Nutritional and biochemical evaluation of vitamin K enriched dietary sources

By

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IN

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Muhammad Yasin
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Abstract

The present investigation was an effort to explore the health claims of vitamin K enriched dietary sources i.e. cooked spinach and fermented soybean/natto against the menace of vitamin K deficiency. Initially, spinach and soybean were characterized with special reference to vitamin K contents followed by product development and finally bioefficacy study for the management of blood coagulation and vitamin K dependent proteins. The nutritional analysis indicated that spinach has ample amount of moisture followed by protein and fiber whereas soybean contains higher amount of protein and fat contents. The high performance liquid chromatography (HPLC) quantification of vitamin K revealed that phylloquinone was higher in spinach as compared to soybean. Amongst antioxidant extracts, methanolic extracts of spinach and soybean showed higher total phenolic, 2,2-diphenyl-1 picrylhydrazyl (DPPH) scavenging and antioxidant activities. During product development phase, four dietary products i.e. cooked spinach (T1), reconstituted spinach (T2), natto A (T3) and natto B (T4) were formulated. Characterization of soybean based prepared products showed improvement in nutritional status due to fermentation as compared to raw material. In the formulated products, cooked spinach (T1) and natto A (T3) attained higher scores for sensory profile from each category. On the basis of nutritional characterization, vitamin K contents and antioxidant potential, two best products i.e. T1 and T3 one from each raw material were selected for efficacy study. Bioevaluation study was carried out involving New Zealand rabbits through two sequential trials for validity of the results. Accordingly, two types of studies were conducted on the basis of different groups of rabbits i.e. study I (normal rabbits) and study II (vitamin K deficient rabbits). Moreover, three different types of dietary sources namely cooked spinach a source of phylloquinone (D2), natto for menaquinone-7 (D3) and synthetic menadione (D4) along with control (D1) were provided to the respective groups. Feed & water intakes and body weights of rabbits varied significantly (p<0.05) with vitamin K dietary sources in study II while these traits behaved non-significantly in study I. The blood coagulation parameters including bleeding, clotting and prothrombin & partial thrombinplastin times and international normalized ratio (INR) were significantly (p<0.05) reduced due to vitamin K enriched dietary sources in vitamin K deficient rabbits (study II) except fibrinogen level that was improved. The serum phylloquinone levels were increased momentously (p<0.05) during study I & II (trial 1) by 23.78, 16.71 & 10.64 and 47.58, 36.42 & 27.71% in D2, D3 & D4 groups, respectively as compared to control. Similarly, serum menaquinone-7 level was improved in D2, D3 and D4 groups by 2.59, 17.77 & 1.11 in study I and 12.74, 24.50 & 10.78% in study II (trial 1). The vitamin K dependent proteins i.e. osteocalcin was increased whilst, undercarboxylated osteocalcin (ucOC) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) were decreased due to vitamin K enriched dietary sources. Likewise trend for these traits was noticed in trial 2 of both studies. The liver & kidney functioning tests and hematological values were within normal range. From the present exploration, it is concluded that vitamin K enriched dietary sources containing menaquinone-7 and phylloquinone are effective to improve the serum vitamin K status and ameliorate the coagulation and vitamin K dependent proteins related abnormalities.
CHAPTER 1

INTRODUCTION

Escalating incidences of micronutrients deficiencies in the developing economies have motivated the nutritionists to develop such dietary interventions that may tackle the existing dilemma. During the last few decades, ample availability of micronutrients remained the prime issue in relation to human health (Booth, 2012). As the micronutrients are essential for regulating numerous metabolic pathways hence their deficiencies may lead to various physiological threats (Willershausen et al., 2011). Amongst those, inadequacy of vitamin K is generally associated with malnutrition, drugs interaction and sedentary lifestyle. In this context, micronutrients enriched foods are gaining core attention of the consumers as a preventive device against various health infirmities (Urala et al., 2007; Schwager et al., 2008). Provision of ample food containing diversified nutrients is one of the major goals of contemporary nutrition (Bech-Larsen and Scholderer, 2007). Accordingly, diet based therapies including diversification, fortification, supplementation and functional/nutraceutical foods are helpful against the menace of hidden hunger. Principally, dietary modifications are considered as a vanguard remedy to alleviate various lifestyle related disorders (Butt et al., 2009; Yasin et al., 2012).

The concept of vitamin was firstly introduced in 1912 and engrossed great attention of the researchers due to their positive impact on physiological activities. The vitamins requirements are influenced by various factors like presence of antagonists, drugs and body vitamin reserves (McDowell et al., 2006). Nevertheless, vitamin K has lime lighted owing to its active participation in normal blood coagulation, bone strengthening and homeostasis (Truong and Booth, 2011).

Vitamin K exists in two naturally occurring bioactive forms; phylloquinone (vitamin K\textsubscript{1}) and menaquinone (vitamin K\textsubscript{2}). The phylloquinone is the most common form of vitamin K present in green leafy vegetables like spinach, kale, broccoli (Kamao et al., 2007) and certain vegetable oils (Bolton-Smith et al., 2000). Likewise, menaquinone is present in fermented soybean/natto and animal products (Schurgers et al., 2007). The natto is a popular breakfast food of Japanese cuisine made from soybean fermented with Bacillus subtilis containing high protein and menaquinones content. However, differences in the amount and form of menaquinones may vary in different dietary sources. Natto restrains sufficient
amount of menaquinone-7 (MK-7), menaquinone-8 (MK-8) & menaquinone-9 (MK-9) and plays preventive role against vitamin K deficiency (Katsuyama et al., 2002; Wu and Chou, 2009; Tabasum and Qadir, 2010). Likewise, spinach (Spinacia oleracea) belongs to the family Amaranthaceae is also recognized as vitamin K enriched vegetable. Generally, phylloquinone (vegetables) and MK-7 (pulses including fermented soybean) are considered as major sources of vitamin K for the humans (Kamao et al., 2007). Besides, fermented soybean (natto) is also rich in protein, calcium and vitamin K$_2$, involved in the activation of osteocalcin (Booth, 2012).

The contribution of phylloquinone, MK-7 and menaquinone-4 (MK-4) is approximately more than 60, 24 and 7%, respectively of the total vitamin K dietary intake (Iwamoto et al., 2009). Humans and animals have ability to convert phylloquinone into MK-4 by removing integral side chain thereby contributes to bone strengthening (Okano et al., 2008). Physiological requirement of vitamin K for both menaquinones and phylloquinone is about 1.16 µg/kg body weight, normally stored around 90 and 10% in the liver, respectively. However, only 5 to 25% of ingested vitamin K is catabolized to MK-4 followed by conversion of menaquinones in the liver via prenylation (Shearer and Newman, 2008).

Vitamin K deficiency is one of the alarming issues in infants, adolescence and postmenopausal women especially in old age (Shearer, 2009). The lactating mothers and their babies are about 33.3 and 65% vitamin K deficient, respectively (Data et al., 2012). Overall, 59% subjects have low intake of phylloquinone from the recommended dietary guidelines (1 µg/kg body weight/day) however, consumption and serum concentration of vitamin K are higher in male compared to female (Thane et al., 2002). The vegetables contribute about 60% of total phylloquinone where cooked green vegetables provide around 28% (McKeown et al., 2002). The phylloquinone and green leafy vegetables intake has positive correlation with plasma phylloquinone and fasting triglyceride concentrations. Likewise, Rotterdam based study delineated that adequate intake of menaquinones compared with phylloquinone is beneficial against coronary artery disease (Geleijnse et al., 2004). The supplementation of phylloquinone and MK-7 in the diet enhances serum vitamin K level (Schurgers et al., 2007; Novotny et al., 2010).

Excessive use of antibiotics, alcoholism and gastrointestinal disorders are the possible causes of vitamin K deficiency consequently hindering fat incorporation thereby reduce its
absorption (McCann et al., 2009). The deficiency symptoms of vitamin K include simple bruising and delayed time for clotting resulting in blood loss and hemorrhages. Likewise, inflammatory bowel disease patients have high prevalence of vitamin K deficiency that decreases bone mineral density possibly due to its malabsorption (Kuwabara et al., 2009). Similarly, regular utilization of broad spectrum antibiotics suppresses the synthesis of vitamin K in the gut while anticoagulant drugs also act as antagonist to vitamin K functioning thereby delays blood coagulation and modifies certain protein essential for bone health (Booth and Rajabi, 2008).

Osteoporosis and bone fractures are the major threats in elderly women. Earlier, it has been observed that postmenopausal women with fracture problems have higher amount of vitamin K deficiency biomarkers like undercarboxylated osteocalcin (ucOC), protein induced by vitamin K absence or antagonist-II (PIVKA-II) and matrix Gla protein (MGP) in their serum (Feskanich et al., 1999). On global scale, about 30–50% women and 15–30% men become victim of osteoporosis related fractures at least once in their life. However in developing countries like Pakistan, women are facing this disarray at relatively higher rate due to the deficiency of valuable micronutrients in daily diet (Akhter et al., 2004; Fatima et al., 2009). The supplementation of phylloquinone and menaquinones is a pragmatic approach to cope with vitamin K deficiency in old aged population (Booth et al., 2003). During osteoporosis, pharmacological dose of vitamin K 45 mg/day is effective against bone related problems (Shearer and Newman, 2008). In this context, higher osteocalcin carboxylation can be achieved through vitamin K usage, well above the recommended dietary requirement (van Summeren et al., 2009). The phylloquinone and MK-4 treatment reduces serum ucOC however, it does not alter the serum bone-specific alkaline phosphatase and n-telopeptide of type 1 collagen (Binkley et al., 2009).

Among various methods to determine the vitamin K deficiency, plasma phylloquinone fluctuation is a promising indicator (Booth and Suttie, 1998; Booth et al., 1999; Shea et al., 2007). The amount of serum osteocalcin, PIVKA-II and MGP are the sensitive parameters of deprived vitamin K level (Iwamoto et al., 2006; Booth and Rajabi, 2008). Additionally, urinary phylloquinone metabolites concentration is also an important biomarker of vitamin K status as approximately 20% excretion occurs through this route in a dose dependent manner (Harrington et al., 2007). Vitamin K mainly accumulates in the liver, heart and pancreas after administration of vitamin K enriched diet. The long chain menaquinones (MK-6 & 9) are
stored in the liver mitochondria owing to their selective tissue distribution. The hepatic phylloquinone concentration ranged from 6.8-9.0 pmol/g in the vitamin K deficient conditions that subsequently uplifted to 56.9-171.1 pmol/g after supplementation (Shearer, 2009). It has been noticed that menaquinones accounted for a large proportion of total vitamin K in the rat’s liver regardless of gender. Likewise, there are significant gender differences for urinary Gla excretion; less Gla protein excreted by the females indicating low dietary phylloquinone requirements (Huber et al., 1999). However, among 11 different form of vitamin K, phylloquinone and MK-6 are predominately stored in the liver. Nevertheless, phylloquinone, MK-4 and MK-6 are present in the extra hepatic tissues while brain contains only MK-4 and traces of phylloquinone (Huber et al., 1999).

Vitamin K is an indispensable anti-hemorrhagic fat soluble micronutrient required for posttranslational changes of proteins with calcium binding ability. These proteins are normally called as vitamin K-dependent or Gla proteins (Price et al., 2003). The vitamin K-dependent coagulation proteins consist of factors II, VII, IX, and X are synthesized in the liver that play haemostatic role in the body. The glutamate (Glu) residues of vitamin K-dependent proteins undergo carboxylation into γ-carboxyglutamate (Gla) through the action of endoplasmic γ-glutamyl carboxylase (Wajih et al., 2004). Moreover, vitamin K epoxide reductase complex is a membrane intrinsic enzyme responsible for vitamin K reduction from epoxide to hydroquinone. During γ-carboxylation process, vitamin K hydroquinone is oxidized to epoxide (Rost et al., 2004). Abnormal vitamin K-dependent protein molecules are produced at the site of synthesis when the supply of vitamin K is insufficient (Wallin et al., 2010). Furthermore, during deficiency, blood coagulation parameters like clotting time, bleeding time, prothrombin time and partial thromboplastin time are prolonged (Shearer, 2009).

Fermented soybean (natto) water soluble fraction reduces the low density lipoproteins (LDL), triglycerides and total cholesterol in rodents without affecting the growth parameters (Iwai et al., 2002). The antioxidant activity of fermented soybean extract significantly inhibits the formation of serum thiobarbituric acid reactive substances (TBARS) and LDL oxidation (Kim et al., 2008). Furthermore, it also reduces total cholesterol, LDL cholesterol and triacylglycerol in dose dependent manner (Shearer and Newman 2008; Wang et al., 2008). The pharmacological dose of vitamin K$_2$ (1 to 10 mg/kg/day) significantly reduces serum total cholesterol (Eventov-Friedman, et al., 2009). However during deficiency phase, the
PIVKA-II level has linear association with serum conjugated bilirubin, bile acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), prothrombin time, INR, and serum ucOC (Strople et al., 2009). The green leafy vegetables especially spinach contains bioactive molecules that are beneficial against the oxidation of LDL (Chopra et al., 2000). Moreover, it also shows protection against induced malondialdehyde contents by increasing serum glutathione level (Bhatia and Jain, 2004). Additionally, spinach has an ample amount of phylloquinone/vitamin K₁. It has been observed that liver functioning enzymes are within normal ranges after the administration of phylloquinone (Shirakawa et al., 2005). Likewise, creatinine level remained non-significant whereas osteocalcin content increased in phylloquinone supplemented group (Binkely et al., 2009).

Considering the effectiveness of vitamin K for normal blood coagulation and bone related biomarkers, the present project was an attempt forward in this regard. In the first phase, spinach and soybean were characterized with special reference to vitamin K contents. Moreover, vitamin K enriched products i.e. cooked & reconstituted spinach and natto by varying fermentation conditions were formulated. Accordingly, the nutritional profile, vitamin K quantification and hedonic evaluation of the developed products were carried out. The biochemical performance of various vitamin K enriched dietary sources in comparison with synthetic counterpart is the limelight of the study. The objectives set to be achieved are herein:

OBJECTIVES

1. Characterization of vitamin K enriched dietary sources i.e. spinach and soybean
2. Nutritional profiling and HPLC quantification of different forms of vitamin K in the developed products
3. Biochemical evaluation of vitamin K enriched dietary sources through experimental rabbits modeling
CHAPTER 2

REVIEW OF LITERATURE

Vitamins are the bedrock of novel dietary strategies that not only improve and restore the health status of individuals but also tackle various physiological dysfunctions. Globally, the developing countries are facing a serious nutritional crisis due to population burden, poor healthcare practices and economic setbacks. The hidden hunger of micronutrients has contributed to an array of non-communicable diseases. In this milieu, nutrient supplementation is a judicious way to scale up the therapeutic potential of foods. Presently in the developing economies like Pakistan, lack of awareness and escalating treatment cost has declined the efficient consumption of vitamin supplements. Nevertheless, traditional and indigenously available food sources rich in micronutrients have attracted the consumers to incorporate them in everyday diet. Among various bioactive agents, vitamin K is deficient in large segment of the population of developing nations (Kubota and Shimizu, 2009). In this context, vitamin K enriched food is one of the sustainable dietary interventional approaches to overcome the menace (Shearer, 2009). Various epidemiological studies have elucidated that green leafy vegetables like spinach and fermented foods such as natto (fermented soybean) are rich sources of phylloquinone and menaquinone, respectively (Schurgers and Vermeer, 2000; Booth, 2012). The vitamin K and allied bioactive moieties have established coagulating properties as well as imparting affirmative effect on various metabolic pathways of the biological system (Krueger et al., 2009; Viegas, et al., 2009). Taking in consideration the underlying determinants of vitamin K deficiency, the instant research was an endeavor to explore the nutritional and biochemical consequences of indigenously available vitamin K rich dietary sources. Accordingly, a descriptive debate relating to various aspects of current study are explained herein.

2.1. Vitamin K; a preamble
2.2. Dietary sources, requirements and deficiencies
2.3. Vitamin K absorption and metabolic route
2.4. Coagulation activities
2.5. Vitamin K-dependent proteins
2.6. Vitamin K and bone health
2.7. Product development
2.8. Efficacy studies
2.1. Vitamin K; a preamble

The vitamin K is fat soluble vitamin abundantly present in green leafy vegetables animal and fermented products as phylloquinone (vitamin K₁) and menaquinones (vitamin K₂), respectively (Rees, et al., 2010). Among these sources, spinach (Spinacea oleraceae L.) is recognized as a good reservoir of phylloquinone (400 µg/100g), the most common form of vitamin K occurring in green leafy stuff (Bolton-Smith et al., 2000).

Fermented soybean (natto) is one of the richest sources of menaquinones especially menaquinone-7 (882-1034 µg/100g) among other plant and animal based food products (Schurgers and Vermeer, 2000; Schurgers et al., 2007). Menaquinone enriched natto is prepared by the action of bacteria (Bacillus subtilis). They are responsible for the conversion of raw soybean into natto that is a sticky substance mainly due to glutamic residues (Kwaka et al., 2007). Menaquinones are also synthesized by the action of intestinal microflora B. vulgates, B. fragilis and E. coli (Narumi et al., 1998). However, chemically synthesized vitamin K is referred as synthetic menadione (vitamin K₃). Various salts of vitamin K are also available for animal feed supplementation like menadione sodium bisulfate, menadione sodium bisulfite complex and menadione pyridinol bisulfate (Thijssen et al., 2006). Moreover, menaquinones synthesized through fermentation of soybean with B. subtilis are water soluble showing similar behavior like protein apI, accordingly form intracellular complex with protein and release in the extracellular fraction during culture progression (Yanagisawa and Sumi, 2005).

Vitamin K is an essential cofactor in the synthesis of blood coagulation factors II, VII, IX, and X in the liver, osteocalcin in bone and matrix Gla protein (MGP) in blood vessel walls and cartilage (Schurgers et al., 2007a). The vitamin K acts as substrate for the microsomal enzyme that converts protein-bound glutamyl residues to γ-carboxyglutamyl (Gla) residues (Suttie, 2013). The hydroquinone form of vitamin K is indispensable for posttranslational γ-carboxylation of vitamin K dependent proteins by the activation of γ-glutamyl carboxylase (Berkner and Runge, 2004). Hence, these proteins enhance the metal binding properties and modulate the various physiological processes including homeostasis, arterial calcification, bone mineralization, phagocytosis, growth control and signal transduction (Berkner and Runge, 2004).
Deficiency of vitamin K is common in neonates owing to deprived placental transfer, deficit in gut microflora and low vitamin K content in breast milk (Fairfield and Fletcher, 2002; Shearer, 2009). Likewise, vitamin K deficiency is noticed in postmenopausal women (Binkley, et al., 2009). Moreover, deficiency is also reported in coagulopathy, chronic renal failure, hemodialysis, advanced cancer and cephalosporin or cephemycin antibiotics patients (MacLaren, et al., 2001; Małyszko, et al., 2002; Pilkey, et al., 2007; Harrington, et al., 2008; Dougherty, et al., 2010). The cystic fibrosis and pancreatic paucity are also responsible for serum vitamin K deficiency in the humans (Wilson, et al., 2001; Dougherty, et al., 2010).

During the vitamin K deficiency, expression of factors II, VII, IX and X in blood were reduced whilst blood prothrombin time (PT), activated partial thromboplastin time (APTT) and international normalized ratio (INR) were enhanced (Harrington, et al., 2008). Earlier, most common methods used to estimate indigent status of vitamin K in individuals are plasma phylloquinone level, amount of osteocalcin (OC), uncarboxylated vitamin dependent protein or proteins induced by vitamin K absence (PIVKA-II) and undercarboxylated osteocalcin (ucOC) content of serum or plasma (Iwamoto, et al., 2006; Shea, et al., 2007; Booth and Rajabi, 2008). Besides, urinary phylloquinone metabolites profusion is also a considerate marker of vitamin K status (Harrington et al., 2007).

2.2. Dietary sources, requirements and deficiencies

Primarily, natural vitamin K is present in its two bioactive forms; phylloquinone and menaquinone. Nevertheless, menadione (vitamin K₃) is chemically synthesized and used in the feed industry to fulfill dietary requirements of animals.

Among the vegetables, green leafy vegetables including broccoli, spinach and certain lettuces contain higher amount of phylloquinone (Jakob and Elmadfa, 2000; Tikkanen et al., 2000). Currently, Booth (2012) examined the phylloquinone concentration in fresh spinach and reported the value as 380 µg/100g. Earlier, Ruiz et al. (2006) quantified phylloquinone through HPLC and noticed variations from 355±8 to 359±5 µg/100g in the examined samples. It has been observed that fermented soybean contains ample amount of menaquinones especially menaquinones-7 i.e. 850-1038 µg/100g (Schurges et al., 2007).
2.2.1. Spinach: source of vitamin K₁

Green leafy vegetables especially spinach is a rich source of health beneficial nutrients including vitamin K (Gupta and Prakash et al., 2009). In Pakistan, spinach is grown over an area of 7363 hectares with 79698 tones production (Akhtar et al., 2008). The nutritional composition of spinach (Spinacea oleracea) indicated 90.70, 2.54, 0.29, 3.91, 0.7 and 1.9 g/100g of moisture, protein, lipids, carbohydrates, fiber and ash contents, respectively (Bangash et al., 2011). However, dry spinach holds more protein (20.82%), fat (3.325), fiber (4.92%) and carbohydrates (48.82%) as compared to fresh sample (Hussain et al., 2010).

In a study, Bangash et al. (2011) explored the mineral potential of spinach and observed K, Mg, Ca, Na, Fe, Mn, Zn and Cu as 310, 137, 100, 40, 29, 1.52, 1.21 and 0.17 mg/100g, respectively. Likewise, Hussain et al. (2010) noticed appreciable amount of iron, calcium and other nutrients in dry spinach. Moreover, Singh et al. (2001) examined various green leafy vegetables for their mineral profile and reported higher values of copper, manganese and zinc in spinach among the tested samples.

