples of fulfilled requirement of Baudouin test as prescribed by under FSSAI (2011) and Agmark (1991) standard.

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 betel leaves or blend of betel leaves plus liquorice 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.

4.6.2 Sensory Evaluation of Ghee

Sensory evaluation is generally considered to be the most reliable indicator of rancidity. Sensory evaluation mainly depends on human panellists to assess the acceptability and sensory properties of a product. No instrument can replicate or replace the human response, and sensory evaluation is therefore of importance in a quality assessment system for food products (Mehta *et al.*, 2015).

All the fresh samples of ghee were evaluated for various sensory attributes including flavour, texture, colour and appearance and overall acceptability using 9 point hedonic scale. Their results are presented in Table 4.21(a) and colour characteristic of the ghee is depicted in Figure 4.21.

Table 4.21(a): Sensory analysis of fresh ghee samples

Antioxidant used	**Score obtained for sensory attribute of ghee (out of 9)**			
	Flavour	**Texture**	**Colour and appearance**	**Overall acceptability**
Control	9.00	8.80	8.85	8.85
Betel leaves	8.28	8.63	8.77	8.51
Betel leaves + Liquorice	8.00	8.70	8.68	8.43
BHA	9.00	8.58	8.72	8.79
Source of variation				
SEm	0.09	0.06	0.04	0.08
CD (0.05)	0.26	NS	NS	0.23
CV%	1.99	1.39	1.00	1.76

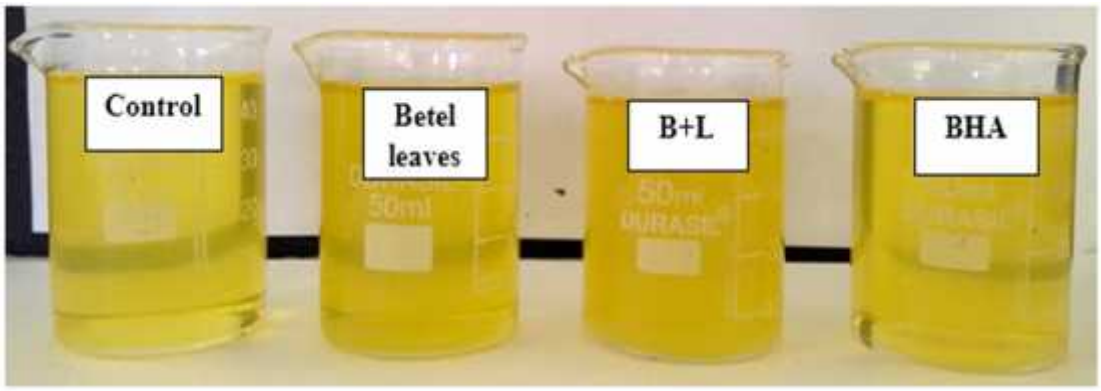


Figure 4.21: Colour of fresh ghee treated with different antioxidants

Where, B+L = Combination of betel leaves and liquorice

4.6.2.1 Flavour

The difference in flavour score of different fresh samples of ghee was statistically significant ($P<0.05$). The results revealed that the treatment of ghee with antioxidants (betel leaves and blend of betel leaves plus liquorice) had significant effect on flavour score of ghee. Flavour score of different fresh samples of ghee was found to vary from 8 to 9. The fresh control sample and BHA added ghee acquired full flavour score (9 out of 9) and considered as like extremely. However, fresh samples of ghee treated with betel leaves or blend of betel leaves with liquorice attained flavour score of 8.28 and 8.00 respectively, which was significantly lower than that of control ghee as well as ghee treated with BHA. Even than flavour score of betel leaves ghee and blend of betel leaves plus liquorice ghee considered as like very much. The lower flavour score of ghee treated with betel leaves or blend of betel leaves with liquorice might be attributed to characteristic aroma of the herbs transmitted in respective ghee sample during treatment.

Patel and Rajorhia (1979) reported initial flavour score of control sample and BHA+BHT (0.02%) added ghee sample was 8.33 and 8.30 respectively. Whereas, initial flavour score of 0.2, 0.5 and 1.0 per cent betel leaves added samples was 8.5, 8.2 and 8.4 respectively. The results obtained in present study differed from the observations reported by Patel and Rajorhia (1979). According to Rangappa and Achaya (1974) ghee flavour is often described as pleasant, nutty, lightly cooked or caramelized aroma. It is best described as lack of oiliness or of blandness, sweetly rather sharply acid. The flavour of ghee is influenced by many factors such as the quality of raw materials, method of preparation, ripening of cream, temperature of clarification. Therefore, the differences noticed between the results obtained in present study and those reported by Patel and Rajorhia (1979) might be attributed to differences in variety of herbs used, form of herb used, rate of addition, stage of addition in preparation of ghee etc.

4.6.2.2 Texture

Texture score of different samples of fresh ghee including control and those treated with BHA, betel leaves and blend of betel leaves with liquorice varied from 8.58 to 8.80. The texture score of all the samples of ghee was statistically at par, there was no significant difference in texture score of different samples of ghee. Texture of ghee

should be firm and non-greasy and grain size should be uniform and large. Thus texture of ghee depends on fatty acid of milk, feed and season. All the ghee samples exhibited granular texture with fine to coarse grains. So it was clear that addition of herbs would not create any problem in texture of ghee.

4.6.2.3 Colour and appearance

Colour and appearance score of different samples of fresh ghee including control and those treated with BHA, betel leaves and blend of betel leaves with liquorice varied from 8.68 to 8.85. The colour and appearance score of all the samples of ghee was statistically at par, there was no significant difference in colour and appearance score of different samples of ghee. The colour of ghee is typical golden yellow. The yellow colour of ghee is attributed to its β-carotene content, which in turn originates from grass fed to the animals. The β-carotene content of milk depends on feed and season. All the ghee samples exhibited typical golden yellow. So it was clear that addition of herbs would not create any problem in colour and appearance of ghee.

4.6.2.4 Overall acceptability

The difference in overall acceptability score of different fresh ghee samples was statistically significant ($P< 0.05$). The results revealed that the treatment of ghee with antioxidants (betel leaves and blend of betel leaves plus liquorice) had significant effect on flavour score of ghee. Overall acceptability of different fresh ghee samples was found to vary from 8.43 to 8.85. However, fresh samples of ghee treated with betel leaves or blend of betel leaves with liquorice attained flavour score of 8.51 and 8.43 respectively, which was significantly lower than that of control ghee as well as ghee treated with BHA. Even than overall acceptability score of ghee betel leaves and blend of betel leaves plus liquorice considered as like very much. The lower overall acceptability score of ghee treated with betel leaves or blend of betel leaves with liquorice might be attributed to characteristic aroma of the herbs transmitted in respective ghee sample during treatment. Thus, results indicated that addition of betel leaves at the rate of 0.3 or blend of betel leaves with liquorice comprising 0.3 per cent of each herb in treatment of ghee did not affect its overall acceptability in sensory evaluation.

4.6.3 Effect of Antioxidants on Oxidative Changes in Ghee on Storage at 80°C

For evaluating the effect of on oxidative changes in ghee during storage at 80°±2°C all the samples of ghee were analysed for peroxide value, carbonyl value, radical scavenging activity and flavour score at regular interval of 2 days till flavour score of almost all the samples of ghee went below the acceptable level (i.e. < 6). The colour characteristic of ghee samples was also recorded.

4.6.3.1 Effect of antioxidants on peroxide value of ghee during storage at 80°C

The results obtained for changes in peroxide value of ghee during storage at 80°±2°C are presented in Table 4.22 and the trend is presented in Figure 4.22.

Table 4.22: Changes in peroxide value of ghee during storage at 80°C after treating with different antioxidants

Storage period (days)	Peroxide value of ghee (meq of O_2 per kg fat)			
	Control	Antioxidant used in ghee		
		Betel leaves	Betel leaves + Liquorice	BHA
0	0.29	0.19	0.10	0.10
2	0.82	0.53	0.34	0.29
4	1.52	0.74	0.60	0.52
6	3.43	1.17	0.80	0.91
8	5.15	1.46	0.95	1.16
10	7.20	1.71	1.20	1.66
12	9.00	2.03	1.48	2.93
14	11.22	2.49	1.75	4.92
16	15.81	2.84	2.18	6.30
18	18.98	3.10	2.46	9.53
20	27.26	3.44	2.70	10.79
22	37.76	3.83	3.24	12.64
24	44.17	4.22	3.73	14.51
26	36.34	4.73	4.49	15.78
28	31.62	5.43	4.82	16.74
30	37.75	5.96	5.40	17.90
32	43.36	6.38	5.78	19.36
34	46.41	6.82	6.51	21.64
Source of variation		**Storage period (P) (days)**	**Treatment (T) (antioxidant)**	**Interaction (P×T)**
SEm		0.32	0.15	0.64
CD (0.05)		0.90	0.42	1.80
CV%		14.46		

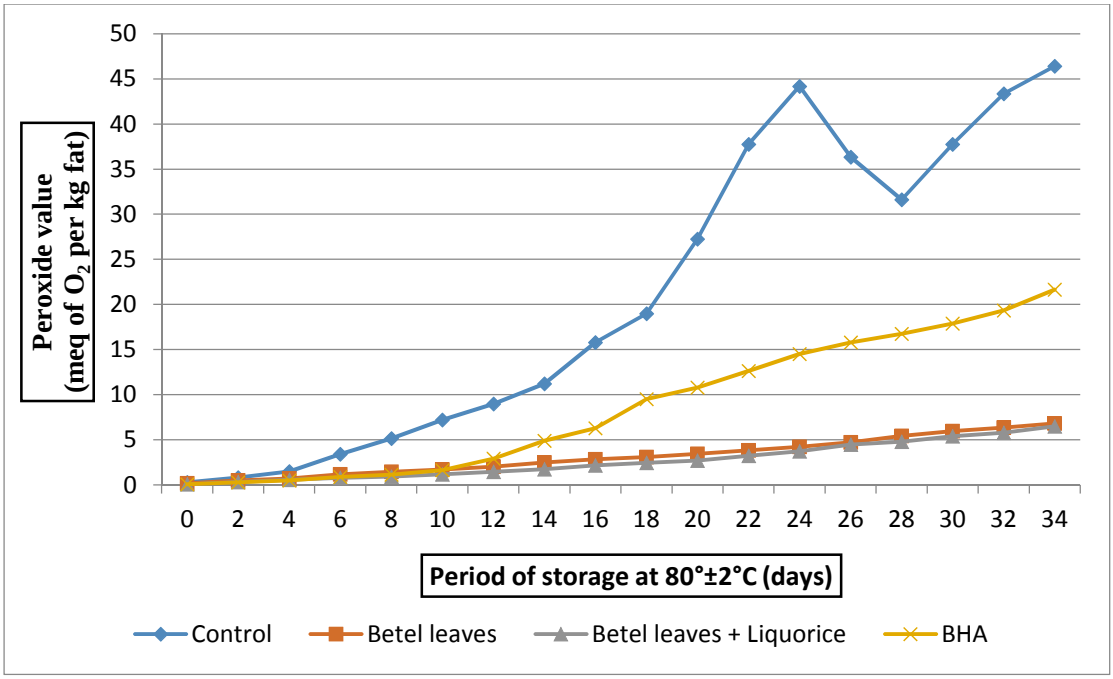


Figure 4.22: Changes in peroxide value of ghee during storage at 80°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in peroxide value of ghee during storage at 80°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other.

The peroxide value of different fresh ghee sample was in the order of control > betel leaves > blend of betel leaves plus liquorice = BHA. However, after the storage at 80°±2°C for 34 days the order of peroxide value of ghee was control > BHA > betel leaves > betel leaves and liquorice.

In control sample of peroxide value rised at a steady rate in the beginning, showed steeped rise in peroxide value on 16th day of the storage, reached to the maximum level (44.17 meq of O_2 per kg fat) on 24th day of the storage, then started falling down for a brief period of 4 days, again regain the momentum of increase in peroxide value on further storage and it reached to maximum level of a peroxide value of 46.41 meq of O_2 per kg fat on 34th day of the storage.

