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Evaluation of cholesterol lowering property of selected herbs in ghee (heat clarified milk fat)

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ABSTRACT

Ghee is a heat clarified milk fat and supply various nutrients. It contains cholesterol which is considered to be associated with various diseases. To the best of our knowledge, the present work focusing on reduction of cholesterol content of ghee using herbs was the first report in literature. In this regard, ghee was treated with various herbs (@0.5%) namely ajwain, betel and curry leaves to evaluate their cholesterol lowering ability. The percent reduction in cholesterol contents in ghee were 4.63%, 10.53% and 4.50% when treated with ajwain, betel and curry leaves respectively. The stage of addition of the herbs in ghee was then standardized. It was observed that the herbs added directly in ghee were able to lower down the cholesterol content from 212.68 mg/100 gm (control) to maximum 175.93 mg/100 gm representing 17.46% reduction in cholesterol content of the ghee sample. Furthermore, among the various stages, herbs which were added in hot ghee at 90 ºC gave maximum reduction in cholesterol content.

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1. Introduction

Ghee is an anhydrous milk fat, occuping a prominent place in the Indian diet. It is manufactured by direct heating of cream or butter churned out of fresh or ripened cream or curd obtained by fermentation of milk (Srinivasan, 1976). Chemically, ghee (99.5 percent 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, carotenoids, carbonyl compounds, hydrocarbons, moisture and trace elements like copper and iron (Sharma, 1981). Ghee exerts different properties such as prompter of memory, intelligence, power of digestion and metabolism, semen and vital essence (Bhatted and Singh, 2002). It is refrigerant and emollient and clarifies the voice and complexion. The ghee has also excellent nutritive, medicinal and therapeutic properties (Bhatted and Singh, 2002). Cholesterol, a steroid alcohol, is the precursor of bile acids, steroid hormones, and provitamin D3 and is found in foods of animal origin, such as eggs, meat, fish and dairy products (Shingla and Mehta, 2018). Substantial amounts of total body cholesterol are obtained from the diet even though a major portion of the cholesterol needed for normal body functions is synthesised endogenously (Sweeney and Weihrauch, 1976). It is essential to our body's cell membrane, to insulate our nerves and for the production of certain hormones. Cholesterol is the major constituent of the unsaponifiable matter of milk fat (Ganguli and Jain 1973). The unsaponifiable matter of milk fat generally falls within the range of 0.40 to 0.5 percent by weight and out of this, 95% is represented by cholesterol , i.e. 0.38-0.47% (Ganguli and Jain 1973). Cholesterol and its esters are associated primarily with milk fat globules. The total cholesterol content in cow and buffalo ghee is 330 mg/100g fat and 275 mg/100 g fat, respectively (Sharma, 1981). In spite of its numerous health benefits, milk fat has received adverse publicity in terms of its effect on blood cholesterol level over the past few years. Cholesterol is associated with coronary heart diseases (CHDs) (Shingla and Mehta, 2018). Cholesterol-lowering