Afterwards, Booth (2010) detected higher phylloquinone content in spinach 380 µg/100g as compared to soybean oil by 190 µg/100g. In another study, Schurgers and Vermeer (2000) carried out a comprehensive profiling of kale & spinach for their phylloquinone content and recorded values as 817 and 387 µg/100g, respectively. The plant maturation, light & dark cycles and climatic variations are the considerate factors that contribute vitamin K contents. In this context, Lester and Makus et al. (2010) explicated the beneficial role of light for vitamin K synthesis. Likewise, Damon et al. (2005) measured the phylloquinone in different processed spinach samples i.e. raw, boiled & microwaved. The cooked spinach indicated the highest amount of vitamin K₁ as 533–547 µg/100g followed by microwaved 348–544 µg/100g and raw 293–441 µg/100g. The authors suggested that the variations among the cooked sample from raw counterparts might be due to leakage of the photosynathatic tissue from the plant matrix, differences in sample lot and analytical variation.

Like other plants, spinach also contains an array of phytonutrients that exhibit good antioxidant activity, in this regard flavonoids, p-coumaric acid, carotene, ascorbic etc. are the important bioactive moieties (USDA Database, 2010). The total phenolic content of spinach
is ranged from 10-12.5 mg GAE/g of fresh weight. Moreover, it has good radical scavenging activity (DPPH) 24.4%, lipid peroxidation inhibition 29.4% and ferrous ion chelating activity (FRAP) 43.9% (Gacche et al., 2010). Later, Sharmin et al. (2011) probed spinach for its phenolics and total antioxidant capacity. They observed values for these traits are 2.58 mgGAE/g and 1422.5 ± 5.6 µmol TE/100, respectively. Previously, Pellegrini et al. (2003) examined the antioxidant potential of 34 tested vegetables through different indices like total antioxidant activity, DPPH and FRAP. They documented higher antioxidant potential of spinach than rest of the others due to its strong polyphenolic profile dominated by flavonoids.

2.2.2. Natto: a source of vitamin K₂

Natto is a Japanese traditional food prepared by soybean fermentation with bacteria i.e. *Bacillus subtilis* that imparts strong flavor, aroma and slippery texture to the end product (Takemura, 2006). Likewise, some other Asian countries also have their traditional fermented soybean products such as “Thua nao” in Thailand, “Kinema or Hawaijar” in India and “Chung kook jang” in Korea (Kwaka et al., 2007; Moktan et al., 2008; Dajanta et al., 2011).

In natto menaquinones-5 (MK-5), menaquinones-6 (MK-6), menaquinones-7 (MK-7), menaquinones-8 (MK-8) are ranged from 7.1–7.8 µg/100g, 12.7–14.8 µg/100g, 882–1034 µg/100g, 78.3–89.8 µg/100g along with 31.2–36.7 µg/100g vitamin K₁ (Schurgers and Vermeer, 2000). Similarly, Berenjian et al. (2007) documented that MK-7 content in fermented soybean is varied from 800-900 µg/100g. Moreover, Booth (2012) also reported that natto contains MK-7 (998 µg/100g).

Recently, Dajanta et al. (2011) characterized the fermented soybean prepared from five different cultivars for their quality traits. They concluded that low fat (15.6%), high protein (45.8%) and small seed size are ideal attributes for natto preparation. Moreover, fermentation with *B. subtilis* exerts better nutritional properties and hedonic acceptability of the developed natto. Likewise, Sharma et al. (2011) expounded that soybean containing 39.4–44.4% crude protein, 14.0–18.7% oil content and 4.3–6.7% starch are ideal for natto preparation.

Nutritionally natto is a good source of protein (40%) and fat (24.68%) depending upon the fermentation conditions (Jeff-Agboola and Oguntuase, 2006). Likewise, the work of
Premarani et al., (2011) elucidated the influence of fermentation conditions on the nutritional composition of natto. They prepared “Hawaijar” (fermented product of soybean) by natural fermentation of soybean and recorded 62.1% moisture, 1.42% ash, 8.2% crude fibre, 26.02% soluble protein, 3.8% free amino acids, 24.36% fat, 0.9% total soluble sugar and 0.23% reducing sugar content however, inoculation with bacteria improves these traits.

Besides the promising source of protein and oil, raw soybean also contains phenolics that enhance its nutritional worth. In this context, Malenčić et al. (2007) evaluated the antioxidant activity of various cultivars of soybean from different origins. They are of the view that climatic variations, topographical conditions and agronomic practices may impart variations in these traits and detected highest antioxidant capacity of Serbian and Chinese soybean cultivars. Later, Kumar et al. (2010) reported variations in the antioxidant activity in terms of ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and DPPH free radical scavenging activity of soybean cultivars depending upon the cultivation conditions.

Earlier, Moktan et al. (2008) evaluated the differences in antioxidant potential of fermented soybean (Kinema) and non fermented cooked soybean. They reveled that fermented product contained higher total phenolics 144%, antioxidant activity 44%, DPPH Value 147% and Fe$^{2+}$-chelating activity 83-92% as compared to non-fermented cooked soybean (Moktan et al., 2008). Alongside, Korean natto (chungkookjang) exhibited 22.19 mg GAE/g phenolic, 77.5% aglycone and 12% malonylglycoside (Kwaka et al., 2007). The soybean fermentation with Bacillus natto resulted higher DPPH activity and total phenolic contents than that of soaked or cooked soybean (Hu et al., 2010).

2.2.3 Vitamin K requirements

Dietary reference intakes (DRIs) are the reference values employed for planning and evaluating of nutrient consumption in healthy individuals. There are three imperative categories of reference values; adequate intake (AI), recommended dietary allowance (RDA) and tolerable upper intake level (UL). According to the Food and Nutrition Board, RDA is the average amount of daily intake of nutrients required by the majority of the population in each age and gender for the defined period. The AI may require or exceed the amount that is obligate for sustaining nutritional adequacy in most of the population. Conversely, UL is the
maximum amount of daily intake of nutrient utilized by an individual without experiencing harmful or toxic effects (Trumbo et al., 2001).

In this context, vitamin K recommended dietary allowance is expressed in micrograms that fulfill the requirements of various biological activities of the body. The Food and Nutrition Board set the RDA level for vitamin K as 80 μg (176 nmol) and 65 μg (143 nmol) for adult men and women, respectively (Institute of Medicine, 2002). The AI of vitamin K from food sources is increased by 120 μg/day and 90 μg/day for men and women, respectively (Food and Nutrition Board, 2001; Schurgers et al., 2004). Infants require about 2 to 2.5 μg/day of vitamin K that gradually increases up to 30 to 55 μg/day in children. The statistical data from eleven different studies of vitamin K revealed that mean intake of phylloquinone/day in young adults is ∼80μg whereas, older adults consume nearly 2 folds more (Booth and Suttie, 1998). In contrast, the mean daily intake of dihydrophylloquinone is ∼ 20 μg/day in adults (Booth et al., 1999).

In the United Kingdom (UK), the instant dietary vitamin K reference value per day is 1 μg/kg body weight regarded as adequate with respect to maintenance of normal coagulation functions (Booth, 2012). The UK Expert Group on Vitamins and Minerals set the guidance level of vitamin K that one may consume is 1000μg/day in addition to food without any harmful effect. Therefore, tolerable upper dietary intake limit cannot be established (Verkerk and Hickey, 2010) thus, dietary recommendations are established on the assumption of optimal vitamin K amount that requires for maintaining normal blood clotting time (Booth et al., 1996). Currently, Beulens et al. (2013) established the new RDA level by considering the menaquinones in the diet. They also reported that menaquinones contributes about 25% of the total vitamin K intake that involve in biological process of the vitamin K.

Nutritionally, the main distributed form of vitamin K in human body is phylloquinone. The concentration of vitamin K$_1$ or phylloquinone during fasting is varied from 0.15 to 1.0 μg/L in adults (Booth and Rajabi, 2008; Shearer, 2009). In another investigation, Shea, et al. (2009) described the normal range of plasma phylloquinone concentration as 1.6 nmol/L and 0.1 to 3.5 nmol/L in men and women, respectively. In human plasma, phylloquinone content differed from 0.22 and 0.56 ng/mL (Otles and Cagindi, 2007). Earlier, Tsukamoto et al. (2000) revealed that serum phylloquinone concentration in healthy adults is 0.94 ng/mL,
increasing gradually from 11.01 to 24.51 ng/mL after seven days of natto intake. Likewise, Hirauchi et al. (1986) documented mean concentration of menaquinone-6 (MK-6), menaquinone-7 (MK-7) and menaquinone-8 (MK-8) as 0.21, 0.37 and 0.20 ng/mL, respectively in humans. Children have comparatively lesser amount of phylloquinone ranged from 0.04 to 0.98 ng/mL in serum and up to 0.34±0.02 ng/mL in plasma (Jakob and Elmadfa, 2000).

Deficiency of vitamin K repercussion includes increase in bleeding tendency, clotting, blood prothrombin (PT) and activated partial thromboplastin times (APTT). For proper functioning of the blood coagulation parameters, activation of procoagulant proteins is necessary (Shearer, 2009). Insufficient concentration of vitamin K also reduces the level of blood coagulation factors. The vitamin K dependent proteins are corboxylated in the presence of bioactive forms of vitamin K. Thus, inadequate amount of vitamin K resulting in elevated level of undercarboxylated protein species released from liver to the blood stream. These forms of proteins are incapable to bind calcium therefore the normal coagulation cascade is hampered (Schaafhausen et al., 2011).

The amount of PIVKA-II (proteins induced by vitamin K absence or antagonist-II) increases in the blood when the serum phylloquinone level is limited (Yoshihiro and Mitsugi, 2010). Importantly, PIVKA-II is detectable in plasma prior to any change occurring in conventional coagulation parameters. It is measured through high sensitivity sandwich ELISA method (Shearer, 2009). The discrepancies of vitamin K in bones enhance the activity of osteoblasts to secrete under-carboxylated species of osteocalcin (ucOC) into the blood. However, the amount of excreted urinary Gla proteins is also decreased. The international normalized ratio (INR) of blood is elevated as well as the blood prothrombin and active partial thromboplastin times are prolonged, may cause hemorrhagic condition (Gundberg et al., 1998).

In developing countries like Pakistan, large segment of population is commonly utilizing anticoagulated drugs such as coumarin or warfarin and heparin against cardiovascular disorders. These drugs suppress the γ-carboxylation process of vitamin K dependent proteins and inhibit vitamin K cycle by acting as substrate, an alternate of vitamin K epoxide reductase enzyme resulting adverse effects on blood coagulation (Schaafhausen et al., 2011)
and enhancing the level of undercarboxylated forms of osteocalcin and matrix Gla proteins (Nimptsch et al., 2009).

Osteoporosis and related fractures are one of the predominant public health problems in elderly people especially women. Approximately, 30–50% of women and 15–30% of men are suffering from osteoporosis related fractures. However in Pakistani women, this disarray has been reported higher due to the deficiency of health benefit nutrients especially calcium and vitamin D in their daily diet (Akhter, et al., 2004; Fatima, et al., 2009). It has been illuminated that postmenopausal women contain higher amount of vitamin K deficiency biomarkers like ucOC, MGP and PIVKA-II in the serum. Thus, supplementation of phylloquinone and menaquinones ameliorates the vitamin K deficiency in the postmenopausal women (Booth, 2012).

Absorption of vitamin K is interrupted due to pancreatic juice scarcity, biliary hindrance and intestinal mucosal abnormality in alcoholism (Lieber, 2000; Leevy and Moroianu, 2005). Accordingly, deficiency of vitamin K and coagulation parameters of blood are managed by consuming vitamin K enriched diet especially green leafy vegetables and fermented soybean (Ansell et al., 2008; Bolton-Smith et al., 2000).

2.2.4. Deficiency of vitamin K

There are multiple indicators for the measurement of vitamin K robust status or deficiency. Accordingly, various biomarkers are practically available that reflect diverse aspects of vitamin K intake, absorption, transport and functions (Booth, 2009). The most frequent method to estimate the deficiency of vitamin K is plasma phylloquinone fluctuation that is also associated with triglycerides concentration (Booth et al., 1999; Booth and Suttie, 1998; Shea et al., 2007). The amount of serum osteocalcin, uncarboxylated PIVKA-II and MGP are sensitive indicators of deprived status of vitamin K (Iwamoto et al., 2006; Booth and Rajabi, 2008). Moreover, high concentration of undercarboxylated osteocalcin (%ucOC) is considered as a poor vitamin K status in body (Gundberg et al., 1998). Additionally, urinary phylloquinone metabolites concentration is also an important biomarker of vitamin K status because excretion of these metabolites is approximately 20% of the daily physiological dose (200 µg and 1000 µg) of phylloquinone (Harrington et al., 2007).
Neonates are categorized in the distinct risk group because of poor placental transport and low concentrations of vitamin K in breast milk (Shearer, 2009; Eventov-Friedman et al., 2009). However, this incidence is curtailed through intramuscular administered phytomenadione or phyloquinone (Chawla et al., 2007). Infants with cholestasis receiving whey based hydrolyzed formula need supplementation of vitamin K (van Hasselt et al., 2010). Dietary vitamin K inadequacy, vitamin K deficiency induced by anticoagulant (warfarin/coumadin) therapy, genetic loss of important vitamin K-dependent proteins and human polymorphisms or mutations are linked to the age related disorders like bone fragility and arterial calcification associated with cardiovascular complications (McCann and Ames, 2009). The general risk factors for vitamin K deficiency in the hospitalized patients include inadequate dietary intake, antibiotic therapy, malabsorption syndromes (during cholestatic liver disease) and renal insufficiency (Fusaro et al., 2011).

2.3. Absorption and metabolic route

Phyloquinone and MK-7 in healthy volunteers are well absorbed with peak serum concentrations at 4 hr after the intake (Schurgers et al., 2007). Furthermore, vitamin K$_2$ is more stable in serum owing to its long half-life and accumulated about 7 to 8 folds higher during prolonged intake. In another study, humans showed significant amount of phyloquinone in plasma after single serving of carbon-13 labeled kale (400 g) with vegetable oil (30 g).

The pharmacokinetics study indicated that concentration of 13C-phyloquinone increased swiftly and reached at peak between 6 & 10 hr then quickly decreased with average plasma concentration 2·1 nmol/L. The mathematical modeling explicated that mean bioavailability of phyloquinone from kale is 4·7%, whilst tissue and plasma half times are 215 and 8·8 hr, respectively (Novotny et al., 2010).

Previously, intake of 500 mg pure phyloquinone or 150 g raw spinach exhibits serum concentration of phyloquinone under the curve after 9 hr period in 22 to 30 year old subjects. Moreover, the phyloquinone absorption is dependent on the consumption of spinach. In another investigation, Garber et al. (1999) delineated that absorption of phyloquinone from fresh spinach (165 mg/Kg), broccoli (184 mg/Kg) and romaine lettuce
(179 mg/Kg) did not differ. Furthermore, phylloquinone absorption showed non-significant differences after consuming fresh or cooked green leafy vegetables having 30 or 45% energy. Nevertheless, short term variability of K₁ has non-momentous effect on international normalized ratio (INR) (Schurgers et al., 2004). Furthermore amongst orally, subcutaneously and colorectally single dose of vitamin K, the colonic absorptions of three forms of vitamin K i.e. phylloquinone, menaquinone-4, and menaquinone-9 are tremendously low suggesting that physiologically menaquinones in the colon do not contribute momentously in vitamin K status (Groenen-van Dooren et al., 1995). Orally administered vitamin K₁ is converted to MK-4 in several organs of the rats and mouse during 24 hr of administration (Shirakawa et al., 2005).

One of the researchers group, van Hasselt et al. (2009) reported significant increase in plasma vitamin K level after gastric administration of polymeric micelles of vitamin K (1 mg). The bile duct totally restores the absorption of vitamin after duodenal administration with the combination of bile acids. The absorption of phylloquinone is affected after taking 13C-labelled cosmopolitan and animal oriented meals in healthy non-obese subjects (Jones et al., 2009). In low phylloquinone diets, prompt decline was observed in plasma and liver phylloquinone and menaquinone concentrations (Usui et al., 1990). After supplementation, serum phylloquinone concentration increased about 10 folds (Binkley et al., 2000).

Later, Booth et al. (2008) explicated the phylloquinone is converted to MK-4 with the same dose of another dietary form of vitamin K i.e., 2,3-dihydrophylloquinone in male Fischer 344 rats of different ages groups. The level of MK-4 is enhanced in serum, kidney, spleen, testes, brain myelin fractions and bone marrow irrespective of age group with concomitant intake of diet. However, dihydrophylloquinone is absorbed but its intake resulting less MK-4 in certain tissues due to its side phytyl chain length. It is documented that liver phylloquinone concentration is positively correlated with dietary phylloquinone intake. The serum phylloquinone concentration is enhanced when the liver contains sufficient amount of vitamin for the optimal synthesis of vitamin K dependent proteins (Kindberg and Sàoettie, 1989).

The phylloquinone turnover time is about 1.5 day in human subjects. The exchangeable body pool of phylloquinone is decreased after taking vitamin K restricted diet. Likewise, faecal
excretion of phylloquinone and its metabolites are reduced approximately 32% in restricted diet compared to administered dose (1 µg/kg) of vitamin K for 6 day (Olson et al., 2002). Moreover, Harrington et al. (2010) demonstrated that 5C and 7C aglycone are the urinary metabolites of vitamin K nonetheless, their excretion rate is positively correlated with vitamin K dose.

2.4. Coagulation activities

In addition to osteocalcin and clotting factors II, VII, IX and X, the MGP is carboxylated to \( \gamma \)-carboxyglutamate (Gla) for its activation. This process is catalyzed by endoplasmic \( \gamma \)-glutamyl carboxylase enzyme. Moreover, MGP is unique among the vitamin K-dependent proteins owing to the reorganization of serine phosphorylation. This phosphorylation was carried out by the golgi casein kinase (Price et al., 2003; Wajih et al., 2004). The carboxylated-MGP is fully phosphorylated at each target serine residue for normal inhibitory activity to transport calcium (Vanakker et al., 2009).

Vitamin K epoxide reductase complex (VKORC) is a membrane-intrinsic enzyme of endoplasmatic reticulum responsible for the reduction of vitamin K epoxide to vitamin K hydroquinone. During \( \gamma \)-carboxylation process, vitamin K hydroquinone is oxidised to its epoxide. This activity is carried out by single small protein, VKORC1 (Rost et al., 2004; Li et al., 2004; Li et al., 2009). The primary target of warfarin is VKORC1 protein that is actually an enzyme for the reduction of vitamin K epoxide (Rieder et al., 2005; Geisen et al., 2005).

The vitamin K-dependent proteins are blood coagulation factors II (prothrombin), VII, IX, X, protein C, protein S and protein Z. Considering their essential haemostatic role, these proteins are well characterized concerning vitamin K-dependent modification and mode of action. Additionally, proteins \( i.e. \) OC and MGP involve in calcium homeostasis and ligands for receptor tyrosine kinases (growth arrest specific protein 6 and protein S) containing \( \gamma \)-carboxylated residues. For osteocalcin and MGP, the \( \gamma \)-carboxylation is responsible to confer functional \( \text{Ca}^{2+} \)-binding sites that enable these proteins to attach with phospholipids membranes. The impaired carboxylation specifically reduces functionality of the osteocalcin
resulting decline in bone mineral density (BMD) and higher risk of hip fracture (Bugel, 2003).

Furthermore, in severe bleeding phenotypes most of the vitamin K-dependent coagulation factors are abrogating blood coagulation factors (Sun, 1998; Xue, 1998). Additionally for FII and FX, a varying phenotype from mid-embryonic lethality to post-partial death is observed due to massive haemorrhage (Zhu et al., 2007; Rombouts et al., 2009). Furthermore, high fat diet containing eicosapentaenoic acid and docosahexaenoic acid reduces liver vitamin K concentration and activities of coagulant factors Ilc and VII-Xc (Andriamampandry et al., 1998). The administration of MK-7 @ 45 μg/day caused significant decline in undercarboxylated prothrombin (ucFII) by 40%. Moreover, the lower dose of MK-7 (10 and 20 μg/day) reduces the INR by 40 and 60% of individual, respectively (Theuwissen et al., 2013).

2.4.1. Vitamin K and anticoagulants

Generally for anticoagulation therapy, warfarin, caumadin, dicumarol and clopidogrel are used in developed and developing countries (Chan et al., 2009). Moreover, anticoagulants require careful attention owing to their narrow INR range which is the ratio of time required for blood to coagulate relative to that of reference sample (Wadelius et al., 2009). Thus, frequent use of anticoagulation drugs may shift towards high risk of bleeding whilst low intensity leads to thromboembolism (Rombouts et al., 2009). Recently, Chatrou et al. (2013) expounded that vitamin K antagonists inhibit the post-translational carboxylation of the vitamin K dependent proteins as well as synthesized the other functional extra-heptic vitamin K dependent proteins.

The warfarin inhibits vitamin K-dependent carboxylation of coagulant factors in liver and adversely affects physiological mechanisms of affirmation proteins (Villines et al., 2009). Another investigation elucidated that warfarin inhibits the blood coagulation parameters and reduces the activity of vitamin K epoxide reductase resulting lower concentration of osteocalcin and prothrombin (Nimptsch et al., 2009). Thus, these drugs suppress the γ-carboxylation process (Schaafhausen et al., 2010).
Earlier, Sarode et al. (2006) expounded that there is a non-significant association between vitamin K-dependent procoagulant factors II and X and supratherapeutic international normalised ratio. Besides, coumarin has no direct effect on the clearance of vitamin K1 from either plasma or liver. Whereas, the threshold vitamin K1 dose (150 g/day) significantly lowers the INR value in healthy volunteers. Conversely, concentrations of phylloquinone in plasma, liver homogenate and microsome are uplifted after the dose of vitamin K1 (10mg/kg) in normal rabbits and coumarin treated rabbits (Winn et al., 1988). Later, Zivelin et al. (1993) described that warfarin treated rabbits showed depression in factor X and prothrombin whilst other coagulation factors indicated a declining trend.