In sample of ghee treated with BHA peroxide value rise very gradually up to 10th day of the storage, on further storage its rate of rise in peroxide value was accelerated, much steeped rise in peroxide value was noticed from 14th day of the storage and peroxide value reached to 21.64 meq of O_2 per kg fat on 34th day of the storage. At the end of the storage, BHA treated ghee sample had significantly higher ($P<0.05$) peroxide value than ghee samples added with betel leaves alone and blend of betel leaves plus liquorice.

In samples of ghee treated with betel leaves alone and combination of betel leaves and liquorice peroxide value rise very gradually all throughout the storage. There was no sign of rapid rise of peroxide value was observed in these ghee samples at any stage of the entire period of storage. On 34th day of the storage peroxide value of ghee treated with betel leaves alone and combination of betel leaves and liquorice was 6.82 and 6.51 meq of O_2 per kg fat respectively. These peroxide values of ghee were statistically at par, the difference between these values of peroxide was statistically non-significant. The data indicated that there was only marginal increase in effectiveness to control peroxide formation in ghee when betel leaves were blended with liquorice.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to control formation of peroxide in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone. Even very popular and permitted synthetic oxidant BHA started losing its ability at a faster rate to control formation of peroxide formation beyond 12 days of the storage.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of BHA, betel leaves and blend of betel leaves with liquorice in controlling formation of peroxides in ghee during storage at 80°±2°C.

Therefore, results obtained in the present study could not be compared as such with the reports in the literature.

Amr (1990) studied the effect of addition of four aromatic herbs on oxidative stability of ghee made from Ewe's milk. Aromatic herbs namely, rosemary (*Rosmarinus officinalis*), sage (*Artemisia herballa*), fennel (*Foeniculum vulgare*) and rue (*Ruta graveatons*) were added at 7.5 % level to the ghee, only rosemary showed an antioxidative effect equivalent to that of BHA+ BHT. All these herbs had an antioxidant effect at least for first 24 hrs of storage under accelerated conditions. Patel *et al.* (2014) evaluated antioxidant potential of ashwagandha, clove, coriander, green tea, shatavari and vidarikand extracts as compared to BHA. Clove, coriander and green tea extracts showed significantly higher antioxidant activity than vidarikand, shatavari and ashwagandha extracts. Merai *et al.* (2003) reported that addition of 0.6% of silica gel charcoal treated fraction of Tulsi leaves powder to ghee was more effective than the BHA at 0.02% until the peroxide value of 5 meq of peroxide oxygen was reached. Pawar *et al.* (2012) compared the potential of *Asparagus racemosus* (shatavari) extract with natural (rosemary and green tea) and synthetic (butylated hydroxyanisole and tert-butyl hydroquinone) antioxidants, in ghee using accelerated oxidation tests. The aqueous and ethanolic extracts of shatavari but were found to be less effective than the natural and synthetic antioxidants. Ghee incorporated with steam distilled coriander extract showed a significant rise in peroxide value as compared with its oleoresin counterpart. This indicated that ghee containing oleoresin was more effective in retarding peroxides development than ghee containing steam distilled extract. The peroxide value of ghee containing coriander extracts was significantly higher than BHA throughout 21 days of storage at 80 ± 1 °C. During deep fat frying, steam distilled extracts showed higher antioxidant activity compared to oleoresin and BHA (Patel *et al.*, 2013).

4.6.3.2 Effect of antioxidants on carbonyl value of ghee during storage at 80°C

The methods reported to monitor oxidative deterioration of various fats and oils are based on chemical changes taking place in the primary and secondary stages of oxidative deterioration. The first compounds formed during the primary stage of the oxidation process are peroxides, especially hydroperoxides, which in turn can generate secondary oxidation products, including aldehydes, ketones, hydroxyl compounds, epoxides and polymers (Mehta *et al.*, 2015).

The carbonyl compounds, including aldehydes and ketones, are the secondary oxidation products generated from degradation of hydroperoxides and are suggested to be the major contributors to off-flavours associated with the rancidity of many food products. The intensity of undesirable sensory notes has been positively correlated with the content of carbonyl compounds formed through lipid autoxidation reactions. The carbonyl compounds present have the greatest impact on flavour owing to their low flavour thresholds (Shahidi and Zhong, 2005). The spectrophotometric method is based on the absorbance of the quinoidal ion, a derivative of aldehydes and ketones. This ion is formed from the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl moiety of an aldehyde or ketone, followed by the reaction of the resulting hydrazone with alkali (Ronald, 2001).

The results obtained for changes in carbonyl value of ghee during storage at 80°±2°C are presented in Table 4.23 and the trend is presented in Figure 4.23.

Table 4.23: Changes in carbonyl value of ghee during storage at 80°C after treating with different antioxidants

Storage period (days)	Carbonyl value of ghee (µmol carbonyl per g)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	3.25	3.37	3.16	3.36
2	3.92	3.68	3.52	3.64
4	4.42	3.89	3.87	3.98
6	5.74	4.16	4.18	4.34
8	7.81	4.56	4.52	4.88
10	8.20	5.04	5.00	5.36
12	10.05	5.67	5.67	5.95
14	10.84	6.14	6.04	6.59
16	12.32	6.96	6.56	7.58
18	14.74	7.51	7.03	8.50
20	16.43	7.95	7.57	9.50
22	18.72	8.64	8.22	10.31
24	21.93	9.05	8.68	11.25
26	25.92	9.46	9.36	12.51
28	29.66	9.91	9.93	13.37
30	34.23	10.49	10.59	14.94
32	36.00	11.29	11.36	16.61
34	37.63	12.59	12.21	19.04

Source of Variation	Storage period (P) (days)	Treatment (antioxidant)	Interaction (P×T)
SEm	0.11	0.05	0.22
CD (0.05)	0.30	0.14	0.61
CV%	4.35		

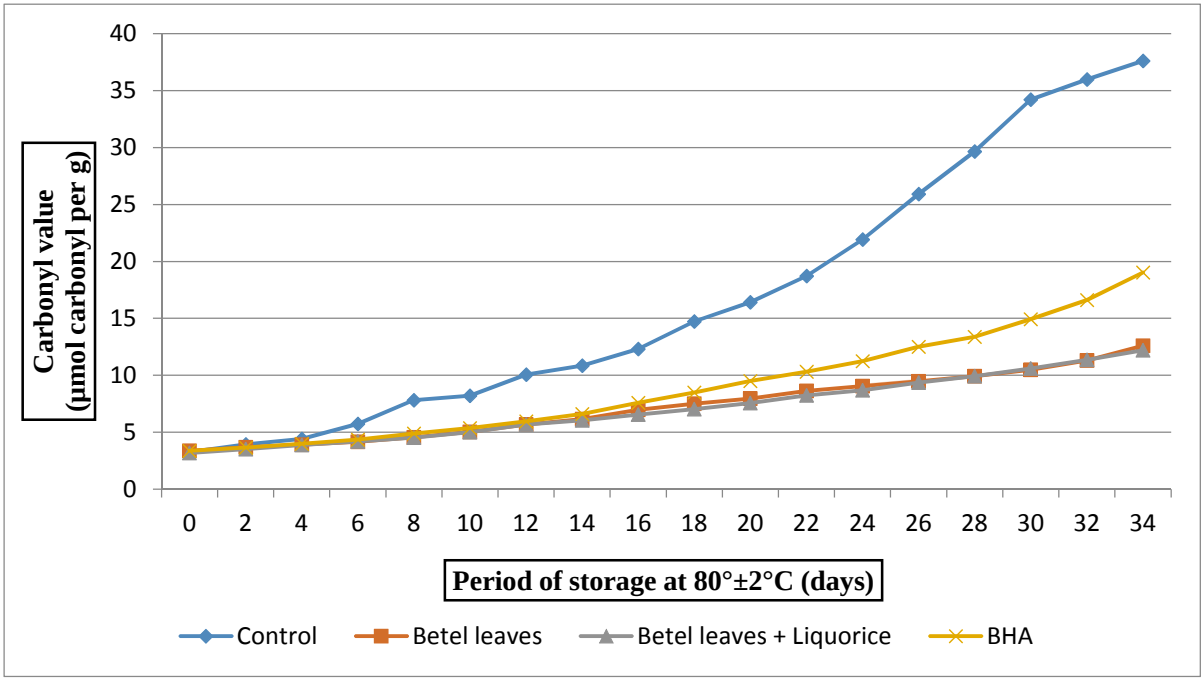


Figure 4.23: Changes in carbonyl value of ghee during storage at 80°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in carbonyl value of ghee during storage at 80°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in carbonyl value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in carbonyl value of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on carbonyl value of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other.

The carbonyl value of different fresh ghee sample was in the order of betel leaves > BHA > control > blend of betel leaves plus liquorice. However, after the storage at 80°±2°C for 34 days the order of carbonyl value of ghee was control > BHA > betel leaves > blend of betel leaves plus liquorice.

In control sample of carbonyl value rise at very rapid from beginning (4th day), showed steeped rise in carbonyl value on 18th day of the storage and it reached to the maximum level of 37.63 µmol carbonyl per g) on 34th day of the storage.

In sample of ghee treated with BHA carbonyl value rise very gradually up to 14th day of the storage and remained quite near to carbonyl values of samples of ghee treated with betel leaves and blend of betel leaves plus liquorice. However, on further storage rate of rise in carbonyl value of the BHA treated ghee was accelerated, started deviating from carbonyl values of samples of ghee treated with betel leaves and blend of betel leaves plus liquorice. The very popular and permitted synthetic oxidant BHA started losing its ability at a faster rate ability to control formation of carbonyl formation beyond 12 days of the storage. The carbonyl value of the control ghee reached to 19.04 µmol carbonyl per g on 34th day of the storage.

On the other hand in samples of ghee treated with betel leaves alone or combination of betel leaves and liquorice carbonyl value rise very gradually all throughout the storage. There was no sign of rapid or steeped rise of carbonyl value was observed in these ghee samples at any stage of the entire period of storage. On 34th day of the storage carbonyl value of ghee treated with betel leaves alone and combination of betel leaves and liquorice was 12.59 and 12.21 µmol carbonyl per g respectively. The difference in carbonyl values of these samples of ghee was significant ($P<0.05$). Thus, the data indicated that when betel leaves were blended with liquorice there was significant increase in effectiveness to control carbonyl formation in ghee.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to control formation of carbonyl in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone; even better than synthetic antioxidant BHA.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of BHA, betel leaves and blend of betel leaves with liquorice in controlling formation of carbonyl in ghee during storage at 80°±2°C. Therefore, results obtained in the present study could not be compared as such with the reports in the literature.

4.6.3.3 Effect of antioxidants on radical scavenging activity of ghee during storage at 80°C

The results obtained for changes in radical scavenging activity in ghee during storage 80°±2°C are presented in Table 4.24 and the trend is presented in Figure 4.24.