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and cholesterol-free products are gaining increasing relevance in the modern era. For this reason, people are prone to eliminate or reduce intake of food in the diet containing cholesterol. To eliminate cholesterol, people are also avoiding the consumption of ghee in the diet. The diet which is completely devoid of ghee is not possible as it plays an important role in Indian diet. The supply of other potent and advantageous nutrients like conjugated linolenic acid, fat soluble vitamins, essential fatty acids etc. are not readily available if diet is devoid of milk fat/ghee. There are many methods of reducing cholesterol content in foods including blending in vegetable oils (Dunkley, 1982; Belichovska et al., 2017), extraction using organic solvents (Larsen and Froning, 1981; Gema and Manuel, 2018), cholesterol degradation by cholesterol oxidases (Watanabe et al., 1989), removal via supercritical carbon dioxide extraction (Ong et al.,1990) and use of β-cyclodextrins (Davidson, 1990; Oakenfull et al., 1991). However, most of these approaches are relatively non-selective methods and require a great deal of investment. They may also have side effects because of the chemical used for removal of cholesterol. β-Cyclodextrin removes cholesterol from ghee by binding it in its hydrophobic core but with cholesterol it also removes fat soluble vitamins (Bhatia et al., 2019). It causes loss to nutritive value of ghee. The other way to lower down the cholesterol level in body is by using cholesterol lowering drugs. Since the usage of the synthetic drug and statins leads to various side effects and due to the various side effects and ineffectivness of many conventional drugs, the search for new drugs from natural origin has gained much interest in recent years (Mohammadhosseini et al., 2019). Plant derivatives have exhibited novel therapeutic characteristics as a result of a large number of scientific investigations over the past few decades. The replacement of chemical and synthesized drugs with natural-based plant products seems highly demanding (Mohammadhosseini et al., 2019). The plants flavonoids with antiartherosclerotic activity gained much attention and were proven to reduce the risk of atherosclerosis in vitro and in vivo based on different animal models (Shamala et al., 2014). The flavonoid compounds also exhibit lipid lowering effects and anti-inflammatory and antiatherogenic properties (Thirumalai et al. 2014). Flavonoids are scientifically substantiated to have remarkable capability to regulate penetrability of blood vessel walls, to improve their elasticity, and to prevent atherosclerotic lesion with cholesterol (Sadovoy et al., 2013). There is a necessity to develop natural standpoints to reduce cholesterol by using herbs in the products. Till date, there is no report available to evaluate herbs for their ability to reduce cholesterol content in ghee. Therefore, the elimination and/or reduction of cholesterol content in food products by natural compounds derived from medicinal plants is unavoidable. Medicinal plants have been of prime importance in the folkloric traditional medicine systems for centuries (Mohammadhosseini, 2017). These plants have many remedial properties (Mohammadhosseini et al., 2017 a,b). The essential oil present in the plants show various medicinal activities (Camilo et al., 2017). The essential oils and extracts of the Achillea species exhibit antioxidant, antibacterial, antifungal, antimicrobial, and herbicidal activities studied by Mohammadhosseini et al. (2017b). Frezza et al. (2017) studied the phytochemical characteristics of Galeopsis ladanum subsp. angustifolia with chemotaxonomic and ethnopharmacological implications. They found various compounds such as verbascoside, martynoside, flavonoids, harpagide, 8-*O*-acetyl-harpagide, chlorogenic acid, quinic acid that exhibit strong antioxidant, antimicrobial, antiinflammatory, antihypertensive, anticancer, antiestrogen, antitumor and antibacterial properties. Recently, Wansi et al. (2018, 2019) reviewed on the bioactive essential oils from Cameroonian rain forest. They have highlighted that effectivity of essential oils. The essential oils isolated from various plant parts, like leaves, bark, fruit, roots and rhizomes are very effective against Plasmodium falciparum, food borne microbes, dermatophytes, the malaria vector Anopheles gambiae, cancer cell lines, river blindness as well as plant pathogen weevils and fungi. Herbs are any plants used for food, flavouring, medicine, or fragrances for their savoury or aromatic properties. Herbs contain biologically active chemical substances such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds which have antioxidants, antimicrobial, antifungal, as well as curative properties. Herbs also have medicinal properties like antiglycemic, hypolipidemic and antihypertensive properties. Herbs have long been used as traditional medicine for the treatment of hyperlipidemia in Indian traditional system of medicine and other parts of the world. The ajwain, betel and curry leaves contain tannins, phenolic compounds carvacrol, flavonoids, saponins, tannins, essential oils, gallic acid, epicatechin, querecetin, glucoside, Onnin, calycosin, hydroxyl chavicol, eugenol, ascorbic acid and beta carotene etc (Gulcin, 2006; Rasineni et al., 2008; Risdian et al., 2011; Igara et al., 2016). Thirumalai et al. (2014) studied the hypolipidemic activity of betel leaves in vivo. They found that the methanolic leaf extract of betel leaves exhibited a significant hypolipidemic effect in high fat diet induced hyperlipidemic rat. A number of epidemiological studies provide evidence that use of fruit and vegetables in our diet is associated with reduced risk of cardiovascular disease. This cardioprotective effect is thought to be due, at least in part, to minor components of fruit and vegetables called flavonoids (Cook and Samman, 1996). Borradaile et al. (1999) have shown that flavonoids and limonoids from citrus, as well as unextracted citrus juices, have cholesterol-lowering properties. James et al. (2007) suggested that both citrus flavonoids and palm tocotrienols reduce cholesterol levels (20-30%) in laboratory animals.