In warfarin treated rats, aortic calcium concentration is higher (3.0mg/g) that subsequently diminished (1.5 mg/g) with the intake of vitamin K. However, vitamin K enriched diets i.e normal (5 µg/g) or high (100 µg/g) decrease the arterial calcium content about 50% and restore arterial distensibility after 6 weeks (Schurgers et al., 2007b). Recently, Passamonti et al. (2010) established that vitamin K-antagonists i.e. warfarin, coumarin and dicumarol decrease the coagulation factors during venous thrombosis treatment. The anticoagulants along with antimicrobials (thiazine), antimalarials and antibiotics require higher dose of vitamin K post-operatively to counteract the donor–acceptor interaction effect (Dozal et al., 2000).

Vitamin K supplementation (daily amount of 150 µg orally) decreases INR in warfarin treated patients compared with placebo after 6 months. The supplementation of vitamin K reduces the relative variability in daily vitamin K intake thus improves the anticoagulation phenomenon (Sconce et al., 2007). The treatment of brodifacoum prolonged the PT and APTT with normal platelets and fibrinogen levels. The intake of vitamin K for several months is effective to curtail the poisoning of the brodifacoum due to its long half life (Yana et al., 2013).

2.5. Vitamin K-dependent proteins

One of the important vitamin K-dependent proteins i.e. matrix Gla protein (MGP) is present in the vessel wall and cartilage that requires vitamin K for carboxylation (Berkner and Runge 2004; Schurgers et al., 2007). It is mainly secreted through osteoclasts, chondrocytes and
vascular smooth muscles cells and then plays a vital role in tissue calcium homeostasis (Krueger et al., 2009; Zhai et al., 2010). Furthermore, phosphorylation of MGP is carried out at 3 serine residues near N-terminus (Price et al., 1994). The protein shows expression in vascular smooth muscle cells & cartilage through binding calcium ions to its carboxylated Gla residues (Zhai et al., 2010). The vitamin K-dependent proteins are involved in an array of processes that include bone mineralization, arterial calcification, signal transduction, homeostasis, phagocytosis, chemotaxis and growth control (Berkner and Runge, 2004).

The MGP together with other vitamin K-dependent proteins participate in the inhibition of vascular calcification. Availability of vitamin K ensures the activation of these proteins through carboxylation (Geleijnse et al., 2004). Although, during deficiency of vitamin K, the concentration of inactive uncarboxylated MGP (ucMGP) is high that accumulates at the sites of arterial calcification. Moreover, complete absence of MGP leads to bone mineralization disturbance and is responsible for rupturing severely calcified aorta (Luo et al., 1997). After a decade, Cranenburg et al., (2008) reported lower level of serum ucMGP in angioplasty, aortic stenosis, hemodialysis and calciphylaxis patients. The MGP is known to hamper the calcification of arteries (Tabasum and Qadir, 2010). Thus, serum ucMGP is an important tool to identify risk for developing vascular calcification and diagnosis of cardiovascular calcification. One of the potential mode of actions of vitamin K-dependent proteins is direct inhibition of calcium precipitated, crystallization that counterbalance the harmful high intracellular/intravesicular calcium concentrations (Zebboudj et al., 2002). Possibly, the proposed mechanism of vitamin K to inhibit calcification is mineralization of bone through the functioning of MGP. The ucMGP is not detected in the inner most lining of the carotid artery of the healthy individuals. In contrary, the majority of MGP is undercarboxylated in the carotid arterial lining of patients with atherosclerosis (Schurgers et al., 2005).

Vitamin K intake has negative association with blood undercarboxylated osteocalcin (ucOC) concentration. Moreover, in postmenopausal women, ucOC concentration is linearly associated with urinary type-I collagen cross-linked-N-telopeptide (NTX) however, non-significant impact on bone mineral density (BMD). The dietary vitamin K intake also affects the serum levels of ucOC in healthy women. Furthermore, ucOC may link with bone biochemical markers (Yamauchi et al., 2010). Previous intervention studies in human
demonstrated significantly higher carboxylated osteocalcin and cOC:ucOC ratio after supplementation of 45 mg MK-7 whilst inactive ucOC is low. Furthermore, MK-7 supplementation improves the circulating concentrations of MK-7 and carboxylated osteocalcin (van Summeren et al., 2009). Recently, Dalmeijera et al. (2013) reported that circulating non-phosphorylated uncarboxylated MGP (dp-ucMGP) is non-invasive biomarker of vitamin K deficiency in healthy individuals. However, low total uncarboxylated MGP and high non-phosphorylated uncarboxylated levels are associated with development of the coronary artery calcification.

Earlier, Binkely et al. (2000) expounded that mean serum phylloquinone concentration is lower in the youngesters than that of old group without gender differences. Among all supplemented groups, mean percent ucOC declined from 7.6% to 3.4% without significant differences by age or sex. Phylloquinone intake decreases serum osteocalcin level however, does not change other biomarkers of bone turnover. Similarly, phylloquinone ingestion (500 μg/day) inversely associated with interleukin-6 (IL-6) and C-reactive protein (CRP). Moreover, serum ucOC concentration is negatively correlated with IL-6 (Shea et al., 2008).

Likewise, pancreatic insufficient cystic fibrosis in children showed significantly lower level of overall undercarboxylated osteocalcin after supplementation of vitamin K₁ 5 mg/day (Drury et al., 2008).

Recently identified vitamin K dependent protein i.e. Gla-rich protein (GRP) has high Gla residues contents and calcium binding capacity (Viegas et al., 2009). Moreover, Gas6 protein is identified as vitamin K dependent protein comprising of N-terminalg-carboxy-glutamic acid domain (Gla-domain). Regardless of some structural resemblance of Gas6 with protein S, it has different functional properties. Whereas, the main function of protein S is anticoagulation however, the functions of Gas6 are platelet aggregation, leukocyte sequestration & migration and phagocytosis (Tjwaaw et al., 2009). The protein S is plasma glycoprotein that acts as antagonist for blood coagulation. It function as essential cofactor for activated protein C in degradation of coagulation factors FVa and FVIIIa thus operates at central node in the coagulation cascade (Burstyn-Cohen et al., 2009).
2.6. Vitamin K and bone health

Osteoporosis related fractures are the major public health problem affected about 30–50% women and 15–30% of men. In this context, Pakistani women have been reported to bear this risk as high as 83% (Akhter et al., 2004; Fatima et al., 2009). In China, India and Pakistan approximately 163, 25 and 9.91 million population, respectively are suffering in this menace (Malhotra et al., 2008; Mithal and Kaur, 2012). However, Indian and Pakistani women have lower bone mineral density (BMD) than American, placing them at a greater risk of osteoporosis (Hafeez et al., 2009). The under recognition of osteoporosis is mainly due to lack of awareness among the population. In most of developing countries, yet there are no approved national guidelines for the diagnosis and treatment of bone related disorders (Mithal and Kaur, 2012).

The vitamin K supplementation is proved effectual to regulate bone metabolism and consider safe regarding lifestyle related disorders. Moreover, vitamin K is thought to enhance the protein $\gamma$-carboxylation in human (Sogabe et al., 2011). Earlier, Troy et al., (2007) expounded the association between dihydrophylloquinone intake and BMD measured by dual-energy X-ray absorptiometry of the hip and spine in human during cross sectional Framingham Offspring Study. Similarly, long term intake of phylloquinone (PK: 600 mg/kg) increases bone mineral density and menaquinone-4 (MK-4: 600 mg/kg) improves the bone strength parameters like width, trabecular, cancellous ash weight & BMD and body composition. Additionally, serum calcium contents are decreased significantly whilst glucose and total cholesterol varied non-significantly in 6 week old female Sprague Dawley rats (Sogabe et al., 2011).

Calcium phosphate and pyrophosphate dihydrate crystals are most concerned calcium containing crystals in the pathogenesis of osteoarthritis and cartilage calcification (Ea and Liote, 2009). The bone calcium content, femoral dry weight, deoxyribonucleic acid (DNA) content and alkaline phosphatase activity in the metaphyseal and diaphyseal tissues of rats are increased by the oral supplementation of Zn (1.0 mg/100 g) plus MK-7 (0.5 mg/100g) once a day for one week. In this context, Zhong et al. (2001) delineated the synergistic activity of vitamin K (MK-7) and zinc on bone components and their preventing role against osteoporosis through modulating calcium deposition in the bones.
Conversely, one of the researchers groups Apalset et al. (2010) established week association of vitamin K2 intake with bone mineral density in 47 to 50 years old women and men. Similarly, vitamin K1 supplementation (600 μg/day) up to 6 months showed non-significant correlation with regional BMD and serum osteocalcin concentrations in pre- and peri-menopausal women whilst, urinary N-telopeptide concentration was improved significantly in the treated group (Volpe et al., 2008).

Vitamin K1 and vitamin K2 supplementation reduces serum undercarboxylated osteocalcin (ucOC) level regardless of dose though it shows inconsistency with serum total osteocalcin level and bone resorption. The meta-analysis conducted by Iwamotoa et al., (2009) showed that high doses of vitamin K1 and vitamin K2 improve indices of bone strength in the femoral neck and reduce the incidence of clinical fractures other than BMD and bone turnover in postmenopausal women. Likewise, menaquinone is helpful to mitigate bone disorders in women with liver cirrhosis through activation of MGP that helps in calcium plunking towards bones (Tabasum and Qadir, 2010). Furthermore, ucOC is inversely correlated with lumbar BMD. The low vitamin K intake and high level of undercarboxylated osteocalcin (ucOC) are the risk factors for hip fractures (Yamauchi et al., 2010).

All homologs such as phytonadione (vitamin K1), menatetrenone (vitamin K2) and menadione (vitamin K3) are promoted in vitro mineralization by human primary osteoblasts cells. The vitamin K1 induced mineralization is highly sensitive to warfarin when compared with K2 and K3. Similarly, it has been observed that vitamin K plays an important role in the improvement of bone metabolism (Sogabe et al., 2011). Earlier, Atkins et al. (2009) suggested the possible route through which vitamin K optimizes bone formation and integrity by promoting osteoblast-to-osteocyte transition and decreasing the osteoclastogenic potential in the cells. Thus, supported evidences suggest that higher intake of vitamin K is required for optimal bone and vascular health. The synthetic short chain vitamin K1 is commonly used in food supplements however, natural long chain MK-7 is also available (Schurgers et al., 2007). Subclinical vitamin K deficiency is also correlated with higher risk of knee osteoarthritis and cartilage lesions estimated through magnetic resonance imaging (MRI) technique. For the management of the menace, vitamin K has prophylactic potential for osteoarthritis (Misra et al., 2013).
Japanese fermented soybeans “natto” contains large amount of menaquinone-7 that may help to prevent the development of osteoporosis. Previous, Ikeda et al. (2006) found linear association between natto intake and rate of changes in BMD in the postmenopausal women. Conversely, non-fermented soybean products provide non-significant effect on BMD changes in the postmenopausal women. The natto intake may help to prevent post menopausal bone loss through the effects of menaquinone 7 or bioavailable isoflavones that are abundant in natto. The higher intake of natto reduces serum ucOC level in human and associates with bone health in elderly men primarily due to its MK-7 content (Fujita et al., 2010). The increased ucOC/cOC ratio (≥20%) and impairment of γ-carboxylation indicate poor vitamin K status in the population. The vitamin K\textsubscript{2} treated groups has better biomechanical performance indicating its effectiveness against osteoporosis (Tasci, et al., 2011).

2.6.1. Socio-demographic studies and vitamin K

In North America, double-blind, placebo-controlled study was conducted on postmenopausal women administrated orally phylloquinone and MK4 @ 1 mg and 45 mg/day, respectively for 12 months along with vitamin D and calcium supplementation. The phylloquinone and MK-4 treatment decreased serum undercarboxylated osteocalcin concentration (Binkley et al., 2009). Previously, Thane et al. (2002) studied the intake of phylloquinone in various socio-demographic natives of British nationals. They delineated that intake is higher in men than women. The 59% participants have phylloquinone intake below the guidelines of adequacy (1µg/kg body weight per day). Overall, green vegetables contribute 60% of the total phylloquinone intake whereas cooked form around 28%. Afterwards, Gallienia et al. (2013) concluded the from different intervention and double blind studies that vitamin K is positively associated with the BMD and significantly reduces the risk of the clinical fractures of compared to placebo. One of their peers, Iwamoto and Sato (2013) recommended vitamin K especially MK-4 as second-line medicine for postmenopausal osteoporotic women with a higher risk for vertebral fractures.

One of their peers, McKeown et al. (2002) also elucidated lower concentration of vitamin K in men (44%) than women (54%) by estimating through dietary phylloquinone using Food Frequency Questionnaire (FFQ). The phylloquinone and green vegetables intakes are
positively correlated with plasma phylloquinone whilst fasting triglycerides concentration varied non-significantly. Likewise, in earlier study, Geleijnse et al. (2004) noticed that adequate intake of menaquinones is more effective against coronary heart disease than phylloquinone. The provision of intravenous vitamin K$_1$ (10 mg) is associated with reversal of coagulopathies showing variable response in different patients (MacLaren et al., 2001). The vitamin K deficiency has positive association with serum bilirubin, $\gamma$-glutamyltransferase ($\gamma$-GT), alkaline phosphatase (ALP), alanine aminotransferase (ALT) level. Moreover, it is inversely associated with plasma carboxylated osteocalcin concentration (Fisher et al., 2009; Nomoto et al., 2011).

### 2.7. Product development

Different diet based approaches are practice to address the micronutrient deficiencies. Amongst the threat of hidden hunger, vitamin K deficiency can be managed by formulating vitamin K enriched dietary products. In this context, selection of suitable materials, stability, economics, socio-stratum, marketability and hedonic response are the cardinal factors. Different leafy vegetables and fermented soybean prepared by adopting various processing techniques are proved effectual to alleviate the scarcity of vitamin K.

In this context, conventional, pressure and microwave cooked green leafy vegetables showed non-significant variations for vitamin K and minerals contents (Bangash et al., 2011). In leafy vegetables, spinach has exceptionally higher total phenolic contents followed by swamp cabbage, kale, shallots and cabbage (Ismail et al., 2004). After cooking, total antioxidant activity decreased depending on the type of vegetable and methods of cooking (Turkmen et al., 2005). Therefore, processed spinach products are varied significantly for inhibiting thiobarbituric acid reactive substances (Castenmiller et al., 2002).

Vitamin K$_2$ content and superoxide dismutase activity of the soybean are increased through fermentation process at 40-45°C. Moreover, bioactive constituents i.e. daidzein and genistein aglycone are also increased after fermentation with *Bacillus subtilis* BCRC 14715. The functional properties of soybean can be improved further through fermentation with *B. subtilis* (Wu and Chou, 2009). One of the researchers, Takemura (2006) expounded the higher productivity of vitamin K$_2$ especially menaquinone-7 after fermentation of soybean.
with *Bacillus subtilis* strain MH-1. Likewise, Sato *et al.* (2001) revealed maximum concentration of MK-7 as 62.5 mg/L in fermented soybean prepared through fermentation process at 37°C for 24 hrs. The isoelectric point of water soluble MK-7 produced by *Bacillus subtilis* natto showed resemblance with protein. Therefore, MK-7 forms an intracellular complex with protein and then releases in the extracellular fraction during culture process (Yanagisawa and Sumi, 2005).

### 2.7.1. Hedonic response

Sensory response including color, odour, flavor, texture and overall acceptability are the main contributing factors for the acceptance of new products. The sensory response of cooked green vegetables was assessed using ranking test and quantitative descriptive analysis (QDA). The parameters tested were color, aroma, taste and texture. The comparison between conventional and microwave methods showed that color of microwave cooked spinach was better and obtained higher scores. The aroma and texture of cooked spinach in pressure cooking is considered relatively inferior than that of conventionally cooked. It has also been established that conventionally cooked spinach has better taste and overall quality (Kala and Prakash, 2004). Likewise, results of the sensory evaluation of spinach revealed that color is the only attribute that varies to a larger extent due to cooking. The color of conventionally and microwaved cooked spinach is better than that of pressure cooking (Bangash *et al.*, 2011). Moreover, boiled spinach has better sensory response compared to the raw sample (Donadinia *et al.*, 2012).

Sensory evaluation of fermented soybean was performed using 7-point hedonic scale. The prepared natto obtained higher scores for appearance, color, taste and viscosity compared to non-fermented cooked soybean (Kim *et al.*, 1997). Similarly, chungkukjang a traditional Korean bacillus fermented soybean is evaluated for sensory response including taste, flavor and overall acceptability using 9-point hedonic scale (Lee *et al.*, 2007). They noticed that fermented soybean has lower bitterness and high savory flavor. Afterwards, Azokpota *et al.*, (2010) documented the panelists acceptance regarding fermented soybean using 10-points scale. The prepared natto obtained higher scores for flavor as compared to conventional fermented soybean. Similarly, Luo *et al.* (2010) evaluated the sensory attributes *i.e.* appearance, odor, taste and stickiness of douchi (Chinese traditional soybean fermented
product) prepared from different cultivars of the soybean using 5-point score card and found non-significant variations.

Different peers, Youn et al. (2002) and Lee et al. (2005) expounded that chungkukjang made from pure culture of microorganism showed higher preference scores in color, flavor and taste than traditional prepared chungkukjang. Moreover, soybean fermented with B. subtilis TN51 has better aroma than conventionally fermented soybean (Dajanta et al., 2011). In another study, sensory attributes of natto including color, aroma, stickiness, bitterness, sweetness, sourness and chewiness were estimated using continuous linear intensity-scale (10 cm) though various demographic panelists (Japanese, Chinese and American). There were non-significant differences in flavor, sweetness or sourness among the experimental and commercial reference natto are noticed (Wei et al., 2004).

Likewise, Lee et al. (2005) determined the sensory response of fermented soybean using 5-point scale with the levels 5, 4, 3, 2 and 1 indicating very strong or very good, strong and good, moderate, weak or poor and very weak, respectively. The sensory evaluation of bitter, sweet & savory tastes, flavor, color and overall preferences of chungkukjangs prepared with 6 different bacterial strains was conducted. Amongst those, B. subtilis inoculated chungkukjangs was stronger in sweet & savory taste, color and general preference.

2.8. Efficacy study

One of the major causes of under nutrition is the poor dietary intake with lesser level of bioavailable micronutrients. Generally, lifestyle preferences and poverty are the main factors responsible for inadequate intake of fruits, vegetables and animal products leading to the deficiency of various micronutrients like vitamin K that can be improved through diet based strategies (Allen and Gillespie, 2001).

The supplementation of vitamin K in the form of phyloquinone and MK-7 significantly enhances the serum vitamin K\(_1\) level (Schurgers et al., 2007; Novotny et al., 2010). Earlier, Vervoort et al. (1997) expounded that vitamin K\(_1\) and vitamin K\(_2\) are protecting from melanaldehyde products generated during lipid peroxidation of liver microsomes by enhancing the activity of epoxide reductase and \(\gamma\)-glutamylcarboxylase. The liver functioning enzymes \(i.e.\) aspartate transaminase (AST) and alanine aminotransferase (ALT) activities are
recorded in the normal range after the intake of vitamin K₁ in rats (Shirakawa et al., 2005). Similarly, Binkely et al. (2009) revealed that ucOC content decreased after the oral supplementation of phylloquinone (1 mg/day) and MK-4 (45 mg/day). However, creatinine varied statistically non-significant whereas, osteocalcin content was increased in phylloquinone supplemented groups compared to placebo. The vitamin K₂ (30 mg/kg/day) treatment has no effect on ALP with considerable biomechanical performance of bone indicating its effectiveness against osteoporosis (Tasci et al., 2011).

Meanwhile, Sogabe et al. (2011) conducted a long term study of phylloquinone and MK-4 consumption in rats and found increased serum triglycerides in phylloquinone and MK-4 administrated groups. There were non-significant differences in the levels of serum phosphorus, alkaline phosphatase, growth hormone and insulin-like growth hormone-1. Earlier, Booth et al. (2004) observed a positive correlation between serum phylloquinone and triglycerides level. They noticed higher triglycerides in men 142±97 mg/dL as compared to women 130±72 mg/dL due to higher phylloquinone intake. Likewise linear association between these traits has been reported by the various scientific groups including McKeown et al. (2002) and Booth et al. (2004).

Afterwards, Thane et al. (2006) observed a correlation between higher phylloquinone level and lipid profile indicators. They disclosed that cholesterol & LDL concentrations were decreased non-significantly. Moreover, vitamin K at 1 to 10 mg/kg/day for a period of 56 days significantly reduces the total cholesterol (Eventov-Friedman et al., 2009). During vitamin K deficient conditions, ucMGP and PIVKA-II concentrations are increased. These proteins have linear association with total cholesterol (Nimptsch et al., 2009). Furthermore, higher PIVKA-II level resulting elevation in serum conjugated bilirubin, bile acids, aspartate aminotransferase, alanine aminotransferase, PT, INR, and serum ucOC however, vitamin K provision cause normalization in these parameters (Strople and Heubi, 2009).

The vitamin K supplementation does not impart any deleterious effect on tested animal organs. Moreover, body weight, weight gain/feed ratio and hematological parameters like hemoglobin and bilirubin levels are varied non-momentously in control and menadione relied groups. The weight of liver, kidney and spleen as well as the morphological structure of
parenchyma cells of the menadione fed animals did not show any modification compared to control (Marchetti et al., 1995).