Table 4.24: Changes in radical scavenging activity of ghee during storage at 80°C after treating with different antioxidants

Storage period (days)	Radical scavenging activity of ghee (% inhibition)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	43.36	61.98	67.08	61.35
2	42.06	60.68	66.06	59.14
4	40.99	57.92	64.37	57.11
6	38.44	56.06	61.91	54.35
8	34.85	54.27	59.36	52.46
10	32.87	52.63	57.25	49.71
12	29.53	49.19	54.10	47.12
14	26.10	47.55	51.69	44.24
16	23.92	44.08	49.29	41.86
18	21.52	41.38	46.25	37.75
20	18.11	39.01	43.88	35.54
22	14.44	35.19	40.64	31.75
24	12.21	31.49	37.65	26.97
26	9.93	28.52	34.41	23.20
28	7.69	25.89	29.30	20.01
30	5.94	23.84	26.14	16.71
32	4.60	21.77	23.84	12.10
34	3.33	19.53	21.24	8.61

Source of variation	Storage period (P) (days)	Treatment (T) (antioxidants)	Interaction (P×T)
SEm	0.58	0.27	1.17
CD (0.05)	1.63	0.77	3.25
CV%	6.28		

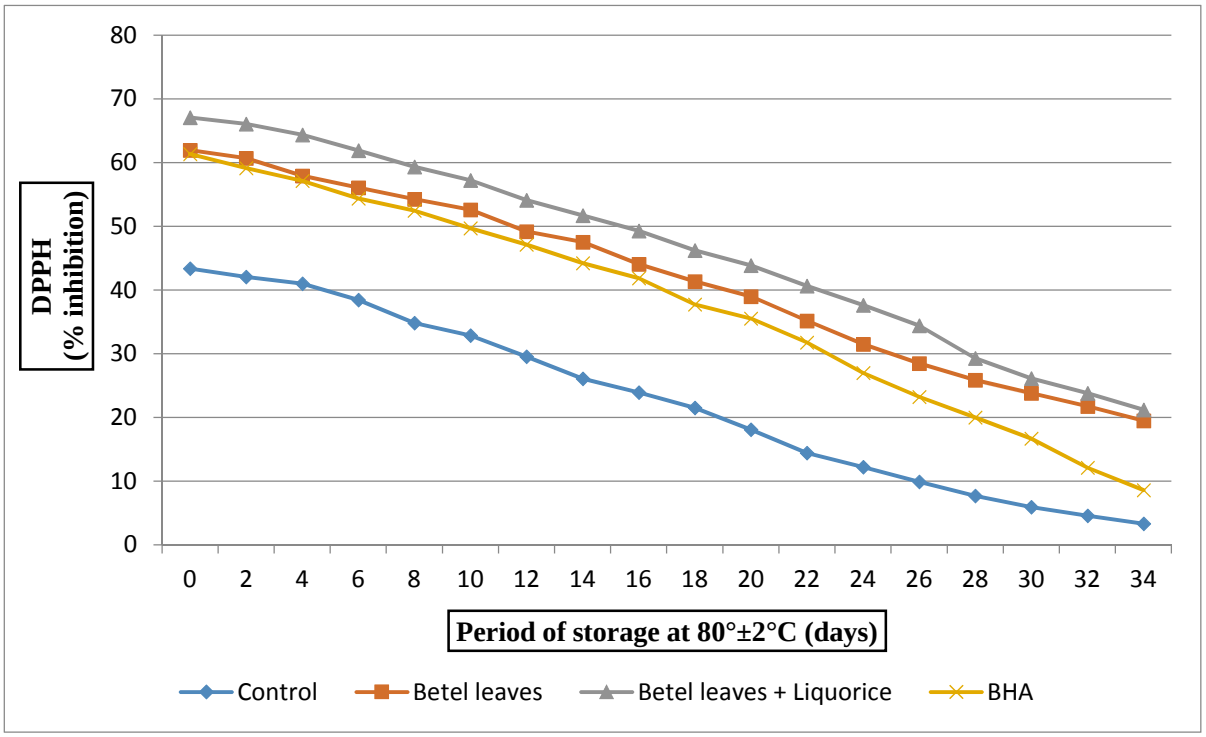


Figure 4.24: Changes in radical scavenging activity of ghee during storage at 80°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in radical scavenging activity of ghee during storage at 80°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in radical scavenging activity of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in radical scavenging activity of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on radical scavenging activity of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other for changes in radical scavenging activity of ghee over a period of storage.

The radical scavenging activity of different fresh ghee samples was in the order of blend of betel leaves plus liquorice > betel leaves > BHA > control. Moreover, after the storage at 80°±2°C for 34 days the order of radical scavenging activity of ghee remained same as that of fresh samples of ghee.

The data revealed that there was significant ($P < 0.05$) increase in radical scavenging activity in ghee when antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) were used in ghee, Among the different fresh samples of ghee, ghee treated with blend of betel leaves plus liquorice had the highest radical scavenging activity. It was significantly ($P < 0.05$) higher than that of the betel leaves treated ghee as well as BHA treated ghee. On the other hand radical scavenging activities of fresh samples of betel leaves treated ghee as well as BHA treated ghee were statistically at par, there was no significant ($P < 0.05$) difference between them.

On the storage radical scavenging activity of control ghee decreased at almost constant rate and reached to a level of 3.33 per cent on 34^{th} day of the storage. In sample of ghee treated with BHA radical scavenging activity decreased very gradually up to 16^{th} day of the storage and remained quite near to radical scavenging activities of betel leaves treated ghee sample. However, on further storage rate of decline in radical scavenging activity of the BHA added sample of ghee was accelerated, started deviating from radical scavenging activities of samples of ghee treated with betel leaves as well as blend of betel leaves plus liquorice and reached to a level of 8.61 per cent om 34^{th} day of the storage. Thus, well known permitted synthetic oxidant BHA started losing its activity of radical scavenging earlier than betel leaves and blend of betel leaves with liquorice.

On the other hand in samples of ghee treated with betel leaves alone or combination of betel leaves and liquorice radical scavenging activity decreased very gradually all throughout the storage. There was no sign of rapid or steeped decline in the radical scavenging activity was observed in these ghee samples at any stage of the entire period of storage. The trend of changes in radical scavenging activity of betel leaves alone or combination of betel leaves and liquorice remained parallel throughout the storage. On 34^{th} day of the storage radical scavenging activity of ghee treated with betel leaves alone and combination of betel leaves and liquorice was 19.53 and 21.24 per cent carbonyl per g respectively. The difference in radical scavenging activities of these samples of

ghee was significant ($P<0.05$). Thus, the data indicated that when betel leaves were blended with liquorice there was significant increase in effectiveness to scavenge radical in ghee.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to scavenge radical in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone. The radical scavenging activity of synthetic antioxidant BHA was far behind than that of the betel leaves alone or blend of betel leaves plus liquorice.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of BHA, betel leaves and blend of betel leaves with liquorice in radical scavenging activities in ghee during storage at 80°±2°C. Therefore, results obtained in the present study could not be compared as such with the reports in the literature.

Ghee incorporated with ethanolic extracts of herbs such as vidarikand, shatavari and ashwagandha showed stronger activity in quenching DPPH radicals in system both before and after oxidation as compared to their aqueous extracts and control ghee (Pawar *et al.,* 2014). In a study conducted by Purohit (2011) for evaluating the effect of herb extract *Withania somnifera* (Ashwagandha) incorporation on storage stability of ghee, the samples incorporated with ethanolic extract of ashwagandha was found to be more effective in quenching DPPH radicals than control ghee. Arjuna bark (alcoholic extract) had significant ($P<0.05$) ability to enhance the antioxidant potential of ghee in terms of its DPPH radical scavenging activity (Parmar *et al.,* 2013).

4.6.3.4 Effect of antioxidants on flavour score of ghee during storage at 80°C

The results obtained for changes in flavour score of ghee during storage 80°±2°C are presented in Table 4.25 and the trend is presented in Figure 4.25(a).

Table 4.25: Changes in flavour score of ghee during storage at 80°C after treating with different antioxidants

Storage period (days)	Flavour score of ghee (out of 9)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	9.00	8.28	8.00	9.00
2	8.60	8.38	8.38	9.00
4	8.13	8.28	8.18	9.00
6	6.98	8.10	8.18	8.50
8	**5.45**	7.90	8.13	8.15
10	4.85	7.75	8.05	7.55
12	4.38	7.63	8.05	6.73
14	3.35	7.43	8.03	**5.25**
16	2.90	7.33	7.88	4.13
18	1.88	7.18	7.83	2.93
20	1.43	7.03	7.60	2.43
22	1.25	6.83	7.48	1.88
24	1.05	6.75	7.33	1.65
26	1.00	6.33	7.10	1.33
28	1.00	6.10	6.75	1.25
30	1.00	6.00	6.43	1.25
32	1.00	**5.43**	6.18	1.13
34	1.00	5.00	**5.68**	1.00

Source of Variation	Storage period (P) (days)	Treatment (T) (antioxidants)	Interaction (P×T)
SEm	0.10	0.05	0.21
CD (0.05)	0.29	0.13	0.57
CV%		7.21	

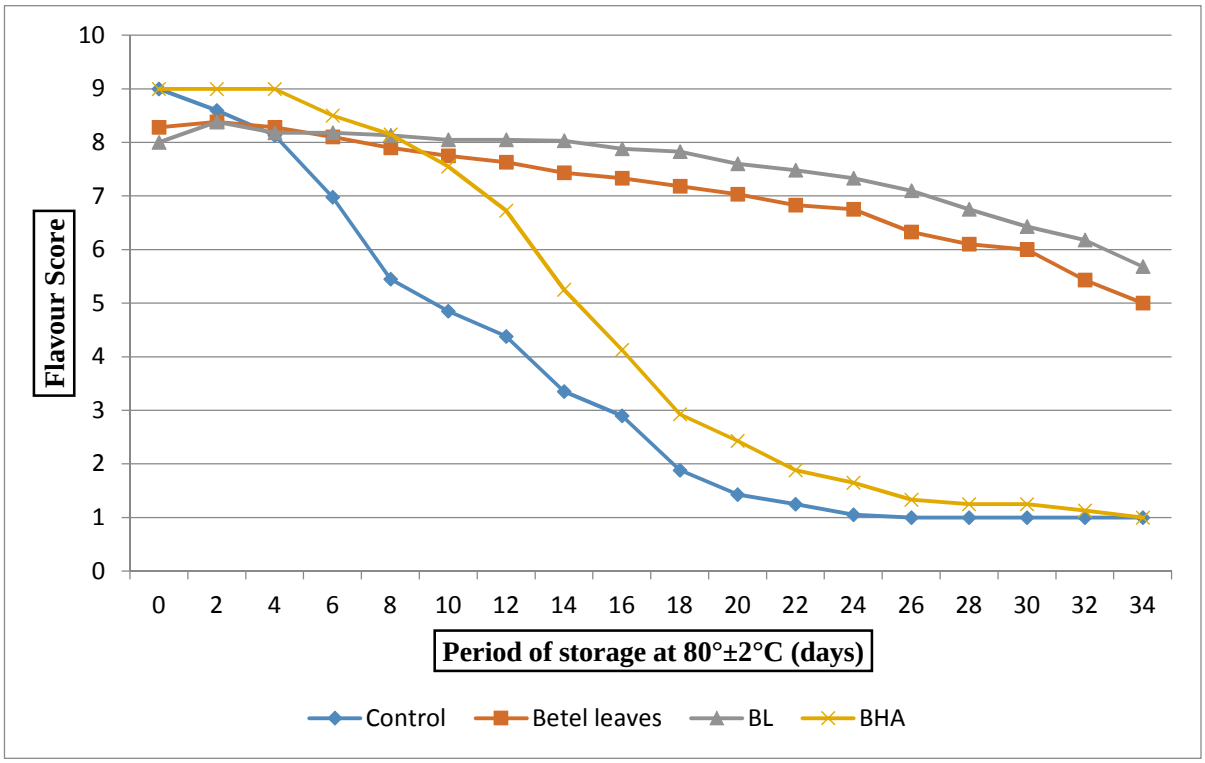


Figure 4.25(a): Changes in flavour score of ghee during storage at 80°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in flavour score of ghee during storage at $80^{o}\pm2°C$ that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between storage period as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, storage period also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other for changes in flavour score of ghee over a storage period.

As stated earlier (Section 4.6.2.1) fresh samples of control ghee and the ghee added with BHA received full flavour score (9 out of 9). Whereas, flavour scores of samples of ghee treated with betel leaves and that treated with blend of betel leaves plus liquorice were significantly ($P < 0.05$) lower compare to the flavour score of control ghee and ghee added with BHA. The flavour score of different fresh ghee sample was in the order of control = BHA > betel leaves > blend of betel leaves plus liquorice. Thus, data revealed that there was significant ($P < 0.05$) decrease in flavour score of fresh ghee when treated with betel leaves or blend of betel leaves with liquorice when compared with the flavour score of control ghee.

On the storage flavour score of control ghee sample decreased at rapid rate from the beginning, the rate of decrease became very sharp from 4^{th} day of the storage and the score reached to a minimum level (1 out of 9) on 34^{th} day of the storage. In sample of ghee treated with BHA flavour score remain constant up to first 4 days of the storage and remained quite above the flavour score of samples of ghee treated with betel leaves and blend of betel leaves plus liquorice. However, on further storage flavour score of the BHA added ghee sample started declining at rapid rate and sharp decline in flavour score was noticed from 10^{th} day of the storage. Its flavour reached score to a minimum level (1 out of 9) on 34^{th} day of the storage. Thus, well known permitted synthetic oxidant BHA started losing its flavour score quite earlier than betel leaves and blend of

betel leaves with liquorice. Its flavour score reached to a minimum level (1 out of 9) on 34^{th} day of the storage.