The quest for finding the new safe and effective drugs for dyslipidaemia in order to protect against cardiovascular disease (CVD) is going to be a continuous process amongst the scientific community. The antihyperlipidaemic activity of plants plays an important role in the reduction of CVD. Herbs have been used as food and for medicinal purposes for centuries. Thus, we need to focus on lipid-lowering activity of herbs and to adopt a new approach to the protective role of these medicinal plants which depends on the reduction of cholesterol. Apart from the synthetic modern drugs like clofibrates, statins, there are efforts to find out herbal drugs possessing lipid lowering activities. The antihyperlipidemic activity of the medicinal plants among Kadazan and Dusun communities in Malaysia was reviewed by Koriem (2014). However, it appears from the available literature that no attention has been paid so far to the potential use of herbal plants directly in ghee for lowing cholesterol content. Therefore, the present study was undertaken to evaluate the effect of selected herbs on cholesterol level in ghee.

2. Experimental

2.1. Preparation of ghee

White butter was procured from a commercial dairy plant (Amul Dairy), Anand, Gujarat. Ghee was prepared by creamery butter method (De, 2004) in our laboratory. The white butter was taken in a stainless steel vessel (pan) and clarified (120 °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 °C and filtered through six folds of cloth in dry glass beaker (2000 mL).

2.2. Herbs

The three herbs namely ajwain leaves (Coleus aromaticus), betel leaves (Piper betle), and curry leaves (*Murraya koenigii*) were brought from Directorate of Medicinal and Aromatic Plants Research, Anand Agricultural University, Anand. The herbs were vacuum tray dried in vacuum drier ((Make: Perfect Engineering and Allied Works Pvt Ltd., Baroda, INDIA) at 40 ± 5 °C under vacuum (650 mm of Hg) for 4-5 h, installed at Anubhav Dairy of the college. After drying, samples were manually crushed into coarse size particles and kept in air tight bottle. This dried form of herb was added at the rate of 0.5% (w/w) in ghee.

2.2.1. Evaluating the compatibility of herbs in ghee

The herb was added directly in prepared ghee. The mixtures of ghee and herb were thoroughly mixed using glass rod and allowed to stand at 80 °C for 30 min. in a hot air oven. The treated ghee samples were filtered through six layers of cloth. The sample without addition of herb was considered as the control sample. The resultant ghee samples were subjected to estimation of cholesterol content. Three replications were conducted for evaluating the compatibility of herbs in ghee.

2.2.2. Selection of stage of addition of herb in ghee

For selection of stage for addition of herb in the preparation of ghee, the herbs (ajwain leaves, betel leaves and curry leaves) were individually added (@ 0.5 percent) at three different stages during manufacturing of ghee. These stages were categorized as: Stage 1: Addition at the initial stage of heat clarification of butter, i.e. in melted butter, 50 °C, Stage 2: Addition at the final stage of heat clarification of butter, i.e. at 105 °C temperature and Stage 3: In hot ghee at 90 ºC and allowed to be cooled at 60 ºC. In stage 1, when butter was melted completely, ajwain, betel and curry leaves were added in 3 beakers. For stage 2, ajwain, betel and curry leaves were added separately when butter temperature reached to 105 °C (nearer to heat clarification). In both of the stages, heating was continued till temperature reached to 120 °C. In third stage, herbs were added in hot ghee at 90 ºC and allowed to be cooled at 60 ºC. One sample in each stages was not treated with any herb to serve as a control. In the entire process (from beginning to end) for the preparation of ghee, all samples were mixed gently with stainless steel spatula turn by turn. The content of each beaker was then filtered through 6 folded cloth. The ghee sample was collected in 150 mL glass beakers and analyzed for cholesterol content. Total four replications were conducted.