Later, Rennenberg et al. (2010a) reported that warfarin intake increased serum total cholesterol, LDL cholesterol and creatinine as 5.5 to 5.9 mmol/L, 3.7 to 4.1 mmol/L and 79 to 81 µmol/L, respectively. In another study, Villines et al., (2009) expounded that warfarin significantly enhanced the creatinine concentration whilst, total cholesterol, LDL, triglycerides, calcium and phosphorus levels were varied non-significantly in older people having BMI up to 28 kg/m². The hypertensive patients during vitamin K deficiency showed higher amount of ucMGP, total cholesterol and LDL levels (Rennenberg et al., 2010b). Significant relationship of arterial calcium score and high undercarboxylated osteocalcin (reflecting low vitamin K status) with serum ucMGP level in mild to moderate hypertensive patients stimulates the calcification process (Rennenberg et al., 2010b).

Phytonutrients in spinach considered safe and recommended for curtailing different physiological disparities. The spinach based antioxidants inhibit the activity of lipoxygenase enzyme. The outcomes of different bioevaluation trials using mouse, rat and rabbit proved the antioxidant worth of spinach (Lomnitski et al., 2003).

Likewise, natto inhibits the oxidation of low-density lipoproteins (LDL) alongside plasma triglycerides and total cholesterol reduction in the experimental rats (Iwai et al., 2002). Another researchers group documented the antioxidant activity of fermented soybean that hinders the formation of malondialdehyde and LDL oxidation (Kim et al., 2008). Earlier, Yokota et al. (1996) delineated that natto significantly reduced the serum thiobarbituric acid reactive substances (TBARS), total cholesterol and LDL-cholesterol in dose dependent manner.

The intake of MK-4 did not affect the body weight and food consumption however, mild changes were observed in the calcium concentration, blood chemistry and urine analyses. The hematological variations were limited to significant elevation in platelet count. During necropsy, organ weights including liver, kidneys and spleen of MK-4-treated rats were comparable with that of control nevertheless, histopathology did not reveal any abnormality (Doi et al., 1995). Similar results were also observed by Goldsmith et al. (1995). Moreover,
higher dose of vitamin K showed non-significant differences in body weight but momentous increased platelet count and red blood cells (Pucaj et al., 2011).

The MK-4 has ability to inhibit the synthesis of adipocytes and stimulates the activity of alkaline phosphatase; an early differentiation marker of osteoblast. It also hinders the adipogenesis and osteoclastogenesis in bone marrow cells and favors bone metabolism by modulating cellular differentiation (Takeuchi et al., 2000). The dietary intake of menaquinones estimated through food-frequency questionnaires showed inverse association with prostate and lung malignancies (Nimptsch et al., 2010). The vitamin K₁ and MK-4 block 12-lipoxygenase activation and prevent reactive oxygen spices (ROS) accumulation in developing oligodendrocytes ultimately inhibit oxidative cell death (Li et al., 2009).

Phylloquinone (vitamin K₁) or menaquinone (vitamin K₂) forms of vitamin K are generally considered as safe. Therefore, tolerable upper level (UL) of vitamin K has not been yet established (Food and Nutrition Board, 2001). However, synthetic menadione (vitamin K₃) may interfere with the function of glutathione resulting in oxidative damage to the endothelial cells (Wang et al., 2008). There is no evidence of adverse effect by consuming phylloquinone up to 10 mg/day. Similarly, the Expert Group on Vitamins and Minerals has also established the guidelines for the intake of vitamin K₁ up to 1 µg/kg body weight, regarded as safe (EVM, 2003). Moreover, no specific adverse effects are documented after the administration of phylloquinone (1 mg/day/person) and MK-4 (45 mg/day/person) in humans (Binkley et al., 2009).

Conclusively, from the aformentation discussion it is suggested that vitamin K is essential for the proper blood clotting system and also helpful for the bone related biochemical mechanisms. For the management of blood and bone related abnormalities vitamin K enriched sources such as green leafy vegetables and fermented soybean are helpful. Considering the above mentioned potential of the spinach and fermented soybean, present research was designed to the evaluated the indigenous sources for the menace.
CHAPTER 3

MATERIALS AND METHODS

The present study was carried out in the Postgraduate Research Laboratory, National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad. The vitamin K dependent proteins assays were conducted in the Department of Biochemistry, Maastricht University, the Netherlands. Indigenously cultivated spinach and soybean were used as vitamin K enriched dietary sources for in vitro studies. Moreover, in vivo evaluation of the tested vitamin K sources alongside synthetic menadione was conducted. Materials used and protocols followed are discussed herein;

3.1. Materials

Spinach variety Desi Palak while soybean cultivar namely Faisal Soybean were procured from Ayub Agriculture Research Institute (AARI) Faisalabad. Synthetic menadione was purchased from Sigma Aldrich (Tokyo, Japan). Analytical reagents and HPLC grade standards were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich. For the efficacy trial, male New Zealand rabbits were housed in the Animal Room of the NIFSAT. Diagnostic kits were purchased from Sigma-Aldrich (Bioassays Chemical Co. Darmstadt Germany) and Cayman Chemicals (Cayman Europe, Estonia) for biological assays.

3.2. Characterization of spinach and soybean

Spinach and soybean samples were analyzed for moisture, crude protein, crude fat, crude fiber, ash and nitrogen free extract by using triplicate samples.

3.2.1. Moisture content

Moisture content in spinach and soybean were evaluated by drying sample in Air Forced Draft Oven (Model: DO-1-30/02, PCSIR, Pakistan) at 105±5 °C till constant weight by following the procedure AACC (2000) Method No. 44-15A.

3.2.2. Crude protein

Estimation of crude protein contents of spinach and soybean were carried out through nitrogen determination in the sample multiplying with factor (6.25) using Kjeltech Apparatus

3.2.3. Crude fat

Soxtec System (Model: H-2 1045 Extraction Unit, Hoganas, Sweden) was used to determine the crude fat content in respective samples using hexane as solvent according to the procedure described in AACC (2000) Method No. 30-25.

3.2.4. Crude fiber

Crude fiber content of fat free sample was estimated by simmering initially with 1.25% H₂SO₄ solution for 30 min followed by 1.25% NaOH solution in Labconco Fibertech apparatus (Labconco Corporation Kansas, USA) as method in AACC (2000) Method No. 32-10.

3.2.5. Ash

Ash contents of oven dry samples were calculated through charring followed by direct incineration at 550 °C in Muffle Furnace (MF-1/02, PCSIR, Pakistan) till grayish white residue (AACC, 2000; Method No. 08-01).

3.2.6. Nitrogen free extract (NFE)

Nitrogen free extract (NFE) was estimated by following the expression:

\[
NFE \% = 100 - (\text{moisture} + \text{crude protein} + \text{crude fat} + \text{crude fiber} + \text{ash})\% ......... (1)
\]

3.3. Minerals profile

The samples were subjected to mineral assay through wet digestion considering the protocols of AOAC (2006). For the estimation of sodium and potassium, Flame Photometer-410 (Sherwood Scientific Ltd., Cambridge, UK) was used whilst calcium, iron, magnesium, zinc and copper were measured through Atomic Absorption Spectrophotometer (Varian AA240, Victoria Australia).
3.4. Extraction of vitamin K

3.4.1. Preparation of sample

For the extraction of vitamin K, spinach and soybean were ground separately in a blender to uniform consistency. The 5 g of anhydrous sodium sulfate was added in weighed amount of each raw material and further pulverized. The resultant powder and 20 µL of internal standard (200 ng of dihydrophylloquinone) were transferred to 50 mL centrifuge tube. Afterwards, 15 mL of 2-propanol/hexane (3:2 v/v) and 32 mL of H₂O were added in the mixture and centrifuged (4000 rpm) for 5 min. The supernatant layer containing phylloquinone and dihydrophylloquinone was transferred to clean amber color tube and evaporated under nitrogen stream. The residue was redissolved in 10 mL hexane with further purification through solid-phase silica gel.

3.4.2. Quantification of vitamin K

Extracted samples were quantified by HPLC (Perkin Elmer, Series 200, USA) using C¹⁸ column (250 mm x 4.6 mm, 5.0 µm particle size). For the intention, 10 µL aliquot of sample was injected via auto sampler (WISP Model 710). The column temperature was maintained at 40 °C. The mobile phase comprised of dichloromethane (100 mL), methanol (900 mL), zinc chloride (1.37 g), sodium acetate (0.41 g) and acetic acid (0.30 g). The flow rate was adjusted at 1 mL/min. Quantification of vitamin K was carried out using UV/vis detector (model 481) at 249 nm (Majchrzak, and Elmadfa, 2001).

3.5. In vitro studies

For in vitro studies, methanol and ethanol extracts of spinach and soybean were tested for their antioxidative properties.

3.5.1. Total phenolics

Total phenolic content (TPC) of each extract was determined by using Folin-Ciocalteu reagent (Singleton et al., 1999). A 50 µL sample was mixed with 250 µL of 2N Folin-Ciocalteu’s reagent. Later, 750 µL of 20% Na₂CO₃ solution and distilled water were added to make the volume 5 mL. After 2 hr, absorbance was measured at 765 nm using UV/vis
Spectrophotometer (CECIL CE7200). Total phenolic content was calculated and expressed as gallic acid equivalent (mg gallic acid/100g).

3.5.2. Antioxidant activity

Antioxidant activity of both spinach and soybean extracts was assessed by coupled oxidation of β-carotene and linoleic acid as described by Taga et al. (1984). Briefly, β-carotene (1.0 mg) was dissolved in 10 mL of chloroform. The 1 mL prepared solution was taken in flask containing linoleic acid (20 mg) and tween 40 (200 mg). The chloroform was removed using Rotary Evaporator at 40 °C. Gradually, 50 mL of distilled water was added to the flask with vigorous shaking to form an emulsion. Subsequently, 5 mL emulsion was mixed with 0.2 mL sample in test tube. After shaking, absorbance was recorded at 470 nm. Test tube was placed in a water bath equipped with agitation at 50 °C and reading was measured after every 10 min interval up to 40 min.

\[
\ln (a/b) \times \frac{1}{t} = \text{degradation rate of sample} \quad (2)
\]

\[
\ln = \text{natural log}
\]

\[
a = \text{initial absorbance (470 nm) at time zero}
\]

\[
b = \text{absorbance (470 nm) after 40 min}
\]

\[
t = \text{time (min)}
\]

Antioxidant activity (AA) was expressed as % inhibition relative to control

\[
AA = \frac{\text{Degradation rate of control} - \text{degradation rate of sample}}{\text{Degradation rate of control}} \times 100 \quad (3)
\]

3.5.3. Free radical scavenging ability

Free radical scavenging activity of spinach and soybean extract was estimated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as described by Muller et al. (2011). Shortly, 125 μL samples were mixed with 4 mL DPPH (1.2 mM) in methanol solution. Absorbance was measured after 30 min at room temperature using Spectrophotometer (CECIL CE7200) at 520 nm. The inhibition of free radicals by DPPH was calculated through following expression:

\[
\text{Inhibition} (%) = [100 \times (A_{blank} - A_{sample}) / A_{blank}] \quad (4)
\]
3.5.4. Ferric reducing antioxidant power (FRAP)

The ability of reduced ferric ions was measured by following the method of Muller et al. (2011). An aliquot of spinach and soybean (50 μL each) was taken with 3 mL of FRAP reagent following incubation at 37 °C for 30 min. The increase in absorbance was noted at 593 nm using Spectrophotometer. The results were compared with the calibration curve, prepared by using various concentrations of trolox as standard.

3.6. Product development

Four different products were developed from the tested raw materials i.e. spinach and soybean. For the purpose, cooked & reconstituted spinach were prepared from fresh and dehydrated spinach, respectively. However, rest two was formulated from soybean using different fermented conditions after inoculation with Bacillus subtilis.

3.6.1. Preparation of cooked and reconstituted spinach

3.6.1.1. Cooked spinach

After cleaning, spinach was washed and cut into small pieces. Cooking condition was estimated with preliminary trails and noted the minimum cooking time for an adequate palatability and taste. For cooking, 100 g cleaned spinach was cooked in covered stainless steel pan for 10 min on moderate flame. The prepared cooked spinach considered as T₁ was stored at refrigeration temperature for further analysis (Sultana et al., 2008).

3.6.1.2. Reconstituted spinach

For drying, 100 g of spinach was spread to a thickness of 1.4 cm on the tray (0.09 m²) in the force air dry cabinet. The drying temperature was maintained at 50 °C with 1.2 m/sec air velocity. The drying process was continued until the moisture content of the sample reached 15 ± 2% (Doymaz, 2009). The dried sample was cooled under room temperature and kept in airtight jar covered with aluminium foil. For reconstitution purpose, 50 g dry spinach was added to 100 mL of boiling water, heated for 10 min and labeled as T₂.

3.6.2. Preparation of Natto

For natto preparation, overnight soaking of soybean was done with 3 times volume of water. Afterwards, beans were steamed for 30-45 min until crushed easily and inoculated with
prepared culture medium of *Bacillus subtilis* gifted from Nattomoto, Yuzo Takahashi Laboratory Co. Japan (0.1 g powder culture dissolved in 10 mL of cooled pasteurized water) at 45 °C in a sterilized bowl. The treated beans were packed in plastic box and covered with sterilized cotton cloth to prevent drying in the subsequent steps. Fermentation of the steamed soybean was done at two different conditions *i.e.* 40 °C for 24 hr (T₃) and 35 °C for 30 hr (T₄) in the incubator.

3.7. Analysis of vitamin K enriched dietary sources

Proximate analysis of cooked and reconstituted spinach and two types of natto were carried out following the instructions of AACC (2000). Mineral content of developed products was determined by the method of AOAC (2006). The phylloquinone and menaquinone were estimated by the respective method as discussed under the section quantification of vitamin K. Moreover, *in vitro* studies including phenolic contents, antioxidant activity, free radical scavenging activity and ferric reducing antioxidant power were also performed through their respective protocols.

3.8. Sensory evaluation

The vitamin K enriched dietary sources (T₁, T₂, T₃ & T₄) were tested for their sensory response using 9-point hedonic scale system *i.e.* 9=like extremely;1=dislike extremely as mentioned in Appendix-I following the instructions of Meilgaard *et al.* (2007). Accordingly, hedonic behavior of vitamin K enriched dietary products was assessed for various quality traits such as color, taste, flavor, texture and overall acceptability. Sensory evaluation of the dietary products was conducted in the Sensory Evaluation Laboratory of the National Institute of Food Science and Technology, University of Agriculture, Faisalabad. For evaluation, vitamin K enriched dietary foods were presented to the sensory panelists in the transparent plates labeled with random codes in separate booths equipped with fluorescent light. Mineral water and unsalted crackers were also provided to the panelist to neutralize their mouths receptors after testing the products. To avoid biasness, samples were offered to the judges randomly and requested to assign scores for mentioned traits.
3.9. Selection of best products

On the basis of HPLC quantification of vitamin K, antioxidant potential and sensory response of formulated products, one best product each from spinach (T2) and natto (T3) were selected for bio-efficacy study.

3.10. Bioevaluation studies

Efficacy studies were carried out to assess the biochemical performance of vitamin K enriched dietary sources. For the first trial, 60 experimental rabbits were housed under control conditions in the Animal Room of NIFSAT, University of Agriculture Faisalabad. The rabbits were acclimatized by feeding the basal diet for a period of one week. The environmental conditions were maintained throughout the trial i.e. temperature (23±2 ºC), relative humidity (50±20%) and air ventilation (10-15 times/day) with 12 hr light-dark cycle. At the initiation of trial, some rabbits were scarified to establish baseline values. In this connection, two types of studies were designed; study I (normal rabbits) and study II (vitamin K deficient rabbits). To induce vitamin K deficiency, warfarin and ciprofloxin were administrated to the target group of rabbits through oral gavage. Each study was subdivided into four groups of rabbits, seven in each. Accordingly, four types of diets including control (D1), spinach based as a source of phylloquinone @ 6 g/day (D2), natto based as a source of menaquinones @ 200 mg/day (D3) and their synthetic counterpart menadione @ 1 µg/ kg (D4) were given to respective groups considering their bioavailability. Feed and drink intakes were measured throughout the experimental period. During 56 days trial, continuous provision of vitamin K enriched diets was assured to evaluate their effect on the blood coagulation assays of respective group of rabbits. The bleeding time (BT) of rabbits was recorded by giving an incision on the ear. Next, whole blood was taken in a test tube for the estimation of clotting time (CT). The experimental animals were scanned for their bone mineral density through dual-energy X-rays absorptionmetry (DXA). For biochemical evaluation, overnight fasted rabbits were sacrificed followed by blood collection in sodium citrate & pro-coagulant coated tubes for plasma & serum analysis, respectively. The plasma tests i.e. prothrombin time (PT), activated partial thromoplastin time (APPT), plasma fibrinogen (PF) and international normalized ratio (INR) level were performed along with vitamin K dependent proteins. Further, serum was separated by centrifugation @ 4000 rpm
for 6 min through Centrifuge Machine (Model: 800, China). The respective sera from each group were analyzed for serum vitamin K content *i.e.* phylloquinone, menaquinone-4 and menaquinone-7. The serum antioxidant status was also estimated though (TBARS) assay. Moreover, serum lipid profile (cholesterol, LDL, HDL & triglycerides), liver & kidney functioning and electrolytes balance were assessed though respective kits using Microlab 300, Merck, Germany. Lastly, the part of blood collected in EDTA coated tubes was analyzed for various hematological parameters as red and white blood cells indices. Likewise study module was followed during trial II to validate the results regarding biochemical performance of developed vitamin K enriched products.

**Study I: Normal rabbits**

In this segment, rabbits were divided into four homogeneous groups and fed on normal feed with simultaneous intake of vitamin K enriched diets (D₁, D₂, D₃ & D₄). Feed and drink intakes of rabbits were measured on daily basis whilst weight on weekly schedule till the termination of trial. To determine the effect of experimental diets, bone mineral density, blood coagulation indicators, vitamin K dependent proteins and serum vitamin K content were assessed.

**Study II: Vitamin K deficient rabbits**

In study II, rabbits were given warfarin (0.1 mg/kg/day) and ciprofloxacin (10 mg/day) for a period of 15 days to induce vitamin K deficiency in addition to basal diet. Similar biochemical and bone biomarkers analyses were performed as mentioned in study I. The warfarin binds the serum phylloquinone that is biochemically active, whereas ciprofloxacin prevent the gut synthesis of the vitamin K through bacteria.

**3.10.1. Physical parameters**

Following physical parameters were measured throughout the experiment.

**3.10.1.1. Feed and water intakes**

Feed intake of individual rabbit was estimated by excluding spilled from the total diet on daily basis during the entire study period. Similarly, water intake was also assessed (Wolf and Weidbrode, 2003).
Table 1: Plan for efficacy study of vitamin K enriched dietary products

<table>
<thead>
<tr>
<th>Groups</th>
<th>Study I</th>
<th>Study II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Vitamin K deficient rabbits</td>
</tr>
<tr>
<td>Groups</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dietary sources</td>
<td>D₁</td>
<td>D₁</td>
</tr>
<tr>
<td>Dietary sources</td>
<td>D₂</td>
<td>D₂</td>
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<td>Dietary sources</td>
<td>D₃</td>
<td>D₃</td>
</tr>
<tr>
<td>Dietary sources</td>
<td>D₄</td>
<td>D₄</td>
</tr>
</tbody>
</table>

The prepared diets were fed to the experimental animals that provide the dose of vitamin K @ 1µg/kg/day
D₁: Control
D₂: Spinach (phytolquinone)
D₃: Natto/Fermented soybean (menaquinones-7)
D₄: Synthetic menadione
3.10.1.2. Body weight gain

In both studies, body weight gain of experimental rabbits was recorded on weekly basis to find out the effect of vitamin K enriched diets.

3.10.2. Coagulation assays

Different coagulation indicators including bleeding & clotting time, blood prothrombin time (PT), activated partial thromoplastin time (APTT), plasma fibrinogen (PF) and international normalized ratio (INR) levels were measured during both studies in all groups to determine the influence of experimental diets.

3.10.2.1. Bleeding & clotting time

Bleeding and clotting times were measured following the guidelines of Hogmoa et al. (2008) and Johnson et al. (2008), respectively. For bleeding time, an incision was made on the back of the rabbit ear. The time in second was recorded from start of incision and termination of bleeding. Likewise for clotting time, freshly drawn blood was taken in the test tube and placed in water bath at 37 °C. After 5 min, test tube was tilted to 45° angle, repeated after every 30 sec until the blood clotted completely.

3.10.2.2. Blood prothrombin time (PT)

Blood prothrombin time was measured in rabbit’s plasma adopting the protocols of Mochizuki et al. (2009). The collected plasma was analyzed by Coagulometer ACL 100 (Instrumental Laboratory, Lexington, MA, USA). In this context, plasma was mixed with tissues factor protein containing phospholipids at 37 °C. Afterward, calcium chloride (25 mM) was also added to initiate the formation of clot and noted the time.

3.10.2.3. Activated partial thromoplastin time (APTT)

Activated partial thromoplastin time (APTT) was recorded by following the protocol of Mochizuki et al. (2009).

3.10.2.4. Plasma fibrinogen level

The level of plasma fibrinogen in all groups was estimated by following the instructions of Mochizuki et al. (2009).
3.10.2.5. International normalized ratio (INR)

The protocol of Mochizuki et al. (2009) was adopted to calculate the INR.

3.10.3. Bone mineral density

Bone mineral density (BMD) of rabbits was measured using dual-energy X-rays absorptionmetry (DXA) following the instruction of Troy et al. (2007). The pre-anesthetized rabbit was laid on DXA scan bed for simultaneous x-ray and laser image. The data obtained from DXA scan software system was presented in term of whole body BMD.