On the other hand in ghee samples treated with betel leaves alone or combination of betel leaves and liquorice flavour score decreased very gradually all throughout the storage. There was no sign of rapid or steeped decline in the flavour score was observed in these ghee samples at any stage of the entire period of storage. The trend of changes in flavour score of betel leaves alone or combination of betel leaves and liquorice remained almost parallel all throughout the storage. On 34^{th} day of the storage flavour scores of ghee treated with betel leaves alone and combination of betel leaves plush liquorice were 5.0 and 5.68 respectively. The difference in flavour score of these samples of ghee was significant ($P<0.05$). Thus, the data indicated that when betel leaves were blended with liquorice there was significant increase in effectiveness to flavour score in ghee.

After the storage at 80°±2°C for 34 days the order of flavour score of ghee was just inversed than that of the fresh samples of ghee. Thus, the order of flavour score of ghee different samples of ghee at end of the storage was blend of betel leaves plus liquorice > betel leaves > BHA = control. The flavour score of control ghee went below the acceptable level (< 6) on 8^{th} day of the storage. On the hand flavour score of ghee treated with BHA, betel leaves and blend of betel leaves plush liquorice went below the acceptable level on 14^{th}, 32^{nd} and 34^{th} day respectively.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to flavour score in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone. The flavour score of synthetic antioxidant BHA was far behind than that of the betel leaves alone or blend of betel leaves plus liquorice.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of BHA, betel leaves and blend of betel leaves with liquorice on changes in sensory attributes of ghee during storage at 80°±2°C. Therefore, results obtained in the present study could not be compared as such with the reports in the literature.

4.6.3.5 Effect of antioxidants on colour of ghee on 26th day of storage at 80°C

The colour characteristic of different samples of ghee on 26 day of storage at 80°±2°C are presented in Figure 4.25(b).

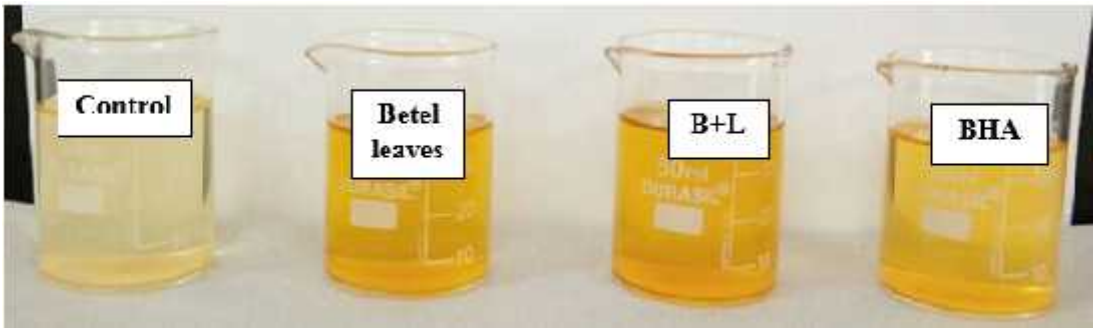


Figure 4.25(b): Colour of ghee samples on 26th day of storage at 80°C

Where, B+L = Combination of betel leaves and liquorice

Ghee samples showed marked variation in their colour after 24 days because of this reason colour characteristic of stored ghee samples compared with the fresh ghee samples on 26th day of storage. Among different samples of ghee, on storage the sample of ghee treated with betel leaves, blend of betel leaves plush liquorice treated were able to retain the inherent yellow colour with slight change in their shade and intensity.

During the storage at 80°±2°C on 26 days, shade of yellow colour of these ghee sample moved from golden yellow colour which was present in fresh ghee samples (Figure 4.21). In the stored ghee samples slight brownish tinge was also observed. From amongst samples of ghee, the sample of ghee treated with betel leaves, blend of betel leaves plush liquorice or BHA treated; intensity of yellow colour was relatively lower in BHA treated ghee compared to remaining two ghee samples (betel leaves and blend of betel leaves plus liquorice treated). On the other yellow colour of the control ghee sample decreased drastically and turned to very light yellow.

The shift in tinge of the ghee from golden yellow to slight brownish yellow might be attributed to formation of caramelization and maillard reactions products. At the same time fading of the golden yellow colour in control ghee sample might be attributed to bleaching of β-carotene, which imparted yellow colour to the ghee. These aspects of changes in colour of ghee on storage are discussed in Section 4.2.5.

4.6.4 Effect of Antioxidants on Oxidative Changes in Ghee on Storage at 35°C

For evaluating the effect of on oxidative changes in ghee during storage at 35°±2°C all the samples of ghee were analysed for peroxide value, carbonyl value, radical scavenging activity (DPPH assay) and flavour score at regular interval of 30 days till flavour score of almost all the samples of ghee went below/reached near the acceptable level (*i.e.* 180 days). The colour characteristic of ghee samples was also recorded at the end of the storage.

4.6.4.1 Effect of antioxidants on peroxide value of ghee during storage at 35°C

The results obtained for changes in peroxide value of ghee during storage 35°±2°C are presented in Table 4.26 and the trend is presented in Figure 4.26.

Table 4.26: Changes in peroxide value of ghee during storage at 35°C after treating with different antioxidants

Storage period (days)	Peroxide value of ghee (meq of O_2 per kg fat)			
	Control	Antioxidant used in ghee		
		Betel leaves	Betel leaves + Liquorice	BHA
0	0.29	0.19	0.10	0.10
30	0.49	0.28	0.21	0.20
60	1.05	0.41	0.43	0.47
90	1.68	0.66	0.64	1.11
120	3.17	1.26	1.06	2.27
150	5.80	1.83	1.70	3.59
180	8.51	2.40	2.35	5.17
Source of variation		Storage period (P) (days)	Treatment (T) (antioxidant)	Interaction (P×T)
SEm		0.07	0.05	0.14
CD (0.05)		0.20	0.15	0.40
CV%		16.81		

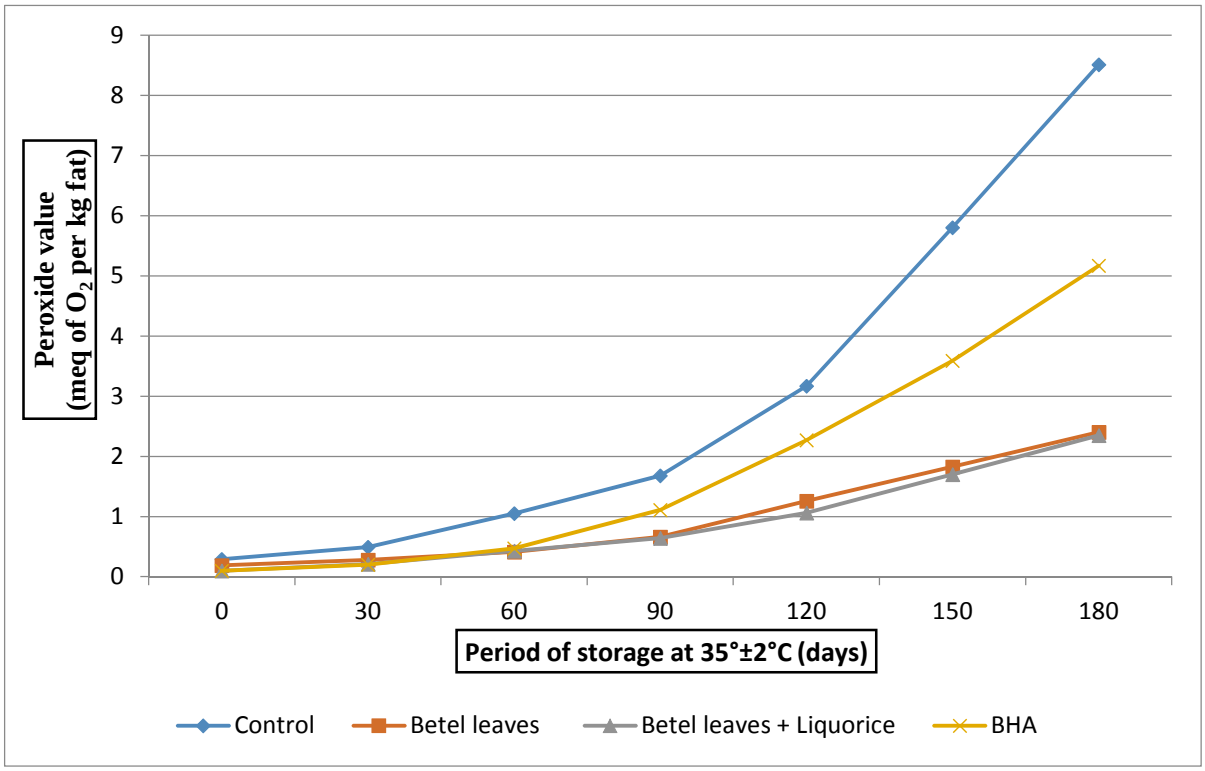


Figure 4.26: Changes in peroxide value of ghee during storage at 35°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in peroxide value of ghee during storage at 35°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in peroxide value of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on peroxide value of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other.

The peroxide value of different fresh ghee samples was in the order of control > betel leaves > blend of betel leaves plus liquorice = BHA. However, after the storage at

35°±2°C for 180 days the order of peroxide value of ghee was control > BHA > betel leaves > betel leaves and liquorice.

Control ghee sample was statistically at par with only the betel leaves treated fresh ghee, statistically higher than ghee samples treated with blend of betel leaves plus liquorice and BHA. On subsequent storage it was significantly ($P<0.05$) higher than corresponding ghee samples. There was some increase in peroxide value observed on 30^{th} day of the storage. However rapid rise in its in peroxide value was noticed after 60^{th} day of the storage and steeped rise in peroxide value became evident on 120^{th} day of the storage. The peroxide value reached to a level of 8.51 meq of O_2 per kg fat on 180^{th} day of the storage.

The sample of ghee containing BHA followed almost similar trend of changes in peroxide value during storage, albeit at somewhat lower rate. There was some increase in peroxide value of this sample of ghee on 30^{th} day of the storage, but significantly ($P<0.05$) lower than the corresponding ghee samples. The rapid rise in its in peroxide value was noticed after 30^{th} day of the storage and steeped rise in peroxide value became evident after 90^{th} day of the storage. The peroxide value reached to a level of 5.17 meq of O_2 per kg fat on 180^{th} day of the storage.

In samples of ghee treated with betel leaves alone and combination of betel leaves and liquorice peroxide value rise very gradually up to 90^{th} day of the storage. There was some increase in rate of peroxide formation in these sample after 90^{th} day of the storage, but this rate was far behind the rates observed in control ghee and ghee containing BHA. The sign of steep rise in peroxide value was not noticed in these ghee samples at any stage of entire period of the storage. On 180^{th} day of the storage peroxide value of ghee treated with betel leaves alone and combination of betel leaves plus liquorice was 2.40 and 2.35 meq of O_2 per kg fat respectively. These peroxide values of ghee were statistically at par, the difference between these values of peroxide was statistically non-significant ($P<0.05$). Even during almost entire storage (except on 120^{th} day of storage) peroxide values of betel leaves alone and combination of betel leaves treated ghee samples were statistically at par. The data indicated that there was only marginal increase in effectiveness to control peroxide formation in ghee when betel leaves were blended with liquorice.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice during the storage at 35°±2°C that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone. Even very popular and permitted synthetic oxidant BHA started losing its ability at a faster rate to control formation of peroxide formation beyond 150 days of the storage.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves when added to melted butter during clarification. Fresh betel leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of betel leaves 0.2, 0.5 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. A mixture of BHA and BHT (1:1) at concentration of 0.02 per cent by weight was also added to a separate lot of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C. The sample were examined for Peroxide value. (meq of 0.002N $Na_2S_2O_3$/g fat). The authors found that peroxide values of ghee samples treated with betel leaves and antioxidants changed very little up to 30 days of storage. The control (without antioxidants) showed a steep rise in peroxide with value after 60 days of storage. Ghee samples added 1.0 per cent betel leaves showed least increase in peroxide value up to 135 days of storage. However, ghee samples treated with betel leaves 1 per cent concentration proved to be most acceptable and stable even up to 147 days of storage at 30°C. The antioxidative effect of various treatments was in order of > 0.2 per cent betel leaves > 0.5 per cent betel leaves > 0.02 per cent BHA+BHT > 1 per cent 1 per cent betel leaves. Thus results obtained in present study were in general agreement with those reported by these authors. Some variations in results between the reported study and the present study might be attributed to differences in variety of the betel leaves used, in which form herb added, stage in preparation of ghee at which betel leaves added, temperature at which ghee was stored, etc.