2.3. Estimation of cholesterol content

The cholesterol content in the ghee samples were estimated using method developed by Fletouris et al. (1998) with modification. The method comprises of (a) saponify the ghee samples followed by (b) extraction of unsaponifiable matter and then (c) color developed by using enzymatic diagnostic cholesterol test kit (Arkray Healthcare Pvt. Ltd, Surat, India). The kit contained cholesterol mono reagent 1 (comprised of cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine) and cholesterol standard reagent 2 (comprised of cholesterol standard 200 mg/dL).

(a) Saponification of ghee

The sample of ghee (0.1-1.5 g) was taken in the test tube with Teflon line screw cap. The five mL of 5% methanolic KOH was added and then the test tube thoroughly mixed. The tube was then incubated in water bath for 90 °C/ 20 min with intermittent shaking at the interval of 5 min. The tube was then cooled to the room temperature. From the saponify ghee, unsaponificable matter was extracted.

(b) Extraction of unsaponifiable matter

After saponification, 1 mL of distilled water was added to the contents followed by the addition of 5 mL of hexane. The contents were then thoroughly mixed using vortex mixer for 1 min followed by centrifugation at 2000 rpm for 2 min. After centrifugation, the upper hexane layer was pipetted out and passed through anhydrous sodium sulphate, to remove traces of moisture (if at all present). Then, 0.5 mL of the prepared extract was transferred to a dry test tube and evaporated at 50-60 °C under nitrogen. The dried unsaponifiable matter obtained was used for color development.

(c) Color development

10 µL portions of ethanol were added to dissolve dried unsaponifiable matter. Immediately after, 1 mL of cholesterol reagent 1 was added. The standard was prepared using 1 mL of cholesterol reagent 1 and 10 µL of cholesterol reagent 2 to another test tube. All the test tubes were then incubated at 37 °C for 10 minutes. The color intensity was measured at 505 nm using spectrophsotometer (Systronics, Ahmedabad, India) and cholesterol content was calculated from following formula (Eqn. 1).

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

(Eqn. 1, 0.02 is the concentration (mg) of cholesterol in 10 µL of standard solution.)

Table 1
Total cholesterol lowering ability of herbs in ghee.

Sr. No	Samples of ghee added with herbs	Cholesterol content (mg/100 gm)	
		Range	Average*
1	Ajwain leaves	199.00-207.00	200.33±2.14
2	Betel leaves	163.00-193.00	187.78±4.43
3	Curry leaves	182.00-218.00	199.67±5.39
4	Control	188.07-233.14	209.07±7.43

*Data presented as means ± SD (n=3).

2.4. Statistical analysis

The collected data were subjected to statistical analysis. The data obtained were analyzed by completely randomized design and critical difference test at 5% level of significance ($P < 0.05$) to compare the different treatments means (Rudolf et al., 2010).

3. Results and Discussion

3.1. Evaluation of the herbs for their cholesterol lowering ability in ghee

In the present study, the three herbs (ajwain, betel and curry leaves) were incorporated in ghee. The resultant samples were subjected to cholesterol estimation and the corresponding results are presented in the Table 1. Upon care examination from the data given in Table 1, it was found that ajwain, betel and curry leaves were able to lower down the cholesterol content of the ghee. The average cholesterol content in control ghee sample was 209.07 mg/100 gm. The highest cholesterol reduction was found in ghee when treated with betel leaves and it was 187.78 mg/100 gm. The percent reductions in cholesterol contents were observed in ghee when treated with betel leaves (10.53%) > ajwain leaves (4.63%) > curry leaves (4.50%). There are no reports available on the potential use of herbs in oils/fat to lower down the cholesterol content. However, in vivo studies were carried out using various herbs for their hypolipidemic activity. Ghasi et al. (2000) reported that moringa leave extract at a concentration of 200 mg/mL for 30 days gave rise to a hypocholesterolaemic action in rat. The potential of licorice extract as an antiatherosclerotic agent was studied in hypercholesterolemic patients (Fuhrman et al., 2002). In this study, supplementation of licorice root extract (0.1 g/day) was given to patients for one month. It was found out that licorice supplementation resulted in the reduction of plasma cholesterol levels by 5.0%. Kassaian et al. (2009) observed 30% reduction in VLDL-cholesterol when fenugreek was soaked in hot water and gave to the patients. Jain et al. (2010) studied the hypolipidemic activity of *Moringa oleifera* in rats. They observed 6-38% reduction in cholesterol level when methanolic extract of moringa fed to rats at the rate of 150-600 mg/kg. Thirumalai et al. (2014) studied the hypolipidemic activity of betel leaves in vivo. They found that the methanolic leaf extract of betel leaves significantly lowers down the total cholesterol content in rat when high fat diet was fed. Sadovoy et al. (2013) studied the mechanism of interaction of red grape flavonoids with cholesterol as a result of researches, they have established that flavonoids rutin, catechine, quercetin, resveratrol, epicatechin, and epicatechin-gallate form a stable complex combination with cholesterol.