3.10.4. Vitamin K dependent proteins

The vitamin K dependent proteins including osteocalcin, undercarboxylated osteocalcin and protein induced vitamin K absence or antagonist-II were estimated through the ELISA method using commercial kits procured from MyBioSource, Inc., San Diego, USA and TalaRa, Takara Bio Europe/SAS France following the method of Martini (2006).

3.10.4.1. Osteocalcin (OC)

For the estimation of osteocalcin (OC), standards were prepared according to the guidelines of MybioSource. In antibody pre-coated microtiter plate, 50 μL plasma was added followed by 100 μL of horseradish peroxidase (HRP)-conjugated antibody, mixed and incubated for 1 hr at 37 °C. The HRP conjugate was used to enhance the detection of OC. Later, the incubated mixture was removed from the well though automated washing buffer (350 μL/well). The substrates A and B were added 50 μL separately to each well. The micortiter plate was covered and incubated for 15 min at 20-25 °C. Finally, 50 μL stop solution/ sulfuric acid (0.18 mol/L) was added in adduct to impede the reaction. Immediately, optical density of the resultant mixture was recorded at 450 nm using microtiter plate reader (BioTek, ELX800, Winoski, USA).

3.10.4.2. Undercarboxylated osteocalcin (ucOC)

The separated plasma sample was also analyzed for ucOC content by following the protocol of TakaRa. Purposely, plasma (100 μL) was taken in microtiter plate well and incubated for 2 hr at 37 °C. The biotin antibody (100 μL) was added in each sample followed by incubation for 1 hr. Further, the sample was washed for 2 min with 200 μL buffer. The HRP-conjugated
antibody (100 μL) was mixed and placed for 1 hr. Subsequently, 90 μL of TMB (3,3',5,5' tetramethyl-benzidine) substrate was added. After 30 min, stop solution (50 μL) was gently mixed and optical density (OD) was recorded at 540 nm with reference wavelength 650 nm.

3.10.4.3. Protein induced by vitamin K absence or antagonist-II (PIVKA-II)

For the assessment of PIVKA-II, 100 μL of plasma sample was incubated at 37 °C for 30 min in microtiter plate. After washing with buffer (200 μL), HRP-conjugated antibodies (100 μL) was added. The mixture was incubated at 37 °C for 30 min. The 90 μL of TMB substrate was added followed by incubation for 20 min. Lastly, stop solution (50 μL) was added gently and mixed until blue color developed. The reading was recorded at 450 nm after 30 min through ELISA Reader (BioTek, ELX800, Winoski, USA).

3.10.5. Serum vitamin K contents

The serum vitamin K contents after the administration of the spinach (D$_2$), natto (D$_3$) and menadione (D$_4$) were quantified through HPLC using C$_{18}$ column (Otiles and Cagindi, 2007). For the extraction of serum vitamin K, 2 mL sample was mixed with 3 mL hexane and 1 mL ethyl acetate. The resultant organic layer was washed by mixing with 10 mL methanol/water (9:1, v/v). Sample was vortexed for 15 min and centrifuged at 2000g for 10 min. The supernatant was evaporated with nitrogen. Immediately, 200 μL methanol was added to the residue and mixed for 5 sec. The sample was transferred to an injection vial for HPLC quantification. The mobile phase comprised of acetonitrile/dichloromethane/methanol (60:20:20 v/v/v) with flow rate of 1 mL/min. The serum vitamin K$_1$, MK-4 and MK-7 concentrations were recorded simultaneously in each sample.

3.10.6. Serum TBARS

Serum lipid peroxidation was estimated by calculating the amount of malondialdehyde (MDA) as per instructions of Choi _et al._ (2010). Shortly, 100 μL serum was mixed well with 500 μL of each 2.5% trichloroacetic acid (TCA) and 1% thiobarbituric acid (TBA). After boiling at 104 °C for 15 min, adduct was cooled at room temperature followed by centrifugation at 3000g for 10 min at 4 °C. The absorbance of supernatant was noted at 532 nm using Spectrophotometer. The results were expressed as nmole of malenaldehyde/mL.
3.10.7. Serum lipid profile

Serum lipid profile including cholesterol, low density lipoproteins, high density lipoproteins and triglycerides were measured with their respective protocols. The further explanation is given below;

3.10.7.1. Cholesterol

Serum cholesterol level was measured by using CHOD–PAP method following the protocol of Lee (2006).

3.10.7.2. Low density & high density lipoproteins

Low density lipoproteins (LDL) was determined by the method of Lee (2006) whilst, high density lipoprotein (HDL) through cholesterol precipitant method as described by Alshatwi et al. (2010).

3.10.7.3. Triglycerides

Triglycerides in serum samples were measured by liquid triglycerides (GPO–PAP) method as outlined by Kim et al. (2012).

3.10.8. Liver and kidney functioning tests

Liver functioning tests including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated using commercial kits (Sigma 59-50 and 58-50, respectively) following the protocol of Basuny (2009). For kidney performance, serum urea concentration was measured by GLDH-method whilst creatinine level through Jaffe-method using commercial kits (Jacobs et al., 1996; Thomas, 1998).

3.10.9. Hematological aspects

Collected blood samples were analyzed for their red and white cells indices. The red blood cell parameters including total red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscle volume (MCV) were estimated. Likewise, white blood cell traits comprised of total white blood cells, neutrophils, monocytes and lymphocytes were assessed using Automatic Blood Analyzer (Nihon Kohden, Japan).
3.10.10. Electrolytes balance

Electrolytes balance indicators *i.e.* Na, K and Ca were estimated by using their respective protocols (Al Haj *et al.*, 2011)

3.10.11. Statistical Analysis

The obtained data were subjected to statistical analysis using completely randomized design (CRD) by Statistical Package (Costat-2003, Co-Hort, version 6.1). Level of significance was determined through analysis of variance (ANOVA) technique following the principles outlined by Steel *et al.* (1997).
CHAPTER 4

RESULTS AND DISCUSSION

In the present investigation, the indigenously grown soybean (Faisal Soybean) and spinach (Desi Palak) were tested with special reference to vitamin K contents. The instant study was divided into three segments, firstly nutritional profile of raw materials was carried out. In the second phase, four vitamin K enriched dietary products were prepared, two from each raw material \textit{i.e.} spinach (cooked & reconstituted) and soybean (natto A & B). On the basis of vitamin K quantification, antioxidant potential and hedonic response, one product from each raw material was selected for bioefficacy studies. Lastly, bioevaluation of vitamin K enriched products was carried out through a model feeding trial, involving normal and vitamin K deficient New Zealand rabbits. Accordingly, blood coagulation parameters, bone mineral density (BMD), vitamin K dependent proteins and serum vitamin K content were estimated. A comprehensive debate regarding the investigated parameters is herein:

4.1. \textbf{Nutritional analysis of raw materials}

4.1.1 \textbf{Proximate composition}

Means for the proximate composition of spinach indicated moisture, crude protein, crude fat, crude fiber, ash and nitrogen free extract (NFE) as 90.71±4.14, 2.03±0.95, 0.32±0.007, 0.58±0.02, 1.24±0.06 and 5.01±0.11%, respectively. Whereas, tested soybean showed the values 8.96±0.45, 32.28±1.99, 18.64±1.02, 2.93±0.16, 3.38±0.19 and 33.79±1.15% for respective traits (Table 2).

The results of instant research are synchronized with the previous findings of USDA (2010), reported 91.40, 2.86, 0.39, 2.2 and 3.63% of moisture, protein, total lipids, fiber and carbohydrates, respectively in raw spinach. Similarly, Hussain \textit{et al.} (2010) investigated the proximate composition of spinach and documented the values for protein, fat, fiber and carbohydrates as 20.82, 3.325, 4.92 and 48.82 %. Likewise, Bangash \textit{et al.} (2011) also carried out a nutritional analysis of Pakistani spinach (\textit{Spinacea oleracea}) and noticed 92.70±0.13, 2.5±0.04, 0.29±0.01, 3.91±0.02, 0.70±0.06 and 1.90±0.002% of moisture, protein, fat, carbohydrates, fiber and ash contents, respectively. The compositional variations
in present study are might be due to varietal differences, climatic changes, different topographic locations and variations in agronomic practices.

The findings of Ren et al. (2012) are in accordance with the present observations for soybean proximate composition, they expounded that soy contains 8.43±0.44, 37.29±1.99, 17.86±1.17, 13.31±0.80 and 4.99±0.23% of moisture, crude protein, crude fat, carbohydrates and ash, respectively. Similarly, Esteves et al. (2010) illuminated that soy has 37.83 to 39.37% protein, 20.83 to 22.57% crude fat, 4.31 to 6.62% ash, 2.10 to 2.11% total dietary fiber and 22.98 to 25.06% carbohydrates. Earlier, Wei and Chang (2004) delineated that proximate composition of soybean is varied from 5.20-7.41, 36.49-40.29, 18.78-25.41, 5.35-5.83 and 30.35-36.04% for moisture, crude protein, crude fat, ash and carbohydrate contents, respectively.

### 4.1.2. Mineral profile

Means for mineral profile (Table 3) indicated potassium (K), zinc (Zn), magnesium (Mg), calcium (Ca), iron (Fe), sodium (Na) and copper (Cu) contents as 515.41±25.77, 80.18±4.01, 712.26±35.61, 87.58±4.38, 2.49±0.12, 76.51±3.83 and 6.92±0.35 mg/100g, respectively in the spinach (Table 5). Besides, respective minerals in soybean were 673.17±31.31, 288.91±14.45, 228.95±11.45, 181.20±9.06, 7.35±0.37, 2.80±0.14 and 1.93±0.03 mg/100g.

The results for calcium, sodium, potassium, magnesium, iron and zinc are in accordance with the work of Bangash et al. (2011), they noticed that spinach contains 100±0.01, 50±0.9, 310±0.11, 137±0.16, 29±0.02, 0.17±0.01 and 1.21±0.02 mg/100g of K, Zn Mg, Ca, Fe, Na and Cu, respectively. Earlier, Singh et al. (2001) determined iron, copper, magnesium and zinc in Indian spinach and reported the values 35.8±0.11, 1.9±0.03, 10.2±0.05 and 6.0±0.04 mg/100g, respectively. The slight differences in mineral profile of soy and spinach are owing to variations in climate, soil and agronomic practices. Likewise, Almeida et al. (1996) delineated that the nutritional composition of green leafy vegetables is influenced by agrotechnics, raining condition, growing method and soil type. The instant results regarding calcium, potassium, sodium and magnesium are in harmony with the previous investigation of Esteves et al. (2010), they noticed variations for aforementioned minerals in soy ranging from 1.70 to 2.08, 17.40 to 18.90, 0.24 to 0.25 and 2.61 to 3.17 g/100g.
<table>
<thead>
<tr>
<th>Proximate composition</th>
<th>Spinach</th>
<th>Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>90.71±4.14</td>
<td>8.96±0.45</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>2.03±0.95</td>
<td>32.28±1.99</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>0.32±0.007</td>
<td>18.64±1.02</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>0.58±0.02</td>
<td>2.93±0.16</td>
</tr>
<tr>
<td>Ash</td>
<td>1.24±0.06</td>
<td>3.38±0.19</td>
</tr>
<tr>
<td>NFE</td>
<td>5.01±0.11</td>
<td>33.79±1.15</td>
</tr>
<tr>
<td>Minerals</td>
<td>Spinach</td>
<td>Soybean</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>K</td>
<td>515.41±25.77</td>
<td>673.17±31.31</td>
</tr>
<tr>
<td>Zn</td>
<td>80.18±4.01</td>
<td>288.91±14.45</td>
</tr>
<tr>
<td>Mg</td>
<td>712.26±35.61</td>
<td>228.95±11.45</td>
</tr>
<tr>
<td>Ca</td>
<td>87.58±4.38</td>
<td>181.20±9.06</td>
</tr>
<tr>
<td>Fe</td>
<td>2.49±0.12</td>
<td>7.35±0.37</td>
</tr>
<tr>
<td>Na</td>
<td>76.51±3.83</td>
<td>2.80±0.14</td>
</tr>
<tr>
<td>Cu</td>
<td>6.92±0.35</td>
<td>1.93±0.03</td>
</tr>
</tbody>
</table>
One of their peers, Paucar-Menacho et al. (2010) evaluated different soybean cultivars for their mineral potential and recorded calcium, iron, copper and zinc as 290.41±1.47 to 335.37±1.27, 22.30±0.07 to 26.46±0.25, 2.88±0.04 to 3.14±0.06 and 7.42±0.16 to 8.27±0.10 mg/100g, respectively. Earlier, calcium and magnesium contents of four soy cultivars were estimated that varied from 175.80 to 227.90 and 251.25 to 283.05 mg/100g, respectively (Wei and Chang, 2004).

**4.1.3. Vitamin K content**

The raw spinach and soybean were subjected to vitamin K quantification through HPLC and results showed phylloquinone concentration in respective materials as 378.09±13.89 and 29.79±1.23 µg/100g (Figure 1). The present finding are in accordance with the previous work of Schurgers and Vermeer (2000), they illuminated the phylloquinone contents of the fresh spinach as 299-429 µg/100g. Later, Kamna et al. (2007) explored phylloquinone concentration of 58 commonly consumed foods of Eastern Japan through HPLC and narrated higher values in spinach (498 µg/100 g) as compared to broccoli (307 µg/100g). Furthermore, Booth (2012) quantified phylloquinone content of soybean and its oil through HPLC and recorded the values 34.74 and 193 µg/100g for respective material. Earlier, Peterson et al. (2002) explored the phylloquinone contents of different oils. They elucidated that its concentration varied from 33.5 to 71.8, 50.1 to 70.3 and 4.8 to 11.1 µg/100g in vegetable, olive and corn oils, respectively. It has been documented that phylloquinone is principally present in the photosynthetic tissue of the plants and its concentration varied among different vegetables (Damon et al., 2005). Furthermore, it is synthesized in the chloroplast membrane of plant cell through dogma reaction in the presence of light. In this context, chorismate is a direct precursor of phylloquinone formed through 1,4-dihydroxy-2-naphthoate that is synthesized from α-ketoglutarate and isochorismate in the presence of Mn^{2+} and thiamine diphosphate (Shimada et al., 2005). Earlier, Koivu et al. (1997) recorded that phylloquinone contents of the vegetables are higher in summer season than that of winter (Koivu et al., 1997).
Figure 1. Phylloquinone (µg/100g) of raw materials (fresh weight basis)

Spinach: 378.09 µg/100g
Soybean: 29.79 µg/100g
4.1.4. Antioxidant potential

Mean squares in Table 4 indicated that antioxidant indices were affected significantly by raw materials and solvents however, their interactive effect was found non-momentous except for total phenolic content. Means for total phenolic content (TPC) of raw materials (Figure 2) showed the highest value 894.16±36.69 mg GAE/100g for methanolic extract of spinach followed by 701.81±23.02 mg GAE/100g in Ethanolic extract of spinach whereas the lowest TPC value 312.39±13.15 mg GAE/100g for Ethanolic extract of soybean. Likewise, the maximum DPPH (1, 1-diphenyl-2-picrylhydrazyl) inhibition was noticed in methanolic extract of spinach (69.25±3.38%) trailed by ethanolic extract of spinach (62.04±3.15%) whereas the minimum for ethanolic extract of soy (29.73±1.66%). Similarly, methanolic extract of spinach exhibited the highest values for β-carotene and FRAP as 62.53±2.79% & 2.36±0.11 μmol trolox Eq/100g followed by ethanolic extract of spinach 55.82±2.64% & 2.2±0.10 μmol trolox Eq/100g whilst, the lowest outputs were recorded for ethanolic extract of soybean 21.49±1.18% & 1.08±0.05 μmol trolox Eq/100g, respectively.

The polyphenolic compounds i.e. phenolic acids, phenols, hydroxycinnamic acid derivatives and flavonoids are the promising phytonutrients that hold strong antioxidant potential. Accordingly, plant derived dietary polyphenols are gaining importance due to their high antioxidant activity. In this context, p-coumaric acid and flavonoids derivatives are the main spinach based antioxidant compounds (Bergman et al., 2001). Furthermore, isoflavones and lignans are the primary polyphenols of soybean that modulate various biochemical processes (Hedlund et al., 2003; Takahashi, et al., 2005). Moreover, soybean also contains highly polymerized procyanidins with significant DPPH activity (Takahata et al., 2001).

The results pertaining to antioxidant potential of the spinach are in line with the findings of Turkmen et al. (2005), they observed 1274.8 ± 94.09 mg GAE/100g & 67.4 ± 7.82% of total phenolic and DPPH activity, respectively. Later, Fan et al. (2011) explored total phenolic contents of the spinach as 125 mg GAE/100g on fresh weight basis. The results of the present study are in harmony with the findings of Slavin et al. (2009), reported 12.1 mg GAE/g of total phenolics in soybean.
Table 4. Mean squares for antioxidant potential of raw materials

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Total phenolic content</th>
<th>DPPH free radical scavenging activity</th>
<th>Antioxidant activity</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials (M)</td>
<td>1</td>
<td>39298.7**</td>
<td>2121.96**</td>
<td>2004.44**</td>
<td>3.141**</td>
</tr>
<tr>
<td>Solvents (S)</td>
<td>1</td>
<td>226.9*</td>
<td>64.30*</td>
<td>32.16*</td>
<td>0.058*</td>
</tr>
<tr>
<td>M×S</td>
<td>1</td>
<td>205.0*</td>
<td>0.54&lt;sub&gt;NS&lt;/sub&gt;</td>
<td>1.05&lt;sub&gt;NS&lt;/sub&gt;</td>
<td>0.001&lt;sub&gt;NS&lt;/sub&gt;</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>21.2</td>
<td>6.98</td>
<td>4.53</td>
<td>0.007</td>
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<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant  
*=Significant  
<sub>NS</sub>=Non-significant
Figure 2. Total phenolic content, DPPH free radical scavenging activity, antioxidant activity and FRAP in different extracts
Earlier, Malenčić et al. (2007) documented the variations in TPC and DPPH values for different soybean cultivars from 2.70±0.15 to 4.72±0.07 g catechin/kg and 22.87±1.32 to 48.17±2.78%, respectively. Previously, Takahata et al. (2001) estimated the polyphenols of black, brown and reddish brown coated soybean cultivars that ranged from 6.55 ± 0.40 to 81.3 ± 5.5 mg catechin Eq/g. Moreover, brown, green and yellow soybean cultivars showed variations in TPC by 0.8 to 2.2 mg GAE/g. Afterwards, Popovic et al. (2010) demonstrated that total phenolic contents and FRAP values are 31.3±2.3 to 59.7±7.1% and 307 to 480 μM Fe²⁺/g of different soybean varieties. Moreover, they also observed antioxidant activity of soybean 54.55 to 65.65% through β-carotene linoleic acid model system.

From the above discussion, it is deduced that raw spinach contains higher amount of phylloquinone as compared to soybean. Moreover, antioxidative characteristics of spinach and soybean extracts are affected by the type of solvents. In this context, methanolic extract performed better than ethanolic extract. In general, spinach showed higher antioxidant activity as compared to soybean.

4.2. Nutritional analysis of vitamin K enriched products

4.2.1. Proximate composition

Mean squares (Table 5) indicated that proximate composition of vitamin K enriched dietary products i.e. cooked spinach (T₁), reconstituted spinach (T₂), natto A (T₃) and natto B (T₄) differed significantly except for crude fiber content.

Proximate composition of prepared vitamin K enriched dietary products depicted that cooked spinach (T₁) contained the maximum moisture 75.64±3.51% followed by reconstituted spinach (T₂) 71.86±2.96% and natto B (T₄) 62.72±3.11% whereas the minimum moisture content was noticed in natto A (T₃) 56.47±2.81%. However, the protein content was highest 15.39±0.65% in T₃ trailed by 13.19±0.54% in T₄ and 6.11±0.21% for T₂ whilst, the lowest value as 5.29±0.18% was recorded in T₁. Crude fat content was observed in vitamin K enriched products as 0.81±0.03, 0.92±0.02, 7.31±0.31 and 7.61±0.32% for T₁, T₂, T₃ and T₄, respectively (Table 6). Moreover, formulated vitamin K rich dietary products showed non-significant differences for crude fiber content that ranged from 1.17±0.04 to 1.42±0.03% in tested products. The maximum ash content was observed in T₂ 6.27±0.31% followed by T₁.
Table 5. Mean squares for proximate composition of vitamin K enriched products

<table>
<thead>
<tr>
<th>SOV</th>
<th>Df</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Crude Fat</th>
<th>Crude Fiber</th>
<th>Ash</th>
<th>NFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>3</td>
<td>756.758**</td>
<td>159.161**</td>
<td>7.387**</td>
<td>0.106&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>287.392**</td>
<td>8.264*</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>13.734</td>
<td>3.226</td>
<td>0.605</td>
<td>0.218</td>
<td>0.672</td>
<td>1.745</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant
*=Significant
NS=Non-significant
Table 6. Means for proximate composition (%) of vitamin K enriched products (fresh weight basis)

<table>
<thead>
<tr>
<th>Proximate composition</th>
<th>T₁</th>
<th>T₂</th>
<th>T₃</th>
<th>T₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>75.64±3.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.86±2.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.47±2.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62.72±3.11&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>5.29±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.11±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.39±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.19±0.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>0.81±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.92±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.31±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.61±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>1.23±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.42±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.41±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ash</td>
<td>5.41±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.27±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.59±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.37±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFE</td>
<td>11.47±0.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.35±0.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.78±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.89±0.54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means carrying same letter in a row differed non-significantly (p>0.05)

T₁: Cooked Spinach  
T₂: Reconstituted Spinach  
T₃: Natto A  
T₄: Natto B
Cooking and fermentation are the important processing steps that inactivate the nutritional inhibitors present in spinach and soybean. The results regarding proximate composition of cooked spinach are in corroboration with the finding of Kuti and Kuti (1999), they elucidated that spinach has 8.67±0.23% crude protein content. Similarly, the values documented by USDA (2010) regarding proximate composition of cooked spinach are supporting the present results. The published values for cooked spinach are 91.21, 2.97, 0.29, 2.4 and 3.75% for moisture, protein, fat, dietary fiber and carbohydrate, respectively. The conventionally cooked spinach has significantly less amount of moisture while protein, lipid, ash and total dietary fiber contents are higher as compared to the raw sample (Kala and Prakash 2004).