4.6.4.2 Effect of antioxidants on carbonyl value of ghee during storage at 35°C

The results obtained for changes in carbonyl value of ghee during storage 35°±2°C are presented in Table 4.27 and the trend is presented in Figure 4.27.

Table 4.27: Changes in carbonyl value of ghee during storage at 35°C after treating with different antioxidants

Storage period (days)	Carbonyl value of ghee (µmol carbonyl per g)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	3.25	3.37	3.16	3.36
30	3.60	3.61	3.37	3.53
60	4.42	4.13	3.91	3.96
90	5.50	4.73	4.43	4.65
120	6.76	5.47	5.06	5.67
150	9.27	6.44	5.91	7.07
180	13.89	7.65	6.83	8.94

Source of Variation	Storage period (P) (days)	Treatment (antioxidant)	Interaction (P×T)
SEm	0.09	0.07	0.19
CD (0.05)	0.27	0.20	0.53
CV%	6.97		

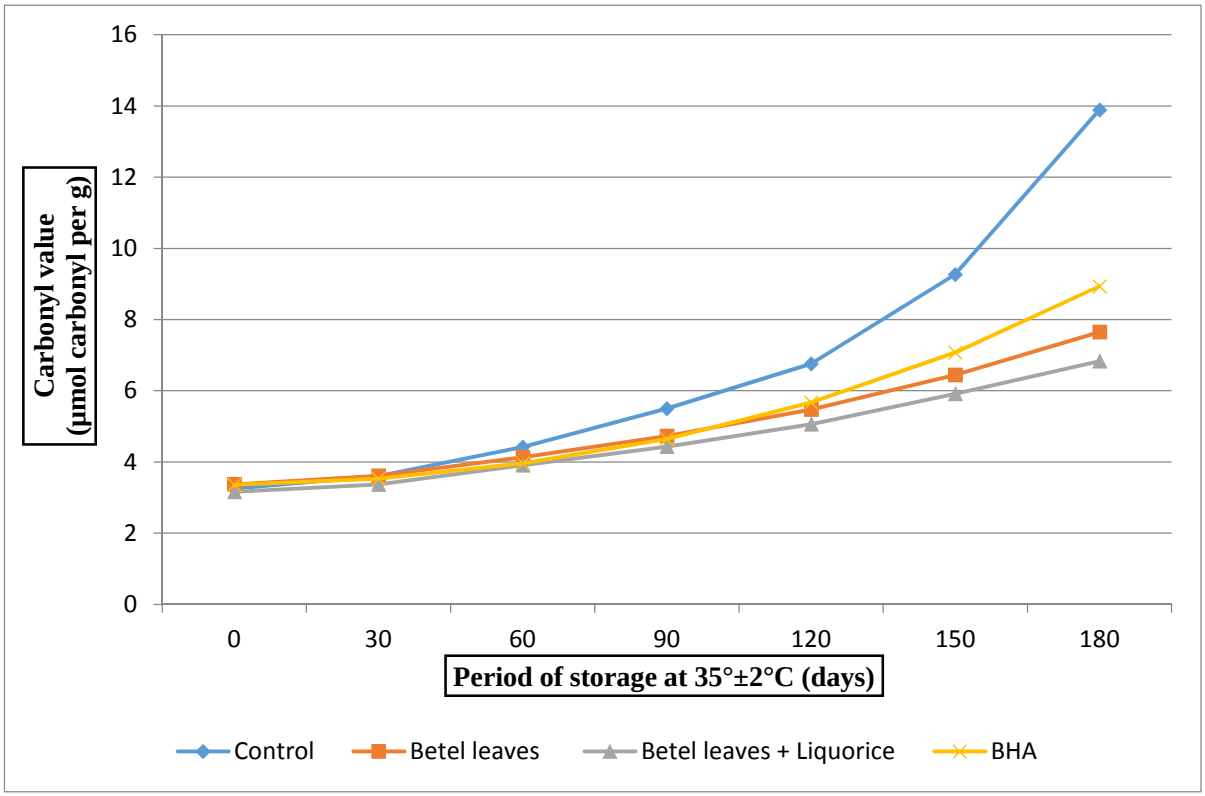


Figure 4.27: Changes in carbonyl value of ghee during storage at 35°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in carbonyl value of ghee during storage at 35°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in carbonyl value of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in carbonyl value of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on carbonyl value of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other.

The carbonyl value of different fresh ghee samples was in the order of betel leaves > BHA > control > blend of betel leaves plus liquorice. However, after the storage at 35°±2°C for 180 days the order of carbonyl value of ghee was control > BHA > betel leaves > blend of betel leaves and liquorice.

In control ghee sample there was increase in carbonyl value on 30th day of the storage and statistically it was significantly ($P < 0.05$) higher than ghee sample treated with blend of betel leaves and liquorice. The rise in carbonyl value of this sample was gradual up to 60th day of the storage, however rapid rise in its in carbonyl value was noticed after 60th day of the storage and steeped rise in carbonyl value became evident on 150th day of the storage. The carbonyl value reached to a level of 13.89 µmol carbonyl per g on 180th day of the storage.

The ghee sample containing BHA followed almost similar trend of changes in carbonyl value during the storage, albeit at somewhat lower rate. There was some increase in carbonyl value of ghee sample on 30th day of the storage, but statistically it was at par with the corresponding fresh samples. The rapid rise in its in carbonyl value was noticed after 90th day of the storage and steeped rise in carbonyl value became evident after 120th day of the storage. The carbonyl value reached to a level of 8.94 µmol carbonyl per g on 180th day of the storage.

In samples of ghee treated with betel leaves alone and combination of betel leaves and liquorice carbonyl value rise very gradually up to 90th day of the storage. There was increase in rate of carbonyl formation in these sample after 90th day of the storage. However, sign of steep rise in carbonyl value was not noticed in these ghee samples at any stage of entire period of the storage. On 180th day of the storage carbonyl value of ghee treated with betel leaves alone and combination of betel leaves plus liquorice was 7.65 and 6.83 µmol carbonyl per g respectively. The difference in these carbonyl values of ghee was statistically significant ($P<0.05$). Even during almost entire storage carbonyl values of ghee treated with combination of betel leaves plus liquorice remained significantly lower than that of the betel leaves treated ghee sample. The data indicated that there was significant increase in effectiveness to control carbonyl formation in ghee when betel leaves were blended with liquorice.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice for controlling carbonyl formation in ghee during the storage at 35^{o}±2°C, that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone.

4.6.4.3 Effect of antioxidants on radical scavenging activity in ghee during storage at 35°C

The results obtained for changes in radical scavenging activity of ghee during storage 35°±2°C are presented in Table 4.28 and the trend is presented in Figure 4.28.

Table 4.28: Changes in radical scavenging activity of ghee during storage at 35°C after treating with different antioxidants

Storage period (days)	Radical scavenging activity of ghee (% inhibition)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	43.36	61.98	67.08	61.35
30	42.72	61.47	66.54	60.69
60	41.56	60.61	65.66	59.75
90	39.37	59.58	64.60	58.27
120	34.27	58.06	62.78	56.05
150	28.80	55.36	59.56	50.72
180	24.00	52.03	55.28	44.01

Source of variation	Storage period (P) (days)	Treatment (T) (antioxidants)	Interaction (P×T)
SEm	0.62	0.47	1.24
CD (0.05)	1.74	1.32	3.49
CV%	4.65		

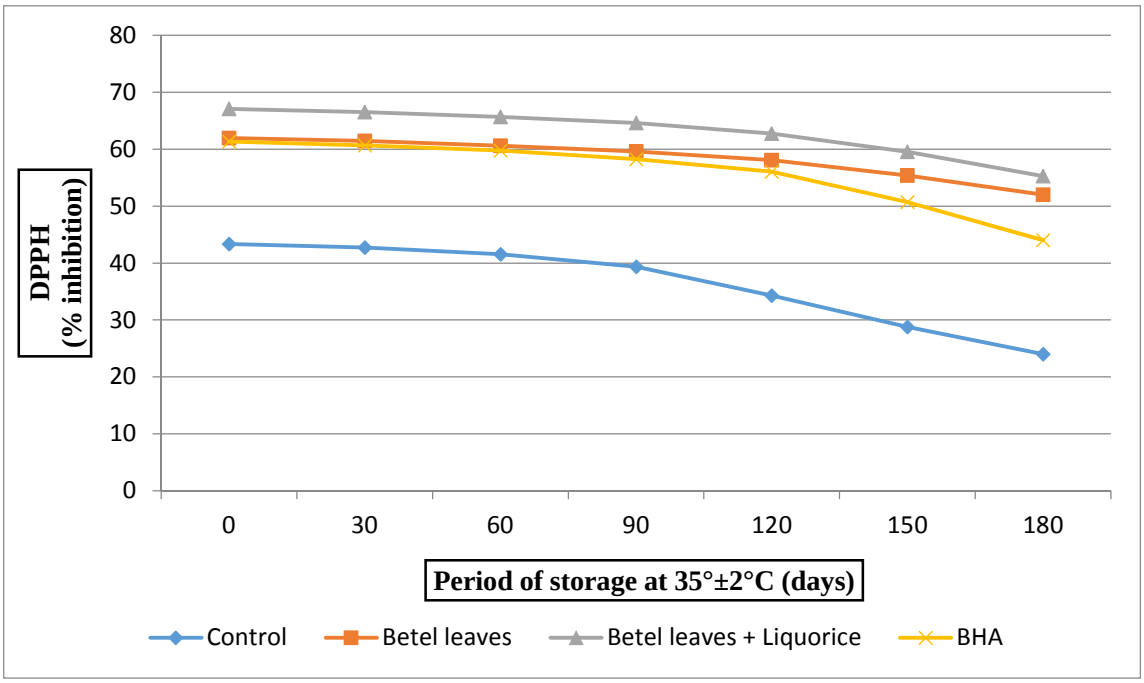


Figure 4.28: Changes in radical scavenging activity of ghee during storage at 35°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in radical scavenging activity of ghee during storage at 35°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in radical scavenging activity of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in radical scavenging activity of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on radical scavenging activity of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other for changes in radical scavenging activity of ghee over a period of storage.

The radical scavenging activity of different fresh ghee samples was in the order of blend of betel leaves plus liquorice > betel leaves > BHA > control. Moreover, after the

storage at 35°±2°C for 180 days the order of radical scavenging activity of ghee remained same as that of fresh samples of ghee.

The data revealed that there was significant ($P < 0.05$) increase in radical scavenging activity in ghee antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) were used in ghee. Among the different fresh samples of ghee, ghee treated with blend of betel leaves plus liquorice had the highest radical scavenging activity. It was significantly higher than that of the corresponding ghee samples. On the other hand radical scavenging activities of fresh samples of betel leaves treated ghee as well as BHA treated ghee were statistically at par, there was no significant ($P < 0.05$) difference between them.

On the storage radical scavenging activity of control ghee decreased at slower rate up to 90^{th} day of the storage, the rate of decline in radical scavenging activity was accelerated after 90^{th} day of the storage and reached to a level of 24.00 per cent on 180^{th} day of the storage.