3.2. Effect of stages of addition of herbs on cholesterol content

The result of cholesterol content of ghee obtained from various stages of addition of herbs are shown in Table 2. Four replications were conducted for evaluating the effect of stage of addition of herbs on cholesterol content in ghee. In stage 1, the highest cholesterol reduction was observed in betel leaves (14.16%) whereas ghee treated with ajwain leaves increased the cholesterol content.

In stage 2, the cholesterol content was also marginally reduced in all the treated ghee samples. The percent reduction in cholesterol content of ghee obtained in stage 2 were very close and the highest reduction was found only 5.97% in ghee when treated with curry leaves. However, when treated ghee obtained from stage 3, the cholesterol contents were significantly reduced. The average cholesterol content was 212.68 mg/100 gm that decreased to 175.93 mg/100 gm in ghee added with ajwain. Thus, the highest reduction found in ajwain (17.46%) > betel leaves (16.99 %) > curry leaves (11.77%). Among all the stages, the maximum reduction in cholesterol contents were observed in stage 3 and hence stage 3 was considered as the best for lowering down the cholesterol content in ghee. Dairy industry would abruptly stop without heating. Heating is a very essential processing step in manufacturing of milk and its relevant products. It is well-known that many properties of food can be significantly lost as a consequence of food processing such as sterilization, pasteurization and dehydration as well as during storage and handling and cooking (Nicoliy et al., 1999). Food processing involves changes in structural integrity of the plant material and this produces both negative and positive effects on their various properties. Thermal processes lead to an either decrease or increase in phenolic compounds (flavonoid) which depend on their magnitude and duration. Gerard and Roberts (2004) observed 50% increase in flavonoid content when apple juice was heated from 40 °C to 70 °C. A roasting of 130 °C, 33 min increases the phenol content of cashew nuts (Chandrasekara and Shahidi, 2011); same results were noticed for peanuts (Yu et al., 2005). In these cases, an increase of temperature improves the extraction of phenolic compounds from foods; other results showed losses of phenolic compounds in different quantities. A loss of about 22% in total flavonoids has been observed in boiled products at a temperature of 50 °C during 90 s (Viña and Chaves, 2008). A few studies identified phenolic compounds in foods and followed their degradation during heat treatment. They noticed that individual phenolic compounds are also subjected to heat degradation. The identification and quantification of these compounds were performed with high performance liquid chromatography. Rutin in buckwheat groats was reported to be more stable to heat then vitexin, isovitexin, homoorientin and orientin during roasting at 160 °C for 30 min (Zielinski et al., 2009). Thermal pasteurization treatments (90 °C, 60 s) for strawberry juices have no effect on quercetin and kaempferol contents (Odriozola-Serrano et al., 2008), whereas daily they reduce naringin, rutin, quercetin and naringenin content for grapefruit juices (Igual et al., 2011). However, the above results may not be comparable, because on the one hand, the food matrix is different from one assay to another and on the other hand, the food matrix can act as a barrier to heat effect or induce the degradation. It is not easy then to dissociate the thermal processing effect from the food matrix effects. Thus, some authors studied the effects of thermal processes on model solutions of phenolic compounds; these studies have led especially to the characterization of flavonoids. The data indicated that flavonoids in aqueous solutions show different sensitivity to heat treatment depending on their structures (Irina and Mohamed, 2012). As it is well-known that ghee is almost an anhydrous milk fat obtained by clarification of milk fat at high temperature, the high heat was applied to cream or butter for removal of moisture. Both are usually clarified at 110 to 120 °C temperature. However, in southern India, clarification is carried out at higher temperature (120 to 140 °C) (Ganguli and Jain, 1973). Exposure of the herbs to such a high temperature may adversely affect the stability of major and/or minor flavonoid components present in the herbs. Even possibility also exists about interaction of phenolic compounds present in herbs with ghee residue, leading to change in cholesterol lowering property. There is the possibility of improvement in extraction of these phenolic compounds due to their better leaching from the herbs to ghee at higher temperature. Therefore, it was necessary to find out appropriate stage for addition of herb in process method adopted for the preparation of ghee. An adoption of third possibility, i.e. herbs added in hot ghee at 90 °C and allowed to be cooled at 60 °C for the treatment of ghee, avoids possibilities of loss of flavonoids due to the destruction by heat or interaction with ghee residue. The addition of herbs at 50 °C in ghee (for stage 1) might not be sufficient in extracting out the flavonoids from ghee, whereas at high heat treatment of 105 °C (for stage 2) might be responsible for destruction of the flavonoids. The addition of herbs in ghee at 90 °C improved the extraction of flavonoids of ghee which is responsible for lowering down the cholesterol content in the ghee. Moreover, adopting the practice of adding herb to the prepared ghee sample is technically the most appropriate strategy in the study particularly when a large number of samples have to be treated alike, to avoid sample to sample variation in intensity of heat clarification. Thus, stage 3 was considered as the best one for lowering down the cholesterol content in ghee.