The instant results are in harmony with the findings of Premarani et al. (2011), they illuminated the values for fermented soybean as 62.1% moisture, 1.42% ash, 8.2% crude fiber, 26.02% soluble protein and 24.36% fat contents. Likewise, Jeff-Agboola and Oguntuase (2006) expounded that natto contains 40% protein and 24.68% fat contents. They were on the view that controlled fermentation process imparts significant differences on the nutritional composition of natto as compared to natural fermentation. However, hawaijar (fermented soybean) prepared from different soybean cultivars exhibited non-significant variations for lipid, fatty acid and amino acid contents (Li et al., 2007). Earlier, Wei and Chang (2004) evaluated natto samples prepared from four different soybean cultivars using B. natto “Itobiki” for moisture, protein, lipid, ash & carbohydrates and recorded the range 59.30-61.24, 36.63-42.72, 19.64-23.09, 4.38-4.97 & 30.80-34.23%, respectively. However, the natto prepared from B. natto NRRL B-3393 showed the values 61.07-61.75, 39.4-44.31, 25.00-27.28, 4.72-4.86 and 25.02-28.91%, for these attributes.

4.2.2. Mineral profile

Mean squares for the mineral profile of vitamin K enriched dietary products explicated significant differences due to treatments (Table 7). In present exploration, spinach based vitamin K enriched products (cooked and reconstituted spinach) have values 514.23±23.78 & 513±24.34, 76.72±3.84 & 65.66±3.28, 646.28±32.31 & 616.08±30.80, 86.14±3.56 &
85.51±3.41, 2.37±0.19 & 2.35±0.15, 72.58±3.81 & 71.74±3.73 and 6.89±0.42 & 6.84±0.45 mg/100g for K, Zn, Mg, Ca, Fe, Na and Cu in T₁ & T₂, respectively (Table 8). However, soybean based vitamin K enriched dietary products i.e. natto A & B exhibited the highest concentrations 671.47±29.74 & 667.15±31.36 mg/100g for K followed by 284.93±13.55 & 279.70±12.83 mg/100g for Zn whilst, the lowest levels 1.87±0.06 & 1.76±0.05 mg/100g were noticed for Cu in T₃ and T₄, respectively.

Lisiewska et al. (2008) explored the mineral profile of cooked spinach and reported that potassium (258.0±51.0 mg/100g), magnesium (19.8±2.2 mg/100g), iron (0.94±0.18 mg/100g) and zinc (0.74±0.06 mg/100g) contents were non-significantly differed from raw spinach i.e. 335.0±24.4, 29.0±3.8, 1.11±0.14 & 0.80±0.11mg/100g, in respective manner. Recently, Sikora and Bodziarczyk (2012) expounded that green leafy vegetables showed variations in the mineral contents after cooking due to handling & processing losses and alteration in the moisture content of end product. The iron was non-substantially reduced to 2.97±0.75 mg/100 in conventionally cooked spinach compared to the raw sample 3.00±0.53 mg/100g (Kala and Prakash 2004).

The current results for potassium, magnesium, copper, iron, zinc, sodium and calcium are in line with the earlier findings of Nikkuni et al. (1995). They delineated the values for tested minerals as 1697, 221, 1.46, 7.2, 4.55, 14.4 and 281 mg/100g for natto. Moreover, kinema (fermented soybean) indicated the values for these minerals as 1768, 252, 1.71, 17.1, 4.52, 27.7 and 432 mg/100g, respectively. Earlier, it was deduced that intake of natto containing poly-γ-glutamic acid resulted high percentage of Ca solubility in the intestine after inhibiting the formation of insoluble Ca complex (Tanimoto et al., 2001). The deviations in mineral contents are supposed to be linked with different geographical conditions, agronomic practices, cultivars and harvesting time.

During fermentation process bacteria are utilizing minerals for their growth thereby alter the mineral profile of resultant product. In the current case, some variations regarding nutritional composition of developed spinach and soybean products from their respective raw material are due to differences in moisture contents of the final product. Nevertheless, the fermented
<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>K</th>
<th>Zn</th>
<th>Mg</th>
<th>Ca</th>
<th>Fe</th>
<th>Na</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>3</td>
<td>423737**</td>
<td>41703**</td>
<td>169043**</td>
<td>6049.92**</td>
<td>22.755**</td>
<td>2.422**</td>
<td>44.0462**</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>530</td>
<td>100.4</td>
<td>559</td>
<td>64.70</td>
<td>0.090</td>
<td>0.015</td>
<td>0.067</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant
Table 8. Means for mineral profile (mg/100g) of vitamin K enriched products

<table>
<thead>
<tr>
<th>Minerals</th>
<th>T₁</th>
<th>T₂</th>
<th>T₃</th>
<th>T₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>514.23±23.78&lt;sup&gt;c&lt;/sup&gt;</td>
<td>513±24.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>671.47±29.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>667.15±31.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn</td>
<td>76.72±3.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>65.66±3.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>284.93±13.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>279.70±12.83&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mg</td>
<td>646.28±32.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>616.08±30.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>225.44±10.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>218.19±10.56&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ca</td>
<td>86.14±3.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.51±3.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>181.69±8.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>173.07±7.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fe</td>
<td>2.37±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.35±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.25±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.18±0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Na</td>
<td>72.58±3.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.74±3.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.72±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.64±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu</td>
<td>6.89±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.84±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.87±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.76±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means carrying same letter in a row differed non-significantly (p>0.05)

T₁: Cooked Spinach  
T₂: Reconstituted Spinach  
T₃: Natto A  
T₄: Natto B
soybean (natto) showed higher amount of protein and fat than that of cooked and reconstituted spinach.

4.2.3. Phylloquinone & menaquinone-7

Mean squares (Table 9) regarding phylloquinone (vitamin K$_1$) and menaquinone-7 (vitamin K$_2$) showed significant variations among vitamin K enriched dietary products. Means relating to phylloquinone exhibited the highest value 368.81±13.96 µg/100g for cooked spinach (T$_1$) followed by 270.07±9.45 µg/100g in reconstituted spinach (T$_2$) whereas, natto A (T$_3$) showed the minimum value 26.90±0.94 µg/100g. However, maximum menaquinone-7 (MK-7) was recorded in T$_3$ 803.82±21.14 µg/100g trailed by T$_4$ 681.35±16.85 µg/100g. In contrary, menaquinone-7 was not detected in the cooked and reconstituted spinach (Table 10).

The results of instant investigation are comparable with the earlier work of Kamao et al. (2007) for phylloquinone and MK-7 contents of spinach and natto. The documented values for phylloquinone and MK-7 were 498 & 939 µg/100g in spinach & natto, respectively. They concluded that vegetables and fermented foods are one of the prime sources of vitamin K in humans. Recently, Booth (2012) explicated the levels of phylloquinone and MK-7 as 380 and 998 µg/100g for spinach and natto, correspondingly. Likewise, Damon et al. (2005) quantified phylloquinone concentration in fresh, boiled and microwaved spinach samples by 293-441, 533-547 and 348-544 µg/100g, respectively. Earlier, Schurgers and Vermeer (2000) delineated that phylloquinone contents are 299-429 µg/100g in spinach and 31.2-36.7 µg/100g in soybean. Moreover, the concentration of MK-7 was reported in natto as 882-1034 µg/100g. Afterwards, Tsukamoto et al. (2001) identified mutant strain of bacteria that has relatively higher productivity of menaquinone-7 i.e. 139-156% as compared to commercial strain that produces 864 µg/100g of MK-7 in natto.

Alongside, natto has almost 2.5 times higher MK-7 level than that of phylloquinone content of spinach (Schurgers and Vermeer, 2000; Booth, 2012). The phylloquinone and menaquinones are heat stable entities however, phylloquinone is more susceptible to light and alkaline conditions (Fu and Booth, 2012). Nevertheless, phylloquinone and menaquinones are quite stable in cooked spinach and fermented soybean, respectively. Generally, green leafy vegetables contribute approximately 60% of the daily phylloquinone.
Table 9. Mean squares for phylloquinone (vitamin K$_1$) and menaquinone-7 (vitamin K$_2$) in vitamin K enriched products

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Phylloquinone</th>
<th>Menaquinone-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>3</td>
<td>1019.80**</td>
<td>3057.02**</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>72</td>
<td>183</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(P<0.05)  
**=Highly significant
Table 10. Phylloquinone and menaquinone-7 (µg/100g) in enriched products (fresh weight basis)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Phylloquinone</th>
<th>Menaquinone-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>368.81±13.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>270.07±9.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>26.90±0.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>803.82±21.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>29.80±1.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>681.35±16.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means carrying same letter in a column differed non-significantly (p>0.05)

ND=Not detected

T<sub>1</sub>: Cooked Spinach  
T<sub>2</sub>: Reconstituted Spinach  
T<sub>3</sub>: Natto A  
T<sub>4</sub>: Natto B
intake. However, allied health claims of long chain menaquinones enhance their importance in the everyday diet (Schurgers et al., 2002). Conclusively, HPLC quantification showed higher phylloquinone content in cooked spinach (T₁) as compared to the reconstituted form (T₂). Similarly, menaquinone-7 was more pronounced in the natto A (T₃) might be due to better fermentation conditions for Bacillus subtilis.

4.2.4. Antioxidant potential

The statistical interpretation showed that both treatments and solvents imparted significant variations in the antioxidant indices of different products however, their interactions proved non-momentous (Table 11).

Means for total phenolic content (TPC) of vitamin K enriched dietary products (Table 12) indicated the highest value 714.94±32.10 mg GAE/100g in T₁ followed by 700.21±24.01 mg GAE/100g in T₂ and 405.83±17.80 mg GAE/100g in T₃ while the lowest TPC value 395.59±14.51 mg GAE/100g was observed in T₄. Likewise, antioxidant activity and DPPH were maximum in cooked spinach (52.70±2.35 & 59.02±2.19%) trailed by reconstituted spinach (51.00±1.99 & 57.42±2.42%) and natto A (25.09±1.11 & 34.05±1.37%) whereas, the minimum values in natto B (23.75±0.92 & 32.35±1.08%). Similarly, the gathered data for ferric reducing antioxidant power (FRAP) of resultant products showed the values 2.04±0.10, 1.99±0.09, 1.27±0.04 and 1.23±0.06 µmol trolox Eq/100g for T₁, T₂, T₃ and T₄, respectively.

The results of the present study are in harmony with the earlier finding of Bunea et al. (2008), they documented phenolic contents of spinach i.e. 1067.4±7.3 to 2108.8±14.9 mg GAE/kg on fresh weight basis. Moreover, Ismail et al. (2004) also determined phenolics and antioxidant activity of raw and cooked spinach ethanolic extracts. They deduced that the raw spinach has higher phenolics and antioxidant activity i.e. 7167±73 mg GAE/100g and 66.4±1.1% than that of cooked treatment; 6168±41 mg GAE/100g and 61.9±0.6%, correspondingly. Furthermore, they also observed that phenolic content has linear association with antioxidant activity.

Similarly, Jimenez-Monreal et al. (2009) observed 11.1 and 31.6% reduction for TPC and DPPH values, respectively in thermally treated spinach. The current outcomes for antioxidant potential of cooked spinach are in coherence with Amin et al. (2006), they found that β-
Table 11. Mean squares for antioxidant potential of vitamin K enriched products

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Total phenolic content</th>
<th>DPPH free radical scavenging activity</th>
<th>Antioxidant activity</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments (T)</td>
<td>3</td>
<td>234710**</td>
<td>671.243**</td>
<td>928.707**</td>
<td>1.184**</td>
</tr>
<tr>
<td>Solvents (S)</td>
<td>1</td>
<td>1595*</td>
<td>186.859*</td>
<td>58.576*</td>
<td>0.138*</td>
</tr>
<tr>
<td>T×S</td>
<td>3</td>
<td>672^{NS}</td>
<td>2.557^{NS}</td>
<td>0.610^{NS}</td>
<td>0.018^{NS}</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>185</td>
<td>13.200</td>
<td>7.059</td>
<td>0.007</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Highly significant
* = Significant
NS = Non-significant
Table 12. Means for antioxidant potential of vitamin K enriched products

<table>
<thead>
<tr>
<th>Products</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Means</th>
<th>Products</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total phenolic content (mg GAE/100g)</td>
<td>DPPH free radical scavenging activity (%)</td>
<td></td>
<td></td>
<td>FRAP (µmol trolox Eq/100g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₁</td>
<td>803.55±35.90</td>
<td>626.34±24.31</td>
<td>714.94±32.10</td>
<td>T₁</td>
<td>62.40±2.39</td>
<td>55.65±2.01</td>
<td>59.02±2.19</td>
</tr>
<tr>
<td>T₂</td>
<td>785.85±37.41</td>
<td>614.58±20.62</td>
<td>700.21±24.01</td>
<td>T₂</td>
<td>60.52±2.06</td>
<td>54.33±1.79</td>
<td>57.42±2.42</td>
</tr>
<tr>
<td>T₃</td>
<td>466.37±21.88</td>
<td>345.30±15.73</td>
<td>405.83±17.80</td>
<td>T₃</td>
<td>36.19±1.83</td>
<td>31.92±1.26</td>
<td>34.05±1.37</td>
</tr>
<tr>
<td>T₄</td>
<td>453.65±17.56</td>
<td>337.53±13.47</td>
<td>395.59±14.51</td>
<td>T₄</td>
<td>34.65±1.05</td>
<td>30.05±1.11</td>
<td>32.35±1.08</td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td>627.35±26.60</td>
<td>480.93±19.53</td>
<td></td>
<td><strong>Means</strong></td>
<td>48.44±2.14</td>
<td>42.98±2.03</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Products</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Means</th>
<th>Products</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antioxidant activity (%)</td>
<td>FRAP (µmol trolox Eq/100g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₁</td>
<td>55.74±2.45</td>
<td>49.67±2.24</td>
<td>52.70±2.35</td>
<td>T₁</td>
<td>2.12±0.09</td>
<td>1.97±0.08</td>
<td>2.04±0.10</td>
</tr>
<tr>
<td>T₂</td>
<td>54.57±2.07</td>
<td>47.43±1.90</td>
<td>51.00±1.99ab</td>
<td>T₂</td>
<td>2.07±0.10</td>
<td>1.92±0.09</td>
<td>1.99±0.09</td>
</tr>
<tr>
<td>T₃</td>
<td>26.76±1.20</td>
<td>23.42±1.01</td>
<td>25.09±1.11b</td>
<td>T₃</td>
<td>1.36±0.06</td>
<td>1.19±0.05</td>
<td>1.27±0.04c</td>
</tr>
<tr>
<td>T₄</td>
<td>24.97±10.98</td>
<td>22.54±0.85</td>
<td>23.75±0.92b</td>
<td>T₄</td>
<td>1.32±0.04</td>
<td>1.15±0.03</td>
<td>1.23±0.06c</td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td>40.51±1.67a</td>
<td>35.76±1.52b</td>
<td></td>
<td><strong>Means</strong></td>
<td>1.93±0.12a</td>
<td>1.82±0.10b</td>
<td></td>
</tr>
</tbody>
</table>

Means carrying same letter in a column differed non-significantly (p>0.05)

T₁: Cooked Spinach; T₃: Natto A
T₃: Reconstituted Spinach; T₄: Natto B
carotene inhibition, DPPH free radical activity and total phenolic content are in the range of 21-43%, 28-75% and 32-68 g GAE/kg, respectively. Moreover, ferric reducing antioxidant power (FRAP) of uncooked spinach is 15.89 mmol Fe$^{2+}$/100g that decreased to 13.89 mmol Fe$^{2+}$/100g after 10 min cooking (Mazzeo et al., 2011). The heat treatment significantly breakdown the lignocellulosic structure of the vegetables through depolymerization and defibration of lignin components that is why antioxidant activity of resultant product was reduce. Consequently, phenolic molecules may leach down from the vegetable matrix to the boiling water (Xu and Chang 2008). The differences in the antioxidant potential of spinach based products are probably due to variations in cooking methods and conditions i.e. time and temperature (Gazzani et al., 1998).

The findings of Yao et al. (2010) are synchronized with the current results that Bacillus sp. fermented Korean soybean has higher values for TPC, DPPH and FRAP activity by 379.7 mg GAE/100g, 58% and 250.04 µg trolox Eq/g as compared to non-fermented soybean 257.6 mg GAE/100g, 42% and 307.03 µg trolox Eq/g FW, respectively. The higher antioxidant activity of fermented soybean is associated with the generation of isoflavones and their glycosides through microorganisms during fermentation. Similarly, DPPH radical scavenging capacity is uplifted by fermentation with various types of microorganisms. In this context, Bacillus sp. yields the highest FRAP activity due to the synthesis of iron chelating compounds during fermentation. Earlier, it has been observed that fermented soybean contains antioxidant peptides responsible for scavenging free radicals and exert inhibitory effect on β-carotene in linoleic acid model system (Wang et al., 2008). The results of present study regarding antioxidant indices are in corroboration with the work of Moktan et al. (2008), revealed that methanolic extract of soybean fermented with B. subtilis has higher metal chelating ability, lipid peroxidation inhibition, DPPH free radical scavenging activity and reducing power as compared to raw soy and suggested that fermentation significantly enhances these attributes. Later, Dajanta et al. (2011) explicated that fermented soybean (Thai Thua nao) has appreciable amount of phenolics and DPPH radical scavenging activity. Besides, its antioxidant activity was estimated as 54.55 to 65.65% through β-carotene assay.

It is deduced that the antioxidant activity of resultant spinach products is better than fermented soybean formulations due to higher polyphenolic contents. Additionally,
fermented soybean exhibited higher antioxidant indices as compared to the raw form probably due to the production of isoflavones and their glycosides during fermentation.

4.3. Sensory evaluation

Mean squares for the sensory response of resultant vitamin K enriched products (Table 13) showed significant variations in the tested traits including color, flavor, taste, texture and overall acceptability.

The highest color score was assigned to T\(_1\) (7.86±0.34) followed by T\(_2\) (6.94±0.26), T\(_3\) (6.84±0.30) and T\(_4\) (6.04±0.25). The observed scores for flavor were 7.96±0.35 (T\(_1\)), 7.34±0.32 (T\(_2\)), 7.02±0.30 (T\(_3\)) and 6.14±0.26 (T\(_4\)). Likewise, taste scores for T\(_1\), T\(_2\), T\(_3\) and T\(_4\) were 7.78±0.24, 7.18±0.27, 7.08±0.25 and 6.98±0.29, respectively. Means for texture in various treatments illuminated variations from 7.56±0.33 to 6.16±0.26 for T\(_1\) to T\(_4\), respectively. Lastly, the recorded scores for overall acceptability of the vitamin K enriched products \textit{i.e.} T\(_1\), T\(_2\), T\(_3\) and T\(_4\) were 7.84±0.34, 7.74±0.34, 7.14±0.31 and 6.74±0.29, respectively (Table 14).

Present results concerning sensory profile of the processed spinach are in harmony with the work of the Donadini \textit{et al.} (2012), they elucidated that boiled spinach has better hedonic response as compared to raw sample in a vegetable liking questionnaire assessment. Earlier, Bangash \textit{et al.} (2011) inferred that amongst tested sensory parameters, color is exceptionally affected due to cooking. Likewise, the color of conventionally and microwave cooked spinach is fair than pressure cooked spinach. Similarly in a study, hedonic attributes of cooked green vegetables were evaluated using ranking test and quantitative descriptive analysis (QDA). The traits analyzed were color, appearance, aroma, taste and texture. The comparison between conventional and microwave methods expounded that color of the microwave cooked spinach is comparatively better. Furthermore, aroma and texture of cooked spinach by pressure cooking is relatively inferior to conventionally cooked sample. Additionally, taste and overall acceptability are appealing for conventionally cooked spinach than pressure cooked sample (Kala and Prakash, 2004).