In sample of ghee containing BHA also radical scavenging activity decreased very gradually up to 90^{th} day of the storage and remained quite near to radical scavenging activities of samples of ghee treated with betel leaves and blend of betel leaves plus liquorice. However, on further storage rate of decline in radical scavenging activity of the BHA added ghee was accelerated after 90^{th} day of the storage, started deviating from radical scavenging activities of samples of ghee treated with betel leaves as well as blend of betel leaves plus liquorice and reached to a level of 44.01 per cent on 180^{th} day of the storage. Thus, well known permitted synthetic oxidant BHA started losing its activity of radical scavenging earlier than betel leaves and blend of betel leaves with liquorice.

On the other hand in samples of ghee treated with betel leaves alone or combination of betel leaves and liquorice radical scavenging activity decreased very gradually all throughout the storage. There was no sign of rapid or steeped decline in the radical scavenging activity was observed in these ghee samples at any stage of the entire storage. The trend of changes in radical scavenging activity of betel leaves alone or blend of betel leaves and liquorice remained parallel up to 120^{th} day of the storage. However, on further storage radical scavenging activity of ghee treated with blend of

betel leaves plus liquorice decreased at a higher rate and difference between radical scavenging activities of betel leaves treated ghee and betel leaves plus liquorice treated ghee became narrow. On 180th day of the storage radical scavenging activity of ghee treated with betel leaves alone and combination of betel leaves and liquorice was 52.03 and 55.28 per cent respectively. The difference in radical scavenging activities of these ghee samples was significant ($P<0.05$). Thus, the data indicated that when betel leaves were blended with liquorice there was significant increase in effectiveness to scavenge radical in ghee.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to scavenge radical in ghee during the storage at 35±2°C that blend of betel leaves and liquorice was the best, which was very closely followed by betel leaves. The radical scavenging activity of synthetic antioxidant BHA was far behind than that of the betel leaves alone or blend of betel leaves plus liquorice.

From survey of literature it appears that no work is reported so far dealing with evaluation for relative effectiveness of BHA, betel leaves and blend of betel leaves with liquorice in radical scavenging activities in ghee during storage at 80°±2°C. Therefore, results obtained in the present study could not be compared as such with the reports in the literature.

Asha *et al.* (2015) evaluated the antioxidant activities of BHA and orange peel powder extract in ghee stored at different storage temperatures (T1:6±2°C; T2:32±2°C and T3:60±2°C) for a period of 21 days. Ghee incorporated with orange peel extract showed stronger activity in quenching DPPH radicals and least development of peroxide value, TBA and FFA than ghee incorporated with BHA and control.

4.6.4.4 Effect of antioxidants on flavour score of ghee during storage at 35°C

The results obtained for changes in flavour score of ghee during storage 35°±2°C are presented in Table 4.29 and the trend is presented in Figure 4.29.

Table 4.29: Changes in flavour score of ghee during storage at 35°C after treating with different antioxidants

Storage period (days)	Flavour score of ghee (out of 9)			
	Control	Antioxidant used		
		Betel leaves	Betel leaves + Liquorice	BHA
0	9.00	8.28	8.00	9.00
30	8.85	8.32	8.10	8.91
60	7.60	7.86	7.95	8.41
90	6.72	7.69	7.81	7.86
120	6.23	7.49	7.63	7.06
150	**5.47**	7.23	7.45	6.58
180	4.05	7.02	7.23	**5.87**

Source of Variation	Storage period (P) (days)	Treatment (T) (antioxidants)	Interaction (P×T)
SEm	0.05	0.04	0.10
CD (0.05)	0.14	0.10	0.28
CV%	2.67		

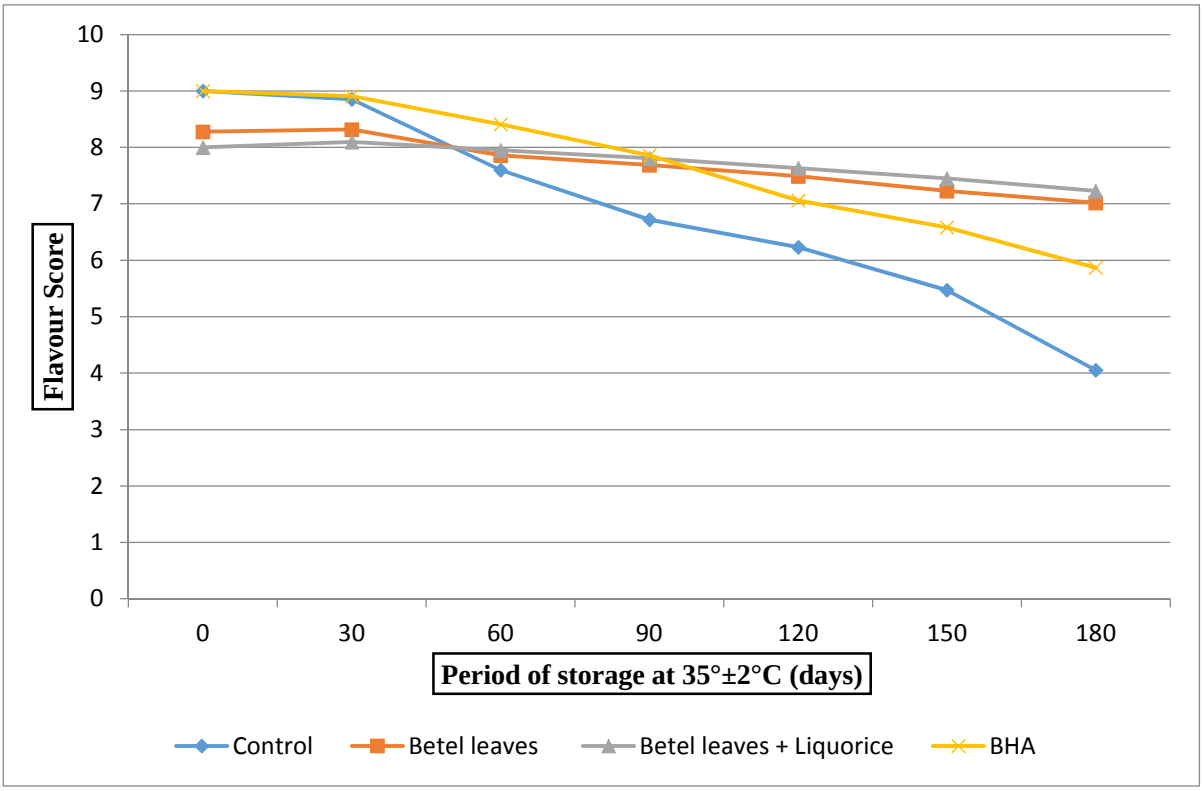


Table 4.29: Changes in flavour score of ghee during storage at 35°C after treating with different antioxidants

It was revealed from statistical analysis of data on changes in flavour score of ghee during storage at 35°±2°C that the treatments (BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants) and period of storage both were significant ($P<0.05$). The interaction between period of storage as well as antioxidants used in treatment of ghee was also significant ($P<0.05$). Therefore, this statistical analysis of the results suggested that different antioxidants (BHA, betel leaves and blend of betel leaves with liquorice) used in treatment of ghee differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee during storage. Similarly, period of storage also differed significantly ($P<0.05$) in their effect on changes in flavour score of ghee. The interaction effect indicated that different antioxidants used in treatment of ghee as well as period of the storage differed significantly from each other in their effect on flavour score of ghee over a period of storage. Thus, it became evident that the effect of different antioxidants used in treatment of ghee and period of storage were dependent on each other for changes in flavour score of ghee over a storage period.

The flavour score of different fresh ghee samples was in the order of control = BHA > betel leaves > blend of betel leaves plus liquorice. As stated earlier (Section 4.6.2.1) fresh samples of control ghee and the ghee added with BHA received full flavour score (9 out of 9). The data revealed that there was significant ($P<0.05$) decrease in flavour score of fresh ghee when treated with betel leaves or blend of betel leaves with liquorice when compared with the flavour score of control ghee and ghee added with BHA.

On the storage flavour score of control ghee sample decreased at somewhat lower rate up to 30th day of the storage. The rate decline in flavour score of control became rapid after 30th day of the storage and very sharp decline was noticed after 120th day of the storage. The score of the control ghee reached below the acceptable level on 150th day of the storage and it reached to a level of 4.05 on 180th day of the storage.

In BHA added ghee there was only slight decrease in flavour score on 30th day of the storage and statistically it was at par with fresh control ghee. Moreover, flavour score of BHA added ghee remained highest among all the ghee samples up to 90th day of the storage. However, on further storage flavour score of the BHA added sample of ghee started declining at rapid rate and became significantly ($P<0.05$) lower than the flavour score of the betel leaves and blend of betel leaves plus liquorice treated ghee sample.

The flavour score of the BHA added ghee reached below the acceptable level on 180th day of the storage with a flavour score of 5.87.

On the other hand in samples of ghee treated with betel leaves alone or combination of betel leaves and liquorice flavour score decreased very gradually all throughout the storage. There was no sign of rapid or steeped decline in the flavour score was observed in these ghee samples at any stage of the entire period of storage. The trend of changes in flavour score of betel leaves alone or combination of betel leaves and liquorice remained almost parallel all throughout the storage. On 180th day of the storage flavour scores of ghee treated with betel leaves alone and combination of betel leaves plush liquorice were 7.02 and 7.23 respectively. Thus, the data indicated that on 180th day of the storage both samples of ghee were rated as like very much, since their flavour score was above 7. Sample of ghee treated with betel leaves was significantly ($P<0.05$) higher than the flavour score of the combination of betel leaves plush liquorice up to 30th of storage then both the samples were statistically at par on 60th day of storage. However, after 60th day of storage ghee treated with combination of betel leaves plush liquorice became significantly ($P<0.05$) higher than the flavour score of the betel leaves treated ghee.

It was evident from the work carried out to evaluate effectiveness of BHA, betel leaves and blend of betel leaves with liquorice used in ghee as antioxidants to flavour score in ghee during the storage that combination of betel leaves and liquorice was the best, which was very closely followed by betel leaves alone. The flavour score of synthetic antioxidant BHA was far behind than that of the betel leaves alone or blend of betel leaves plus liquorice.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) leaves when added to melted butter during clarification. Fresh betel leaves were cut into small pieces separately and added to different lots of melted butter and then heated to 120°C till characteristic ghee flavour developed. The amount of betel leaves 0.2, 0.5 and 1 per cent (w/v). The leaves were filtered off before the storage of ghee. A mixture of BHA and BHT (1:1) at concentration of 0.02 per cent by weight was also added to a separate lot of ghee. Ghee samples were packed, sealed in lacquered tins and stored at 30°C. The sample were evaluated for flavour using 9 point hedonic scale. The authors found that the initial flavour score of control sample and BHA+BHT added ghee sample was 8.33 and 8.3 respectively. Whereas, initial flavour score of 0.2,

0.5 and 1 per cent betel leaves added samples was 8.5, 8.2 and 8.4 respectively. After storage for 147 days the flavour score of control sample and BHA+BHT added ghee sample was 2.16 and 6.15 respectively. Whereas, after storage for 147 days flavour score of 0.2, 0.5 and 1 per cent betel leaves added samples was 2.2, 5.8 and 6.5 respectively. Thus results obtained in present study were in general agreement with those reported by these authors. Some variations in results between the reported study and the present study might be attributed to differences in variety of the betel leaves used, in which form they added, stage in preparation of ghee at which betel leaves added, temperature at which ghee was stored, etc.

Patel and Rajorhia (1979) carried out the study dealing with the antioxidative effects of betel (*Piper betel*) and BHA+BHT leaves when added to butter during clarification. These authors reported that all the samples of ghee were rated excellent at the beginning of the experiment. The judges preferred ghee samples treated with betel and curry leaves as indicated by their highest scores for flavour and colour. The treated samples were also appreciated for slightly higher intensity of colour. Ghee samples treated with BHA and BHT were rated as ordinary. Therefore, findings of the present study were in general agreement with those reported by Patel and Rajorhia (1979).