4. Concluding remarks

Cholesterol-lowering and cholesterol-free products are gaining increasing relevance in the modern era. The physical, chemical and biological methods are used to remove the cholesterol contents from ghee but these are not cost-effective and usually lead to losses of some important nutrients. Certain drugs are also used to control the cholesterol content in human body but these drugs have various side effects. The replacement of chemical and synthesized drugs with natural-based plant products are highly demanding. The selected herbs in the present study were able to lower down the cholesterol content of the ghee in the range of 11.77 to 17.46%. The finding of the research may be useful in production of low cholesterol ghee with relatively chapter rate. The direct use of herbs in reduction of cholesterol content in ghee itself seems to be notable for scientific community who are working on formulating the medicinal and nutraceutical foods. As the future perspectives, it is recommended to carry out further research work on interaction of flavonoids with the cholesterol content as well as effect of heating on extraction of flavonoids from herbs into the ghee.

Conflict of interest

The authors declare that there is no conflict of interest.

Table 2
Changes in cholesterol content of ghee upon treatment with different herbs in various stages.

Stage of addition	Samples of ghee	Cholesterol content (mg /100 gm)	
		Range	Average
Stage-1	Ajwain leaves	194.93-240.96	221.42±11.20^{aA}
	Betel leaves	187.90-189.60	188.88±0.36^{bE}
	Curry leaves	183.30-212.80	194.48±6.36^{bG}
	Control	210.80-238.4	219.50±6.48^{aI}
Stage-2	Ajwain leaves	189.20-232.69	207.47±10.54^{aB}
	Betel leaves	192.45-232.69	208.75±9.16^{aD}
	Curry leaves	195.30-229.76	205.15±8.26^{aH}
	Control	209.38-232.69	218.90±4.92^{aI}
Stage-3	Ajwain leaves	164.32-193.90	175.93±6.38^{bC}
	Betel leaves	165.69-191.54	176.45±5.51^{bF}
	Curry leaves	185.63-188.66	187.06±0.76^{bH}
	Control	215.87-207.00	212.68±2.23^{aI}
Source of variation	Stage of addition	Herbs	Stage of addition x herbs
SEm	3.45	3.98	6.90
CD	9.94	11.47	19.88
CV %		6.22	

$^{a-b}$: values with different letters within a row are significantly different at 5% level of significant (i.e. P < 0.05) for among stage of addition, $^{A-I}$: values with different letters within a row are significantly different at 5% level of significant (i.e. P < 0.05) among herbs, SEM: Standard error of mean; CD: Critical difference; CV: Coefficient of variance. Data presented as means ± SD (n=4).

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