The current results are in accordance with the findings of Youn \textit{et al.} (2002) and Lee \textit{et al.} (2005), they expounded that fermented soybean (chungkukjang) prepared with \textit{B}. 
Table 13. Mean squares for sensory response of vitamin K enriched products

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Color</th>
<th>Flavor</th>
<th>Taste</th>
<th>Texture</th>
<th>Overall acceptability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products</td>
<td>3</td>
<td>2.156**</td>
<td>1.711**</td>
<td>1.862*</td>
<td>1.702**</td>
<td>1.046*</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.083</td>
<td>0.094</td>
<td>0.092</td>
<td>0.094</td>
<td>0.089</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant
*= Significant
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Color</th>
<th>Flavor</th>
<th>Taste</th>
<th>Texture</th>
<th>Overall acceptability</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁</td>
<td>7.86±0.34</td>
<td>7.96±0.35</td>
<td>7.78±0.24</td>
<td>7.56±0.33</td>
<td>7.84±0.34</td>
</tr>
<tr>
<td>T₂</td>
<td>6.94±0.26</td>
<td>7.34±0.32</td>
<td>7.18±0.27</td>
<td>7.14±0.31</td>
<td>7.74±0.34</td>
</tr>
<tr>
<td>T₃</td>
<td>6.84±0.30</td>
<td>7.02±0.30</td>
<td>7.08±0.25</td>
<td>6.94±0.30</td>
<td>7.14±0.31</td>
</tr>
<tr>
<td>T₄</td>
<td>6.04±0.25</td>
<td>6.14±0.26</td>
<td>6.98±0.29</td>
<td>6.16±0.26</td>
<td>6.74±0.29</td>
</tr>
</tbody>
</table>

Means carrying same letter in a column differed non-significantly (p>0.05)

T₁: Cooked Spinach
T₂: Reconstituted Spinach
T₃: Natto A
T₄: Natto B
*licheniformis* attained higher hedonic scores for color, flavor and taste as compared to traditional chungkukjang. Likewise, fermented soybean with *B. subtilis* TN51 showed better performance regarding aroma than conventionally fermented soy (Dajanta *et al.*, 2011). Further, significant differences for aroma and texture were observed in the prepared and commercially available natto. The sensory attributes of natto *i.e.* color, aroma, flavor and texture were within acceptable limit estimated through continuous linear 10 cm intensity scale (Wei *et al.*, 2004). It has been observed that fermentation conditions are the crucial factors affecting sensory response of the fermented products. In this context, controlled fermented soybean attained higher scores for hedonic response as compared to conventionally fermented sample. Additionally, controlled fermentation imparts non-significant variations for appearance, odor, taste and stickiness of natto samples prepared from different soybean varieties (Luo *et al.*, 2010). Earlier, Lee *et al.* (2007) assessed the sensory response of fermented soybean (chungkukjang) including odor, taste and overall acceptability through 9-point hedonic scale and found scores within acceptable range. Previously, Lee *et al.* (2005) determined the hedonic response *i.e.* color, flavor, taste, and overall acceptability of the chungkukjangs prepared from different bacterial strains using 5-point hedonic scale. They noticed better sensory response for chungkukjang prepared from *B. subtilis* than formulated with rest of the bacterial strains.

In the current research, natto based products have attained comparatively less scores for sensory attributes due to their sticky nature, bitter taste and slimy structure as compared to spinach samples. However, considering each raw material, cooked spinach (T1) and natto A (T3) were rated better for hedonic response.

**4.4. Efficacy study**

On the basis of nutritional profile, HPLC quantification of vitamin K content and sensory response, one best vitamin K enriched products from each raw material *i.e.* cooked spinach (T1) and natto A (T3) were selected for bioefficacy trial to elucidate their biochemical performance in normal and vitamin K deficient New Zealand rabbits. The selected treatments *i.e.* cooked spinach and natto A were labeled as D2 and D3, respectively in the bioevaluation trial.
Bioefficacy was conducted using rabbits model rather than humans due to simple & convenient handling, close supervision and controlled diet & environmental conditions. Furthermore, proper animal size facilitates the researchers to collect adequate quantity of blood sample. Additionally, rabbits prominent ear veins provide easy access to conduct bleeding time assay. In the present research, efficacy trial was divided in to two segments involving normal and vitamin K deficient rabbits. In each study, four groups of rabbits were formed relied on D2 (cooked spinach), D3 (natto A) and D4 (menadione) along with control (D1). Principally, three different forms of vitamin K were tested for blood coagulation attributes, bone mineral density, vitamin K dependent proteins and serum vitamin K concentrations. For better understanding, the results of two studies are discussed collectively.

4.4.1. Physical parameters

4.4.1.1. Feed intake

Mean squares (Table 15) explicates that treatments imparted non-significant differences on the feed intake in study I (trial 1 & 2) whereas, in study II this trait showed substantial variations. However, time intervals (weeks) affected the feed intake momentously in both studies.

Means for feed intake (study I; trial1) indicated the values for D1, D2, D3 and D4 groups as 219.49±7.53, 218.53±6.43, 215.04±5.85 and 213.83±7.06 g/rabbit/day. Likewise during trial 2, similar trend regarding feed intake was observed and values recorded as 216.83±6.88 and 215.49±6.77 g/rabbit/day for D1 and D4, respectively (Figure 3).

The feed intakes increased progressively as a function of time and at 1st week were measured as 187.78±5.92, 186.82±5.44, 183.74±5.06 and 182.12±4.98 g/rabbit/day in D1, D2, D3 and D4 groups that momentously elevated to 219.49±7.53, 218.53±6.43, 215.45±5.85 and 213.83±7.06 g/rabbit/day, respectively till the 8th week. Likewise in 2nd trial, vertical trend in feed intake with the passage of time was recorded for D1, D2, D3 and D4 groups and values at the initiation were 185.12±6.54, 185.78±5.53, 184.75±5.62 and 183.78±4.91 g/rabbit/day that increased to 216.83±5.12, 217.49±5.76, 216.46±5.09 and 215.49±5.06 g/rabbit/day, respectively at the termination.

In study II (vitamin K deficient rabbit), relatively less feed intake was noticed as compared to
study I. It is obvious from the Figure 3 that maximum feed intake was recorded for D4 group 201.23±4.94 & 183.45±6.71 g/rabbit/day followed by D3 and D2 group 192.02±4.91 & 194.15±5.63 and 186.24±4.91 & 190.85±5.06 g/rabbit/day, respectively whilst, the minimum values 178.32±5.23 & 179.49±4.99 g/rabbit/day were estimated for D1 group (trial 1 & 2). Similar to study 1, the feed consumption was increased with the passage of time and observed values were 160.11±4.45, 162.16±3.39, 164.38±4.52 and 166.44±4.18 g/rabbit/day (1st week) to 178.32±5.23, 186.24±4.91, 192.02±4.91 and 201.23±4.94 g/rabbit/day (8th week) in D1, D2, D3 and D4 groups, respectively. Similar trend was recorded in the follow up trial.

The results of present investigation are supported by the findings of Chat et al. (2005), noticed significant enhancement in the feed intake of rabbits as a function of time. They observed that normal animals fed on B. subtilis fermented natto exhibited non-significant variations in the feed consumption. However, Sun et al. (2010) expounded that Bacillus subtilis natto mixed milk imparted significant rise in the average daily weight and feed efficiency of the tested animals. Earlier, Marchetti et al. (1995) ascribed that feed intake varied non-significantly among the animal groups fed on diets containing menadione @ 0, 500, 1000 and 2000 mg/kg. During vitamin K deficiency, the feed intake was declined due to physiological abnormalities. Thus, consistent supply of vitamin K along with feed substantially attenuates the dysfunctioning caused by warfarin and modulates the feed intake of tested animals. Conclusively, the non-substantial variations due to treatments among the normal groups of rabbits evinced the suitability and acceptability of the vitamin K enriched products. Nevertheless, in present study, results regarding vitamin K deficient rabbits showed some contradictions. The variations in the instant study regarding feed consumption in the vitamin K deficient rabbits are probably due to malfunctioning of the blood related physiological mechanisms that suppress the feed intake.

4.4.1.2. Water intake

Mean squares regarding water intake (Table 16) in study I showed non-momentous variations due to treatments nonetheless, this trait was significantly affected in study II. In both studies, time period (weeks) exerted significant differences on the water intake.
Table 15. Effect of treatments and study weeks on feed intake (g/rabbit/day)

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Study I (Normal rabbits)</th>
<th>Study II (Vitamin K deficient rabbits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Treatment (D)</td>
<td>3</td>
<td>4.86&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>4.94&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week (W)</td>
<td>7</td>
<td>90.12**</td>
<td>79.65**</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant (p<0.05)
*=Significant
NS=Non-significant
Figure 3. Feed intake in study I & II (g/rabbit/day)
The means pertaining to water intake of rabbits (Figure 4) in both modules showed increasing tendency with the progression of time. In study I (trial 1 & 2) the water intakes at the beginning of the study were 74.92±2.91 & 73.13±2.26, 75.37±2.92 & 74.62±2.26, 75.23±2.92 & 72.47±2.25 and 74.82±2.90 & 76.07±2.22 mL/rabbit/day, respectively for D₁, D₂, D₃ and D₄ groups that increased to 92.24±3.64 & 91.34±2.72, 92.94±3.33 & 92.04±2.71, 92.99±3.73 & 92.09±2.71 and 91.54±3.60 & 90.64±2.67 mL/rabbit/day, correspondingly till the termination of trial.

Vitamin K deficient rabbits (study II) indicated the maximum water intake in D₄ group (90.71±2.33 mL/rabbit/day) at the 8th week followed by D₃ (86.62±3.35 mL/rabbit/day) and D₂ (83.23±2.37 mL/rabbit/day) while, the minimum value was recorded for D₁ group (81.92±3.12 mL/rabbit/day). Likewise in trial 2, the maximum drink intake 89.39±3.82 mL/rabbit/day was noticed in D₄ whilst, minimum value i.e. 81.14±2.38 mL/rabbit/day was recorded for D₁ group.

In the instant investigation, increase in water consumption was observed as a function of time in both studies. Earlier, Yamashita et al. (2003) reported that natto containing ample amount of vitamin K₂ showed non-significant impact on water consumption throughout the model study. In the present case, treatment showed non-momentous differences on the water intake of normal rabbits. Likewise, non-substantial effect on water intake in animals fed on vitamin K enriched dietary sources was reported by Tschudin et al. (2011). However, warfarin induced deficiency significantly reduces daily water intake due to less production of liver coagulation proteins that are possibly involve in the normal metabolic activity of the body. In this context, vitamin K enriched products are effective to maintain the drink intake by soothing liver coagulation factors and proteins.

### 4.4.1.3. Body weight gain

Mean squares regarding body weight of rabbits showed non-momentous variations due to treatments in study I whereas, substantial differences were observed in study II. Similarly, study intervals imparted significant impact on the body weight during both studies (Table 17).

At the initiation of study I (trial 1), body weights for different groups i.e. D₁, D₂, D₃ and D₄
Table 16. Effect of treatments and study weeks on water intake (mL/rabbit/day)

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Study I (Normal rabbits)</th>
<th>Study II (Vitamin K deficient rabbits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Treatment (D)</td>
<td>3</td>
<td>2.10&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>2.81&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Week (W)</td>
<td>7</td>
<td>120.77&lt;sup&gt;**&lt;/sup&gt;</td>
<td>109.04&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**=Highly significant  
*=Significant  
<sup>NS</sup>=Non-significant
Figure 4. Water intake in study I and II (mL/rabbit/day)
were 1564.95±57.87, 1559.23±57.69, 1556.25±57.58 and 1549.72±56.95 g/rabbit, respectively that progressively increased to 1884.38±69.71, 1878.47±69.49, 1892.54±69.52 and 1850.33±68.82 g/rabbit, respectively at the end of trial. The observed values regarding body weights in trial 2 for D₁ group at 1st and 8th weeks were 1547.12±57.25 & 1859.55±58.30 g/rabbit, correspondingly whilst D₂, D₃ and D₄ groups depicted body weights 1542.49±57.06 & 1853.14±58.65, 1539.49±56.95 & 1864.27±58.54 and 1552.78±56.32 & 1845.56±58.23 g/rabbit, correspondingly.

In study II, the maximum weight gain was observed from 1st to 8th week for D₄ group 1516.89±55.10 to 1783.33±69.71 g/rabbit (trial 1) and 1521.99±52.51 to 1752.67±65.71 g/rabbit (trial 2), in respective manner. The remaining groups showed increasing tendency in weight gain during trial 1 from 1527.99±51.95 to 1746.32±45.33 (D₃), 1523.63±54.80 to 1709.53±59.52 (D₂) and 1536.32±54.18 to 1693.71±41.82 g/rabbit (D₁) at respective intervals. Similar trend was noticed during the subsequent trial (Figure 5).

The results of present study are supported by the findings of Pucaj et al. (2011), they reported that vitamin K enriched diet imparted non-significant variations on body weight of normal rats as compared to control. Similarly, encapsulated synthetic MK-4 oral administration @ 0, 20 and 200 mg/kg/day for 3 months showed non-momentous variations for body weight among the normal beagle dogs (Goldsmith et al., 1995). One of their peers, Doi et al. (1995) carried out a 13 weeks trial to evaluate the effect of vitamin K₂ supplementation on the body weight. Purposely, they provided 30 mg/kg/day MK-4 to the normal Sprague-Dawley rats and inferred that feed consumption and body weights were affected non-significantly. Furthermore, all animals received MK-4 survived during the entire study elucidated that vitamin K provision is safe for consumption. Likewise, Vanatta et al. (1995) expounded that oral supplementation of MK-4 @ 20, 200 and 2000 mg/kg/day to the rats for the period of one year did not alter weight gain, food and water consumption. Later, Wang et al. (2008) demonstrated that fermented soybean or douchi flour @ 2 and 4% in the diets have non-significant influence on body weight gain among the treated groups of normal wistar rats during 90 days. Afterward, Choi et al. (2010) delineated that intake of green vegetables imparts non-significant variations on weight gain of rabbits whereas this trait was affected substantially with respect to time interval. Earlier, Breitbart et al. (2001) reported that
<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Study I (Normal rabbits)</th>
<th>Study II (Vitamin K deficient rabbits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Treatment (D)</td>
<td>3</td>
<td>2.46\textsuperscript{NS}</td>
<td>2.51\textsuperscript{NS}</td>
</tr>
<tr>
<td>Week (W)</td>
<td>7</td>
<td>90.13\textsuperscript{**}</td>
<td>88.43\textsuperscript{**}</td>
</tr>
<tr>
<td>Error</td>
<td>213</td>
<td>\text{}</td>
<td>\text{}</td>
</tr>
</tbody>
</table>

\textsuperscript{**}=Highly significant
\textsuperscript{*}=Significant
\textsuperscript{NS}=Non-significant
Study I

Figure 5. Body weight in study I & II (g/rabbit/week)
spinach imparts non-momentous effect on the body weight at the end of study. Similar trend was observed in the present investigation regarding body weight of normal rabbits.

The findings of the instant research are in harmony with work of Mochizuki et al. (2009), they reported that anti-coagulant drug (phenobarbital) significantly decreased the body weight of male Japanese rabbits. In this context, vitamin K administration effectively controls the decline in body weight. In contrary, Booth et al. (2008) explicated that phylloquinone or dihydrophylloquinone enriched diets caused a non-momentous rise in the body weight and feed consumption of vitamin K deficient animals. Likewise, normal animal also showed non-substantial variations. Earlier, Kawashima et al. (1997) also carried out a model study to evaluate the effect of vitamin K$_2$ on body weight and feed consumption. They inferred that menatetrenone (MK-4) @ 1, 10 and 100 mg/kg/day raise the body weight and feed intake non-significantly in the hypercoagulabilic and hypercholesterolemic rabbits. Present results for body weight of vitamin K deficient rabbits showed some contradictions with the previous findings probably due to variations in the vitamin K deficiency induction mechanism.

4.4.2. Blood coagulation

4.4.2.1. Bleeding time

It is evident from the F values (Table 18) that vitamin K enriched dietary sources imparted significant variations on bleeding time during both studies.

Means for bleeding time in study I (normal rabbits) showed the values 1.81±0.07, 1.77±0.06, 1.75±0.07 and 1.70±0.04 min for D$_1$, D$_2$, D$_3$ and D$_4$ groups, respectively (trial 1). Similar pattern was noticed in trial 2, the bleeding time decreased momentously from 1.65±0.06 (D$_1$) to 1.56±0.05 min (D$_4$). Next in study II (trial 1), the highest bleeding time (2.42±0.08 min) was observed in D$_1$ that substantially suppressed in D$_2$ (2.31±0.07 min), D$_3$ (2.17±0.07 min) and D$_4$ (2.08±0.06 min). Likewise in the subsequent trial, significant decline was recorded from 2.59±0.09 to 2.18±0.07 min in D$_1$ to D$_4$, correspondingly.

The bleeding defects are classified into primary (disorders of platelets and von Willebrand factor) and secondary hemostatic (coagulation factor deficiencies) that may be acquired or congenital (Kamal et al., 2007). The results of present exploration are synchronized with the
Table 18. Effect of vitamin K enriched dietary sources on bleeding time (min)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>1.81±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.77±0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>1.65±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.62±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>2.42±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.31±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>2.59±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.44±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means carrying same letter in a row differed non-significantly (p>0.05)
**= Highly -significant
*= Significant

Study I : Normal rabbits
Study II : Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phylloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
work of Cornelissen et al. (1997), reported that vitamin K imparts significant effect on blood coagulation parameters thereby reduces the bleeding incidence by promoting the synthesis of blood coagulation factors.

The elevation in blood coagulation time is attributed to inhibition of vitamin K epoxide reductase (VKOR) activity that is essential for proper blood coagulation cascade. For normalization of coagulation time, bioavailability of vitamin K is varied among different vegetable sources (Holmes et al., 2012b). The synthesis of clotting factors is enhanced with increased availability of vitamin K to the VKOR (Price et al., 1996). It is evident from the exploration of Shea et al. (2010) that extraheptatic and fasting serum phylloquinone concentrations are maintained in the well nourished individuals after warfarin therapy. In acute vitamin K deficiency, bleeding is one of the life threatening consequences that can be minimized by vitamin K supplementation (Holmes et al., 2012b). It is deduced from the preceding discussion that vitamin K enriched dietary sources i.e. cooked spinach (D2) and natto A (D3) are effective to alleviate bleeding and coagulation cascade related malfunctionings. Considering the facts, they are suitable to incorporate in the diet based regimen to minimize the threat of hemorrhages.

4.4.2.2. Clotting time (CT)

The F values in Table 19 showed substantial effect of treatments on clotting time in normal and vitamin K deficient rabbits (study I & II). Means pertaining to clotting time (CT) in study I (trial 1 & 2) exhibited the maximum value for group D1 12.42±0.43 & 12.02±0.59 min that momentously reduced in D2, D3 and D4 as 12.19±0.51 & 11.76±0.54, 11.98±0.56 & 11.54±0.49 and 11.78±0.61 & 11.32±0.45 min, respectively. Likewise in study II (trial 1), the recorded values were 14.34±0.68, 13.89±0.59, 13.46±0.53 and 12.99±0.48 min for D1, D2, D3 and D4 groups, correspondingly. Similarly during trial 2, the highest value for CT was recorded in D1 (14.96±0.71 min) followed by D2 (14.59±0.65 min) and D3 (14.08±0.57 min) whilst, the lowest time was noticed for D4 (13.71±0.42 min).

It is obvious from the Figure 6 that vitamin K enriched dietary sources i.e. cooked spinach (D2), natto A (D3) and menadione (D4) resulted 1.72, 3.44 and 5.40% decline in CT, in respective manner (trial 1). However in 2nd trial, the selected dietary sources caused 2.20,
4.01 and 5.82% reduction. Likewise in study II (trial 1 & 2), D₄ exhibited the highest reduction as 9.41 & 8.11% followed by D₃ 6.14 & 5.88% and D₂ 3.14 & 2.49%.

The results of instant study are in corroboration with the outcomes of Center et al. (2000) for reduction in abnormally high clotting time after the provision of vitamin K. They suggested that vitamin K is helpful against hemorrhagic disease and its disastrous consequences. The anticoagulant drug warfarin enhances clotting time by the inactivation of vitamin K dependent proteins especially protein induced by vitamin K antagonist (PIVKA) and interacts with factor X that converts prothrombin to thrombin. Likewise, Sørensen et al. (2009) delineated that vitamin K along with aspirin and clopidogrel increase the incidence of bleeding by 5.1 and 12.3%, respectively. Earlier, Johnson et al. (2008) explicated that provision of warfarin @ 0.5 mg/kg prolonged the clotting time 24.6 min as compared to 13.60 min for normal rabbits.

In this context, clotting parameters are used to identify the vitamin K deficiency through tissue-factor pathway or prothrombin time. Furthermore during vitamin K deficiency, activated partial thromboplastin time (represents overall blood clotting time) increases however, platelet counts, fibrinogen, factor V and VIII are suppressed. The degradation products of the fibrin including D-dimers are enhanced that indicate swift fibrinolytic activity. Earlier, Booth et al. (1999) assessed the bioavailability of phylloquinone and MK-4 after oral and colorectal administration. They documented that tested vitamin K forms significantly reduce the prolonged blood clotting time. Likewise, Groenen-van Doorena et al. (1993) reported that phylloquinone and MK-4 substantially improve the concentrations of vitamin K dependent coagulation factors.

Later, Gijsbers et al. (1996) observed the bioavailability of vitamin K from different preparations of spinach. They concluded that synthetic forms of phylloquinone have 7.5 and 24.3 times higher bioavailability than that of the spinach with or without butter. It is evident from the above debate that dietary vitamin K sources have potential to be used as dietary intervention against elevated blood clotting time and other coagulation related abnormalities.
Table 19. Effect of vitamin K enriched dietary sources on clotting time (min)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>12.42±0.43⁺</td>
<td>12.19±0.51⁻⁺</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>12.02±0.59⁺</td>
<td>11.76±0.54⁻⁺</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>14.34±0.68⁺</td>
<td>13.89±0.59⁻⁺</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>14.96±0.71⁺</td>
<td>14.59±0.65⁻⁺</td>
</tr>
</tbody>
</table>

Means carrying same letter in a row differed non-significantly (P > 0.05)

**= Highly significant
*= Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
Figure 6. Percent decrease of clotting time compared to control
4.4.2.3. Prothrombin time (PT)

The F values in Table 20 explicated that treatments imparted significant differences on prothrombin time (PT) in study I & II.

In study I, the maximum PT value was noticed in D1 12.41±0.63 sec followed by D2 12.07±0.53 and D3 11.93±0.68 sec whereas, minimum level 11.78±0.57 sec was recorded for D4 (trial 1). Likewise pattern was observed in trial 2 for tested vitamin K enriched dietary sources in normal rabbits. Moreover means for PT in study II (trial 1), exhibited the highest value for D1 (15.01±0.80 sec) that declined momentously to 14.14±0.96, 13.43±0.77 and 13.14±0.88 sec in D2, D3 and D4, respectively. Similar diminishing tendency was observed during trial 2 and the PT values were reduced from 15.29±0.95 to 13.42±0.98 sec in D1 to D4 group.