Sharma (1997) isolated the antioxidant principles of Tulsi (*Ocimum sanctum* Linn.) leaves via a pre-extraction. The anti-oxygenic compounds of Tulsi leaves were extracted into methanol and then vacuum dried. The dried materials were further fractionated into water insoluble fraction which was then treated with mixture of silica gel and charcoal and designated at SCF. Addition of SCF pre-extract at the level of 0.6 % (w/v) was found to be more effective than the addition of BHA at the level of 0.02 %. The phenolic compounds appeared to be the main contributory factors in enhancing the oxidative stability of ghee.

Dinesh *et al.* (2000) isolated the antioxidant principles namely phenolics and phospholipids from MSKP (Dried mango seed kernel powder) using organic solvents. These compounds were dissolved in ghee to prepare phenolic and phospholipids extracts separately and in combination. Addition of extract in combination was more effective than individual extract. Moreover the phenolics were more effective than phospholipids in prolonging the induction period of ghee. Addition of extracts either individually or in combination at a level of 5 % or above were more effective in increasing the stability of ghee than addition of BHA at a 0.02 % level. It was concluded

that the phenolic compounds in MSKP seemed to be the main anti-oxidative compounds which along with phospholipids gave the maximum stabilizing effect to ghee against oxidative deterioration.

Kaur *et al.* (2001) studied the use of Sorghum (*Sorghum bicolor* L.) grain powder in enhancing the oxidative stability of ghee. Direct addition of Sorghum grain powder (SGP) at different levels in ghee was elevated the phospholipids as well as water extractable phenolic compounds of ghee. The results also revealed that addition of SGP at a level of 1 % (w/v) and above have higher effect than the addition of permitted level of BHA. The proactive action of SGP in ghee could be attributed to the transfer of phospholipids and phenolic compounds present in SGP.

The present study was conducted to evaluate potential of selected herbs as an antioxidant in ghee to extend the shelf-life by retarding oxidative reactions during its storage. In preliminary screening of 15 common herbs tested betel leaves, curry leaves and liquorice were found promising. Optimum rate for use of these herbs in treatment of ghee was found 0.3 per cent. In preparation of ghee addition of betel leaves and curry leaves at final stage of clarification was more efficient, whereas, for liquorice addition at initial stage of clarification was more efficient. While evaluation of betel leaves, curry leaves and liquorice as well as their binary and ternary blends; betel leaves and its blend with liquorice were found highly effective. In comparison of betel leaves, blend of betel leaves plus liquorice and BHA for effectiveness in controlling oxidative deterioration during storage, the effectiveness of betel leaves plus liquorice blend or even betel leaves alone was significantly higher than that of the BHA. The samples of ghee treated with betel leaves or blend of betel leaves plus liquorice met all the quality parameters of ghee as prescribed under FSSAI and AGMARK standards. Similarly, in sensory evaluation of ghee treated with betel leaves or blend of betel leaves plus liquorice was very well acceptable. Hence, betel leaves alone and along with liquorice during preparation of ghee may be used as natural antioxidant enhance the stability of ghee against oxidative deterioration during storage.

CHAPTER 5

SUMMARY AND CONCLUSION

Ghee has reasonably good shelf-life in comparison to other indigenous milk products, under ambient conditions of storage it undergoes oxidative deterioration. One of the most common approach to extend shelf-life of ghee is addition of synthetic antioxidants. However, the use of these synthetic antioxidants has been questioned due to their potential health risks and toxicity. Hence, this has directed the attention towards the use of natural antioxidants as resources of safer alternative to synthetic antioxidant. Herbs are derived from plants and are considered among the best sources of natural antioxidants. Very limited work has been reported for utilization of herbs as a possible antioxidant to control oxidative deterioration of ghee. Therefore, the present study is contemplated with a view to evaluate the potential of common herbs as a natural antioxidant for preventing oxidative rancidity in ghee. Keeping the said idea as a central goal, the study was planned with distribution of the work in to 6 different phases: (1) assessment of compatibility of the common herbs as an additive in ghee, (2) evaluation of the compatible herbs for their antioxidant activity in ghee, (3) selection of stage in preparation of ghee for addition of selected herbs in ghee (4) optimization of rate for addition of the selected herbs in ghee (5) combination effect of selected herbs in ghee (6) comparison for effectiveness of the selected herbs as antioxidant for ghee with permitted synthetic antioxidant (BHA) and analysis of ghee samples for quality parameters prescribed under FSSAI regulations and AGMARK standards.

5.1 Compatibility Assessment of Herbs for Use in Ghee

For application of herbs in ghee, two key points of consideration were acceptability herbs for consumption and their compatibility for use in ghee. Therefore, very commonly used herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, drumstick leaves, fenugreek leaves, jequirity leaves, lemon grass, liquorice, mint, shatavari and tulsi) were shortlisted for assessment of their compatibility in ghee. Ghee was prepared by from white butter by heating to final clarification temperature of 120°C and filtered. The shortlisted herbs were added (@ 0.5% w/w of the expected yield of ghee) to the ghee at 80ºC for 30 min and filtered through 6 layers of muslin cloth. Ghee samples were evaluated by judges of sensory

panel for acceptability of their flavour. All the ghee samples treated with herbs were found acceptable. The ghee samples were also examined for their colour characteristics by visual observation. Control ghee and ghee sample treated with ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dill, dodi, fenugreek leaves, lemon grass, liquorice, mint, shatavari and tulsi acquired yellow to golden yellow colour. The ghee samples treated with drumstick leaves and jequirity leaves treated sample acquired dark olive green colour and greenish yellow colour respectively.

5.2 Evaluation of Herbs for Their Antioxidant Potential

All the shortlisted herbs were evaluated for their antioxidant potential in terms of their total phenolic content and radical scavenging activity as well as their effect on changes in peroxide value and flavour score of ghee during storage at 80±2ºC. Total phenolic content (TPC) was estimated using Folin-Ciocalteau method and expressed as gallic acid equivalents (GAE). TPC (mg GAE/100 g of dried herbs) was in decreasing order of betel leaves (3653.33 ± 18.86), curry leaves (1913.33 ± 57.35), tulsi (1863.33 ± 49.22), jequirity leaves (1846.67 ± 67.99), centella (1740.00 ± 58.88), drumstick leaves (1673.33 ± 24.94), mint (1640.00 ± 32.66), dill (1613.33 ± 65.99), fenugreek leaves (1266.67 ± 57.35), coriander leaves (1140.00 ± 32.66), shatavari (1106.67 ± 73.64), liquorice (1030.00 ± 69.76), dodi (673.33 ± 24.94), lemon grass (660.00 ± 28.28) and ajwain leaves (0396.67 ± 16.99). Radical scavenging activity of herbs measured by DPPH assay (% inhibition) was betel leaves (87.31 ± 0.77), centella (86.64 ± 0.52), mint (85.76 ± 0.28), curry leaves (84.07 ± 0.93), dill (83.62 ± 1.13), liquorice (83.20 ± 1.29), fenugreek leaves (79.72 ± 0.82), coriander leaves (79.40 ± 0.76), jequirity leaves (65.24 ± 1.44), ajwain leaves (63.52 ± 1.47), dodi (61.37 ± 0.13), tulsi (39.24 ± 1.46), lemon grass (37.05 ± 1.21), shatavari (28.41 ± 0.89) and drumstick leaves (16.39 ± 0.77).

The oxidative stability of ghee was determined by Schaal oven test using determination of peroxide value. Samples of ghee treated with herbs (@ 0.5% w/w of the expected yield of ghee) were prepared by procedure described earlier, stored at 80°±2°C and analysed for peroxide value at an interval of every 3 days for a period of 12 days. At the end of the 12 days storage the peroxide value of ghee samples (meq of O_2 per kg fat) was in decreasing order of drumstick leaves (40.99), jequirity leaves (40.37), shatavari (38.37), lemon grass (38.02), tulsi (33.35), dodi (31.49), centella (20.46), dill (19.77), mint (14.81), ajwain leaves (14.25), coriander leaves (13.00), fenugreek leaves

(11.66), curry leaves (8.92), control (7.88), liquorice (5.65), betel leaves (4.99). The samples of ghee stored at 80°±2°C were also evaluated flavour score by sensory evaluation panel of judges using 9 point hedonic scale. At the end of the 12 days storage the flavour score obtained by samples of ghee (out of 9) was in decreasing order of betel leaves (6.46), liquorice (6.37), curry leaves (6.24), ajwain leaves (4.56), centella (4.47), mint (4.31), dodi (4.06), fenugreek leaves (4.00), coriander leaves (3.92), control (3.83), dill (3.61), jequirity leaves (3.44), tulsi (2.89), drumstick leaves (2.22), lemon grass (2.11) and shatavari (1.89). Out of 15 herbs used in the study 6 herbs (dill, drumstick leaves, jequirity leaves, lemon grass, shatavari and tulsi) showed negative effect on flavour of ghee during storage, as flavour score of these samples was lower than that of the control ghee. Remaining 9 herbs (ajwain leaves, betel leaves, centella, coriander leaves, curry leaves, dodi, liquorice, fenugreek leaves and mint) were able to retard the deterioration in flavour of ghee during the storage. The most promising herbs able to retain the flavour score at acceptable level (≥ 6) up to 12^{th} day of the storage were betel leaves, curry leaves and liquorice. Colour characteristic of the ghee samples after storage at 80^{o}±2°C for 12 days were examined by visual observation. The golden yellow colour of control ghee became slightly darker and acquired fain brownish tinge after the storage. Among fifteen herbs used in the study to treat the ghee, only the samples of ghee treated with betel leaves and liquorice were able to retain the original colour without any change in its shade and intensity. The samples of ghee treated with ajwain leaves, coriander leaves, curry leaves and fenugreek leaves also retained their golden yellow colour, with slight change in shade and intensity in of the colour, up to the end of the storage study. In samples of ghee treated with centella, dill, dodi, drumstick leaves, jequirity leaves, lemon grass, mint, shatavari and tulsi there was drastic reduction in intensity of their colour.

From the work carried for evaluating computability and antioxidant potential of 15 very commonly used herbs; 3 herbs namely betel leaves, liquorice and curry leaves were selected for further study.

5.3 Selection of Stage in Preparation of Ghee for Addition of Herbs

In manufacturing process of three stages were envisaged to add herbs: (1) initial stage of heat clarification (*i.e.* in melted butter), (2) final stage of heat clarification (*i.e.* near 105°C temperature) and (3) after the heat clarification (*i.e.* in hot ghee). Since, third stage for addition of herbs (in hot ghee at 80°C) was used during initial phases of study

for evaluating antioxidant potential of herbs. Hence work was taken up to evaluate initial stage of heat clarification (*i.e.* in melted butter) and final stage of heat clarification (*i.e.* near 105°C temperature). The selected herbs (betel leaves, curry leaves and liquorice) were added individually at the rate 0.5 per cent of the expected yield of ghee in two different stages of clarification (initial and final). All 7 samples of ghee were stored 80°±2°C and analysed for peroxide value as well as evaluated for flavour score at an interval of two days for a period of 12 days.

After 12 days of the storage peroxide values of controlled ghee was 9.33 meq of O_2 per kg fat. At the same time peroxide values of ghee treated with selected herbs at the initial stage of clarification and the final stage of clarification were 7.09 and 5.05 meq of O_2 per kg fat for betel leaves, 14.76 and 8.13 meq of O_2 per kg fat for curry leaves 4.04 and 4.93 meq of O_2 per kg fat for liquorice respectively. Similarly, after 12 days of the storage flavour score of control ghee was 2.4 out of 9. At the same time on 9 point hedonic scale flavour scorer of ghee treated with selected herbs at the initial stage of clarification and the final stage of clarification were 6.20 and 6.35 for betel leaves, 2.10 and 6.18 for curry leaves, 6.90 and 6.65 for liquorice respectively. Thus, in treatment of ghee with betel leaves and curry leaves their addition at final stage of clarification gave better performance in controlling peroxide formation and flavour deterioration in ghee during the storage. Whereas, in treatment of ghee with liquorice its addition at initial stage of clarification gave better performance in controlling peroxide formation and flavour deterioration in ghee during the storage. From the work carried for selecting stage in preparation of ghee for addition of selected herbs (betel leaves, liquorice and curry leaves), for addition of betel leaves and curry leaves in treatment of ghee final stage of heat clarification (*i.e.* near 105°C temperature) was selected for further study. Whereas, for addition in liquorice treatment of ghee initial stage of heat clarification (*i.e.* in melted butter) was selected.

5.4 Optimization of Rate for Addition of Herb in Ghee

Since, in studies carried for compatibility assessment of herbs in ghee, evaluation of herbs for their antioxidant potential and selection of stage for addition of herbs in ghee preparation rate of herbs was kept at 0.5 per cent of the expected yield of the ghee. In resultant samples of ghee very strong characteristic aroma of the herbs was perceived. Therefore, work was carried out to optimize the rate of the selected herbs (betel leaves, curry leaves and liquorice). Accordingly the concentration range of 0.1 to 0.4 per cent

was chosen for the study. For evaluating different rates of betel leaves, curry leaves and liquorice ghee was prepared by adding the herbs at the rate of 0.1, 0.2, 0.3 and 0.4 per cent of the expected yield of ghee at respective stages as decided in previous work. All the 13 samples of ghee were stored 80°±2°C and analysed for peroxide value as well as evaluated for flavour score at an interval of 2 days till flavour score of most of the samples went below acceptable level (*i.e.* 22 days).

Ghee samples treated with betel leaves at the rate of 0.0 (control), 0.1, 0.2, 0.3 and 0.4 per cent on 22nd day of the storage peroxide values were 35.80, 18.95, 17.16, 3.51 and 11.91 meq of O_2 per kg fat respectively. Moreover, during the storage flavour scores of the ghee samples treated with curry leaves at the rate of 0.0 (control), 0.1, 0.2 and 0.4 per cent went below the acceptable level (< 6) on 8th, 10th, 14th and 16th day respectively. However, flavour score of the ghee sample treated with 0.3 per cent betel leaves was within acceptable level even on 22nd day of the storage. The result suggested that treatment of ghee with different rate of betel leaves, the rate 0.3 per cent was found most effective in reducing peroxide formation and flavour deterioration in ghee during storage 80^{o}±2°C. In ghee samples treated with curry leaves at the rate of 0.0 (control), 0.1, 0.2, 0.3 and 0.4 per cent on 22nd day of the storage peroxide values were 35.80, 27.56, 17.67, 9.44 and 15.83 meq of O_2 per kg fat respectively. Moreover, during the storage flavour scores of the ghee samples treated with curry leaves at the rate of 0.0 (control), 0.1, 0.2, 0.3 and 0.4 per cent went below the acceptable level (< 6) on 8th, 8th, 12th, 14th and 13th day respectively. The result suggested that treatment of ghee with different rate of curry leaves, the rate 0.3 per cent was found most effective in reducing peroxide formation and flavour deterioration in ghee during storage 80^{o}±2°C. In samples of ghee of samples treated with liquorice at the rate of 0.0 (control), 0.1, 0.2, 0.3 and 0.4 per cent on 22nd day of the storage peroxide values were 35.80, 15.81, 15.08, 5.95 and 11.10 meq of O_2 per kg fat respectively. Moreover, during the storage flavour scores of the ghee samples treated with liquorice at the rate of 0.0 (control), 0.1, 0.2, 0.3 and 0.4 per cent went below the acceptable level (< 6) on 8th, 10th, 14th, 18th and 15th day respectively. The result suggested that treatment of ghee with different rate of liquorice, the rate 0.3 per cent was found most effective in reducing peroxide formation and flavour deterioration in ghee during storage 80^{o}±2°C. From the study carried to optimize rate of betel leaves, curry leaves and liquorice for treatment of ghee it was found that for all three herbs a rate of 0.3 was optimum. The variation in rate of the

herbs on any side (towards lower or higher) resulted in to reduction in the effectiveness of the treatment.

5.5 Combinations of the Selected Herbs as Antioxidants in Ghee

Plant-based antioxidants may possess similar, complementary or different effects in their activities. Their combination may exhibit additive, synergistic or antagonistic interactions and result into different antioxidant activities. Therefore, binary blends (betel leaves + curry leaves, betel leaves + liquorice and curry leaves + liquorice) as well as and ternary blend (betel leaves + curry leaves + liquorice) were evaluated for their antioxidant action. Each herb was added at the optimized rate (*i.e.* @ 0.3% of the expected yield of ghee) and at the appropriate (initial or final) stage in preparation of ghee for each case. All the 8 ghee samples were stored 80°±2°C and analysed for peroxide value as well as evaluated for flavour score at an interval of every 2 days till flavour score of most of the samples of ghee went below acceptable level (*i.e.* 29 days).

In control ghee samples peroxide value was 27.75 meq of O_2 per kg fat on 29^{th} day of the storage. In samples of ghee treated with betel leaves, curry leaves, liquorice, betel leaves + curry leaves, betel leaves + liquorice, curry leaves + liquorice and betel leaves + curry leaves + liquorice on 29^{th} day of the storage peroxide values were 6.08, 10.75, 27.13, 11.28, 5.74, 9.64 and 6.93 meq of O_2 per kg fat respectively. The effectiveness of selected herbs and their different combinations in controlling formation of peroxide in ghee during the storage can be arranged in the order of betel leaves and liquorice < betel leaves < betel leaves, curry leaves and liquorice < betel leaves and curry leaves < curry leaves and liquorice < liquorice < curry leaves. Similarly, during flavour score of control ghee went below the acceptable level (< 6) on 10^{th} day of the storage. Moreover, during the storage flavour scores of the ghee samples treated with curry leaves, liquorice, betel leaves + curry leaves, curry leaves + liquorice and betel leaves + curry leaves + liquorice went below the acceptable level (< 6) on 13^{th}, 20^{th}, 22^{nd}, 23^{rd} and 28^{th} day respectively. However, flavour scores of the ghee samples treated with betel leaves and betel leaves + curry leaves + liquorice were within acceptable level even on 29^{th} day of the storage. Thus, effectiveness of the selected herbs and their different combinations in retaining the flavour of ghee during the storage can be arranged in the order of betel leaves and liquorice > betel leaves > betel leaves, curry leaves and liquorice > curry leaves and liquorice > betel leaves and curry leaves > liquorice > curry leaves. Results of the work carried out to evaluate effectiveness of selected herbs and

their different combinations to control peroxide formation and flavour deterioration in ghee during the storage suggested that combination of betel leaves and liquorice was most effective, which was followed by betel leaves alone.

5.6 Comparison of Selected Herbs with Synthetic Antioxidant (BHA)

After selecting the potential herbs, stage in ghee preparation for addition of herbs and optimum rate for addition of herbs as well as evaluating combinations of the herbs as antioxidants in ghee, work was carried out to compare the performance of the selected herbs with synthetic antioxidant (BHA) permitted under FSSAI regulation. The fresh ghee samples were also analysed for quality standards as prescribed under FSSAI and AGMARK.

Based on the performance in reducing the oxidative deterioration of ghee during the storage, betel leaves (@ 0.3%) and combination of betel leaves (@ 0.3%) + liquorice (@ 0.3%) were selected for the comparison with BHA (@ 0.02%). The sample of without addition of any antioxidant was also prepared simultaneously to serve as a control. All the 4 samples were divided into 2 halves. One part of the samples was stored at 80°±2°C, whereas, second part of the samples was stored at 35°±2°C. The stored ghee samples were analysed for peroxide value, carbonyl value, radical scavenging activity and also evaluated for flavour score at an interval of every 2 days till flavour score of all the samples of ghee went below acceptable level (*i.e.* 34 days in case of 80°C and 180 days at 35°C). All the fresh ghee samples were analysed for various quality parameters as specified under FSSAI and AGMARK standards (moisture content, BR reading at 40°C, RM value, Polenske value, FFA content and Baudouin test) evaluated for sensory attributes (flavour, texture, colour and appearance and overall acceptability). All the ghee samples were also examined for colour characteristic (*i.e.* 0 day, 27 days in case of 80°C and 180 days at 35°C).

Among the quality parameters of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA; moisture content was 0.15, 0.14, 0.17 and 0.18% respectively; BR reading at 40°C was 42.16, 41.05, 40.61 and 41.57 respectively; RM value was 33.52, 33.91, 32.31 and 34.07 respectively; Polenske value was 1.38, 1.45, 1.50 and 1.53 respectively and FFA content was 0.25, 0.23, 0.21 and 0.24% oleic acid respectively. Moreover, Baudouin test was negative in all the fresh ghee samples. Thus, all ghee samples prepared in this study fulfilled

requirement of all the quality parameters prescribed under FSSAI and AGMARK standards. The use of betel leaves or blend of betel leaves plus liquorice did not affect any of the quality parameters.

Among the sensory attributes score (out of 9) of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA; flavour score was 9.00, 8.28, 8.00 and 9.00 respectively; texture score was 8.80, 8.63, 8.70 and 8.58 respectively; colour and appearance score was 8.85, 8.77, 8.68 and 8.72 respectively and overall acceptability score was 8.85, 8.51, 8.43 and 8.79 respectively. The results suggested that flavour scores and overall acceptability scores of both the fresh ghee samples (betel leaves treated and betel leaves + liquorice treated) were rated as like very much, but their scores significantly lower compared to the scores of fresh samples of control ghee and ghee treated with BHA. Moreover, texture scores as well as colour and appearance scores of all the ghee samples were statistically at par and rated as like very much or like extremely. Thus, samples of ghee treated with betel leaves alone or in combination betel leaves with liquorice were very well acceptable in sensory evaluation.

After storage at 80°±2°C for 34 days peroxide values of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 46.41, 6.82, 6.51 and 21.64 meq of O_2 per kg fat respectively. Similarly, after storage at 80°±2°C for 34 days carbonyl values of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 37.63, 12.59, 12.21 and 19.04 µmol carbonyl per g respectively. Again after storage at 80°±2°C for 34 days radical scavenging activity of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 3.33, 19.53, 21.24 and 8.61 per cent inhibition respectively. In the same line, during the storage flavour score of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA went below the acceptable level on 8^{th}, 32^{nd}, 34^{th} and 14^{th} day respectively. Among different samples of ghee, on 26^{th} day of the storage intensity of golden yellow colour in control ghee decreased drastically due to extensive fading of the colour (because of bleaching of β-carotene as discussed in section 4.2.5). Even in sample of ghee treated with BHA some decrease in intensity of its yellow colour was noticed. On the other hand samples of ghee treated with betel leaves or blend of betel leaves + liquorice were able to retain their characteristic yellow color. Thus,

work carried to compare betel leaves, blend of betel leaves + liquorice and BHA for their effectiveness in controlling development of peroxides and formation of carbonyl as well as other associated changes in ghee during storage at 80°±2°C; both betel leaves and its blend with liquorice were far superior compared to BHA.

After storage at 35°±2°C for 180 days peroxide values of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 8.51, 2.40, 2.35 and 5.17 meq of O_2 per kg fat respectively. Similarly, after storage at 35°±2°C for 180 days carbonyl values of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 13.89, 7.65, 6.83 and 8.94 µmol carbonyl per g respectively. Again after storage at 35°±2°C for 180 days radical scavenging activity of control ghee, ghee treated with betel leaves, ghee treated with betel leaves + liquorice and ghee treated with BHA were 24.00, 52.03, 55.28 and 44.01 per cent inhibition respectively. In the same line, during the storage flavour score of control ghee and BHA added ghee went below the acceptable level on 150^{th} and 180^{th} day respectively. However, flavour score of samples of ghee treated with betel leaves and ghee treated with betel leaves + liquorice were very well above the acceptable level. On 180^{th} day of the storage flavour scores of these samples of ghee were 7.02 and 7.23 respectively. Thus, even on 180^{th} day of storage these sample were rated as like moderately. Thus, work carried to compare betel leaves, blend of betel leaves + liquorice and BHA for their effeteness in controlling development of peroxides and formation of carbonyl as well as other associated changes in ghee during storage at 35°±2°C; both betel leaves and its blend with liquorice were far superior compared to BHA.

Therefore, present study entailed conclude that addition of 0.3 per cent betel leaves along with 0.3 per cent liquorice or even 0.3 per cent betel leaves alone during preparation of ghee help to enhance stability of ghee against oxidative deterioration during storage.

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