The Figure 7 illustrated that D4 (menadione) caused maximum reduction in PT followed by D3 (natto A) and D2 (cooked spinach). In study I (trial 1& 2) vitamin K enriched dietary sources i.e. D2, D3 and D4 showed 2.73 & 2.95, 3.86 & 4.34, 5.07 & 5.81% reduction in PT, respectively. Contrarily in study II (trial 1& 2), comparatively higher decline was noticed in D4 (12.45 & 12.23%) followed by D3 (10.52 & 11.24%) and D2 (5.79 & 6.54%). The current results are strengthened by the work of Thijssen and Reijnders (1994) regarding prothrombin time in vitamin K deficit and supplemented groups. They explicated that vitamin K deficiency significantly increases PT that moves toward its normal value by vitamin K administration. Later, Sokoll et al. (1997) noticed a decline in PT from 12.6 to 12.4 sec after supplementation of phylloquinone for 5 consecutive days. The functions of extrinsic pathway of blood coagulation factors synthesized in the liver are generally reflected by the prothrombin time (Grimaudo et al., 2005).

In contrary, consumption of natto (2 to 100 g) imparted non-significant decline in the PT value in healthy individuals (Hiroyuki 1999). During chronic gastrointestinal ailment, main factors contributing towards vitamin K deficiency are inadequate diet, nutrient loss and interference of drugs (Alpers et al., 2009). They also expounded that abnormal prothrombin production ultimately increases PT during vitamin K deficiency. The supplementation of
Table 20. Effect of vitamin K enriched dietary sources on prothrombin time (sec)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>12.41±0.63ᵃ</td>
<td>12.07±0.53ᵃ</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>12.20±0.55ᵃ</td>
<td>11.84±0.57ᵃ</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>15.01±0.80ᵃ</td>
<td>14.14±0.96ᵇ</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>15.29±0.95ᵃ</td>
<td>14.29±0.85ᵇ</td>
</tr>
</tbody>
</table>

*= Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
Figure 7. Percent decrease of prothrombin time (PT) compared to control
Phyloquinone significantly reduces the abnormal prothrombin moieties and prothrombin time.

Earlier, Purkins et al. (2003) observed significant upsurge in PT level after consumption of warfarin. Moreover, it further increased when warfarin was taken along with voriconazole antibiotic.

In another study, Mochizuki et al. (2009) compared the PT declining behavior after simultaneous administration of vitamin K and anticoagulant. For the reasons, two experiments based on phenobarbital and phenobarbital plus vitamin K2 (MK-4) were designed separately. They inferred that PT was shortened after MK-4 along with phenobarbital consumption as compared to phenobarbital treated animal group. Earlier, Sato et al. (2001) explicated that prolonged PT during vitamin K deficiency shifts towards normal state after the provision of phyloquinone, menaquinone-4 and menaquinone-7. Furthermore, they deduced that counter effect of MK-7 on extended prothrombin time is higher in comparison to phyloquinone and MK-4. Later, Hirayama et al. (2007) expounded that PT and APTT times are extremely prolonged after the provision of vitamin K deficient diet. The intragestric vitamin K3 (menadione) administration significantly shorten the prothrombin time by the activation of vitamin K dependent proteins, essential for normal blood coagulation cascade. From the present exploration, it is suggested that vitamin K enriched dietary sources especially natto is helpful to attenuate the elevated PT level due to unhealthy dietary practices and anticoagulant usage.

4.4.2.4. Activated partial thromoplastin time (APTT)

The F values indicated that vitamin K enriched dietary sources imparted significant differences on activated partial thromboplastin time (APTT) in study II however, study I showed non-momentous variations (Table 21). The means (study I; trial 1) showed APTT value 17.43±0.66 sec for D1 group that non-substantially declined to 17.17±0.44, 17.04±0.56 and 16.85±0.41 sec in D2, D3 and D4, respectively. Similarly in trial 2, D1 exhibited the highest APTT value (16.99±0.49 sec) that diminished non-significantly in D2 (16.64±0.60 sec), D3 (16.33±0.35 sec) and D4 (16.18±0.64 sec).

Likewise in study II (trial 1), mean APTT values for D1, D2, D3, and D4 were 22.17±0.81,
19.64±0.55, 19.15±0.69 and 18.18±0.45 sec, respectively. During trial 2, the highest value for APTT was observed in D1 (21.97±0.72 sec) followed by D2 (20.03±0.61 sec) and D3 (19.35±0.74 sec) whereas, the lowest was recorded in D4 (18.67±0.58 sec).

The Figure 8 revealed the percent decline in APTT among various groups of rabbits. In study I (trial 1 & 2) non-momentous decrease by 1.52 & 2.05%, 2.26 & 3.91% and 3.33 & 4.47% in D2, D3 and D4 was noticed as compared to control. Nonetheless, significant decline for APTT was noticed during study II (trial 1 & 2) by 11.40 & 8.85% in D2 (cooked spinach), 13.60 & 11.95% in D3 (natto A) and 17.98 & 15.04% in D4 (menadione).

The activated partial thromboplastin time (APTT) is an indicator of intrinsic and normal coagulation pathways. Besides identifying the blood clotting abnormalities, it also reflects the effect of anticoagulants (Fragaakis and Thomson, 2007). The present results for APTT reduction by vitamin K are in harmony with the findings of Chen et al. (2012), documented a decline in plasma APTT by oral vitamin K supplementation. They also reported that anticoagulant (dabigatran) treatment enhanced the plasma APTT by disturbing factor VIIa activity that was reduced substantially with phylloquinone provision @ 10 mg.

Previously, Sakaguchi et al. (2008) explicated that anticoagulant (Na dehydroacetate) affects the activity of vitamin K epoxide reductase and significantly prolongs blood coagulation parameters (PT and APTT) as compared to control. They conferred that vitamin K2 (1 mg/kg) reduces the blood coagulation time. One of their peers, Mochizuki et al. (2009) evaluated the influence of Phenobarbital on blood coagulation traits. They found that APTT was increased 21.4 to 27.3 sec in a dose dependent manner. The prolonged APTT (> 300 sec) during vitamin K deficiency was shortened to 49.5 sec by the provision of menadione (1 µg/day) to germ free rats (Hirayama et al., 2007). Moreover, antibiotic (latamoxef) induced coagulopathy was ameliorated by vitamin K supplementation for 10 days (Tabata et al., 1995). They also assessed reduction in APTT from 37 to 18 sec after the administration of vitamin K @ 200 µg/kg. Moreover, Booth et al. (1999) conducted a randomized crossover study involving healthy individuals to evaluate the effect of broccoli on PT & APTT and recorded non-momentous differences for these traits. From the aforementioned discussion, it is inferred that vitamin K is valuable against blood coagulation complications owing to its positive influence on carboxylation of vitamin K dependent proteins.
Table 21. Effect of vitamin K enriched dietary sources on APTT (sec)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>17.43±0.66</td>
<td>17.17±0.44</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>16.99±0.49</td>
<td>16.64±0.60</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>22.17±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.64±0.55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>21.97±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.03±0.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant  
NS= non- significant

Study I : Normal rabbits  
Study II : Vitamin K deficient rabbits

D₁: Control  
D₂: Spinach (phyloquinone)  
D₃: Natto/fermented soybean (menaquinone-7)  
D₄: Synthetic menadione (K₃)
Figure 8. Percent decrease of APTT compared to control
4.4.2.5. Plasma fibrinogen

The F values depicted that plasma fibrinogen level in different groups varied non-substantially due to treatment in study I however, significant effect was noticed for this trait in study II (Table 22). In study I (trial 1 & 2), the plasma fibrinogen levels were 335.33±14.95 & 337.71±9.81, 345.28±15.12 & 341.71±10.42, 347.27±15.21 & 344.29±10.91 and 350.25±15.66 & 347.57±11.21 mg/dL in D1 (control), D2 (cooked spinach), D3 (natto A) and D4 (menadione) groups, respectively. Similarly, vitamin K deficient rabbits (study II; trial 1 & 2) had minimum plasma fibrinogen concentration 307.09±15.98 & 310.43±13.72 mg/dL in D1 group that enhanced significantly in D4 334.24±15.73 & 337.83±15.27 mg/dL followed by D3 329.52±15.18 & 331.86±15.21 mg/dL and D2 324.67±15.01 & 326.57±12.16 mg/dL.

The current findings for plasma fibrinogen concentration in normal rabbits are in line with the research work of Kristensen et al. (2008), reported non-significant effect of vitamin K supplementation on fibrinolytic activity especially for plasma fibrinogen level (3.46 to 3.48 g/L) in normal subjects during 6 weeks trial. They deduced that phylloquinone intake manages the coagulation process properly especially fibrinogen-c and fVIIc concentrations under normal condition therefore its consumption is considered safe. In another study, Zivelin et al. (1993) documented that warfarin reduces the plasma fibrinogen (50%), factor VIII (42%) and factor V (35%) in rabbits by interfering the activity of plasma protein C & S. Furthermore, anticoagulant inhibits the thrombin and serine related protease coagulation enzymes resulting lower plasma fibrinogen concentration (Hirsh and Fuster, 1994). Later, Li-Saw-Hee et al. (2000) expounded that warfarin significantly reduces the plasma fibrinogen from 2.96±0.9 to 2.46±0.7 g/L. They further illustrated that warfarin effectively diminishes the excessive fibrin turnover. For the purpose, multiple forms of vitamin K are effectual to modulate the abnormal plasma fibrinogen level (Altay et al., 2012). Previously, Kawashima et al. (1997), illuminated the protective role of vitamin K on fibrinogen level in hypercholesterolemic rabbits. They noticed improved plasma fibrinogen concentration in rabbits fed on various levels of vitamin K2 enriched diets as compared to normal rabbits. It is deduced that cooked spinach and natto are effective to tackle the coagulation associated abnormalities i.e. prolonged bleeding, clotting, prothrombin & activated partial thromboplastin
<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<td></td>
<td>D₁</td>
<td>D₂</td>
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<td>341.71±10.42</td>
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<tr>
<td><strong>Study II</strong></td>
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<td></td>
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<tr>
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<td>307.09±15.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>324.67±15.01&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>(Trial 2)</td>
<td>310.43±13.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>326.57±12.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant
NS= Non-significant

Study I : Normal rabbits
Study II : Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phylloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
times and suppressed plasma fibrinogen level during vitamin K deficiency. It is interesting to mention that menaquinone-7 showed better performance to modulate blood coagulation parameters than phylloquinone.

4.4.2.6. International normalized ratio (INR)

The statistical interpretation exhibited that vitamin K enriched dietary sources imparted significant effect on INR in study II whereas, study I showed non-momentous variations (Table 23).

The means compiled for INR in D₁, D₂, D₃ and D₄ groups were 1.64±0.06 & 1.66±0.09, 1.63±0.07 & 1.65±0.04, 1.61±0.05 & 1.63±0.02 and 1.59±0.07 & 1.61±0.01, respectively in study I (trial 1 & 2). However in vitamin K deficient rabbits (study II; trial 1), D₁ illustrated maximum INR value 2.45±0.10 that substantially reduced to 2.37±0.14, 2.33±0.13 and 2.28±0.11 in D₂ (cooked spinach), D₃ (natto) and D₄ (menadione) groups, respectively. Similar declining tendency for INR was observed in the follow up trial. The maximum INR value 2.49±0.12 was recorded for D₁ that significantly suppressed in D₂ 2.39±0.17, D₃ 2.32±0.11 and D₄ 2.29±0.12.

The unstable international normalized ratio is the indicator of thrombosis and bleeding events. The variability in the intake of vitamin K may lead to INR fluctuation. The INR is a mathematical expression that eliminates the differences among sensitivity of various PT reagents (Marlar and Gausman 2011). The supplementation of vitamin K₁ stabilizes the INR value. Earlier, Khan et al. (2004) probed that INR has inverse relation with the intake of vitamin K. Afterwards, Pereira et al. (2005) explicated that consistent provision of vitamin K suppresses the elevated levels of INR and PIVKA-II from 2.2 to 1.0 and 10 to 3.3 AU/mL, respectively.

The coagulation proteins including procoagulant & vitamin K dependent factors are decreased during liver related abnormalities. These factors also depend upon the micorsomal γ-glutamyl carboxylation process, required for activation of zymogens that alter the prothrombin and activated partial thromboplastin times (Pereira et al., 1996). During short
Table 23. Effect of vitamin K enriched dietary sources on INR

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>1.64±0.06</td>
<td>1.63±0.07</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>1.66±0.09</td>
<td>1.65±0.04</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>2.45±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.37±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>2.49±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.39±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant
NS = Non-significant

Study I : Normal rabbits
Study II : Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phylloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
bowl syndrome, vitamin K significantly reduces the INR values from 4.7 to 2.2 (Aljarallah et al., 2012). During anticoagulant intake, the activities of extrahepatic coagulation factors are minimized after the consumption of different dietary vitamin K forms. In this context, progressive increase of phylloquinone (50–500 μg) stabilizes the INR value after warfarin intake. The threshold dose of vitamin K (150 μg/day) significantly reduces the elevated INR level (Schurgers et al., 2004). In another study, Schurgers et al. (2007) examined the effect of phylloquinone and MK-7 in individuals taking anticoagulants. They observed that MK-7 is more efficient in declining INR value than phylloquinone due to its higher efficacy for carboxylation of vitamin K dependent proteins in the liver. Furthermore, MK-7 is comparatively better to mitigate nutritional vitamin K deficiency than its counterparts (Schurgers and Vermeer 2002). Decisively, vitamin K enriched dietary sources have potential to manage abnormal blood coagulation factors and INR level however, synthetic menadione has proven more promising in the current case.

4.4.3. Bone mineral density (BMD)

The F values indicated that treatments exerted non-significant variations on bone mineral density in study I & II (Table 24). Means regarding BMD in study I (trial 1 & 2) depicted the values 0.242±0.007 & 0.246±0.008 g/cm² in D₁ group that increased non-momentously in D₂ 0.244±0.005 & 0.251±0.009 g/cm², D₃ 0.246±0.006 & 0.253±0.008 g/cm² and D₄ 0.245±0.005 & 0.249±0.007 g/cm². Likewise in study II (trial 1 & 2), minimum BMD level 0.222±0.009 & 0.224±0.008 g/cm² was observed in D₁ group nonetheless, vitamin K enriched dietary sources including cooked spinach (D₂), natto A (D₃) and menadione (D₄) non-substantially enhanced this attribute as 0.227±0.010 & 0.230±0.009, 0.231±0.012 & 0.235±0.010 and 0.226±0.011 & 0.228±0.007 g/cm² in respective trials.

The present findings are comparable with the earlier work of Aljarallah et al. (2012), they observed non-momentous improvement in bone mineral density (BMD) of vitamin K deficient volunteers after ingesting vitamin K. They inferred that metabolic bone ailment is due to diet deficient in vitamin K & D, calcium phosphate and protein. The bone mineral density measurement through dual-energy X-ray absorptiometry (DXA) provides a cumulative static estimation of skeletal status (Brown et al., 2009). It has been documented that frequency of osteoporosis is varied among the subjects from various demographic origin
Table 24. Effect of vitamin K enriched dietary sources on bone mineral density (g/cm²)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<tbody>
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<td>$D_1$</td>
<td>$D_2$</td>
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<tr>
<td>Study I</td>
<td>0.242±0.007</td>
<td>0.244±0.005</td>
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<td>(Trial 1)</td>
<td>0.246±0.008</td>
<td>0.251±0.009</td>
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<td>(Trial 2)</td>
<td>0.222±0.009</td>
<td>0.227±0.010</td>
</tr>
<tr>
<td>Study II</td>
<td>0.224±0.008</td>
<td>0.230±0.009</td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>0.224±0.008</td>
<td>0.230±0.009</td>
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<tr>
<td>(Trial 2)</td>
<td>0.224±0.008</td>
<td>0.230±0.009</td>
</tr>
</tbody>
</table>

NS=Non-significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

$D_1$: Control
$D_2$: Spinach (phyloquinone)
$D_3$: Natto/fermented soybean (menaquinone-7)
$D_4$: Synthetic menadione ($K_3$)
of Pakistan. In this reference, the estimated osteoporosis risk in Pashto, Urdu, Balochi and Punjabi population is 13.5, 7.5, 6.9 and 5.1%, respectively (Fatima et al., 2009).

Numerous epidemiological evidences showed a positive association between vitamin K and BMD. In this context, low consumption of vitamin K is linearly correlated with reduced BMD thereby increases the threat of hip fracture (Szulc et al., 1993; Szulc et al., 1994; Feskanich et al., 1999). The possible involvement of vitamin K in the enhancement of bone mineral density is its ability to act as cofactor for vitamin K dependent carboxylase and its participation in the posttranslational modification of Glu to Gla residue of vitamin K dependent proteins (Berkner 2000; Weber 2001). One of their peer, Sogabe et al. (2011) concluded that vitamin K enhances the protein γ-carboxylation. In this connection, ample amount of Gla containing proteins like osteocalcin is present in mature bone tissue (Binkley and Suttie 1995). The fully carboxylated osteocalcin has higher calcium binding ability with its Gla residues and the resultant osteocalcin binds to hydroxyapatite of bone (Shearer and Newman 2008).

The phylloquinone (vitamin K₁), menaquinone-4 (vitamin K₂) and menadione (vitamin K₃) trigger the in vitro mineralization through primary osteoblasts cells. The vitamin K₁ induced mineralization is highly sensitive to warfarin than that of K₂ and K₃. Similarly, it has been observed that vitamin K plays an important role in the improvement of bone metabolism (Sogabe et al., 2011). Earlier, Atkins et al. (2009) suggested that possible routes by which vitamin K optimizes the bone formation and integrity are active osteoblast to osteocyte transition and diminution osteoclastogenic potential. Thus, higher intake of vitamin K is required for optimal bone health related mechanisms (Schurgers et al., 2007). Moreover, Japanese natto contains ample amount of menaquinone-7 is helpful to ameliorate the incidence of osteoporosis. Previously, Ikeda et al. (2006) found linear association between natto intake and BMD level in postmenopausal women. Conversely, non-fermented soybean products showed non-significant effect on BMD.

Earlier, Braam et al. (2003) reported that phylloquinone affects the bone mineral metabolism and imparts positive influence on femoral neck BMD in postmenopausal women. During vitamin K deficiency, vital vitamin D dependent receptors are altered, required for intestinal calcium absorption (Sergeev et al., 1992). In this context, phylloquinone ingestion
diminishes the urinary calcium excretion (Martini et al., 2006). In another study, dietary vitamin K intake also affects the serum ucOC level, a prominent bone biochemical marker (Yamauchi et al., 2010).

Earlier, Troy et al. (2007) conducted a cross section framingham offspring study to evaluate the relationship between vitamin K and BMD. They observed a positive association between dihydrophyloquinone intake and BMD level estimated through dual-energy X-ray absorptiometry. Similarly, long term intake of phylloquinone (600 mg/kg) increases the bone mineral density whereas, menaquinone-4 (600 mg/kg) improves the bone strength parameters like width, trabecular, cancellous ash weight and body composition (Sogabe et al., 2011). The bone calcium, femoral dry weight content, alkaline and metaphyseal phosphatase activities in the metaphyseal and diaphyseal tissues of rats are enhanced by the oral supplementation of MK-7 @ 0.5 mg/100g along with Zn (1.0 mg/100 g). Accordingly, Zhong et al. (2007) delineated the synergistic activity of vitamin K (MK-7) and zinc on bone components and their preventing role against osteoporosis.

Similarly, vitamin K$_1$ supplementation @ 600 μg/day up to 6 months showed non-significant correlation with regional BMD and serum osteocalcin concentrations in pre- and peri-menopausal women whilst, bone health biomarkers like urinary N-telopeptide level improves significantly in the treated group (Volpe et al., 2008).

Afterwards, a meta-analysis conducted by Iwamotoa et al. (2009) showed that high doses of vitamin K$_1$ and K$_2$ improve bone strength in femoral neck and reduce the incidence of fractures. Likewise, menaquinone-7 is helpful to mitigate bone disorders during liver cirrhosis through activation of matrix Gla protein (MGP) that helps in calcium plunking towards bones (Tabasum and Qadir, 2010). From the present results, it is suggested that tested vitamin K dietary sources especially natto containing menaquinone-7 is helpful to improve bone mineral density.

### 4.4.4. Vitamin K dependent proteins

#### 4.4.4.1. Carboxylated osteocalcin (cOC)

The F values in Table 25 indicated significant effect of treatments on plasma carboxylated osteocalcin (cOC) in study I &II. It is evident from the means (study I) that cOC was raised
from 5.08±0.22 ng/mL (D₁) to 5.37±0.29 ng/mL (D₄) while, the values for D₂ and D₃ were 5.21±0.26 and 5.32±0.28 ng/mL, in respective way. Likewise in 2nd trial, the recorded cOC concentration in D₁ was 5.15±0.24 ng/mL that progressively enhanced in the order of D₂, D₃ and D₄ by 5.26±0.27, 5.34±0.32 and 5.42±0.35 ng/mL, respectively. In study II, the lowest plasma cOC values were 3.66±0.11 & 3.89±0.14 ng/mL in D₁ that increased to 4.13±0.13 & 4.23±0.09, 4.26±0.13 & 4.44±0.10 and 4.30±0.14 & 4.57±0.08 ng/mL for D₂, D₃ and D₄ groups, correspondingly (trial 1 & 2).

The graphical representation (Figure 9) illuminated momentous increase in plasma cOC levels in D₂, D₃ and D₄ groups throughout the study I by 2.58 & 2.23, 4.72 & 3.68 and 5.70 & 5.24%, respectively. However, relatively higher percent increase was noticed in study II (trial 1 & 2) by 12.98 & 11.14, 16.57 & 14.13 and 17.68 & 17.39% for respective groups.

The results of instant research are comparable with the earlier observations of Niemeier et al. (2005), they reported that carboxylated osteocalcin contents were enhanced in experimental mouse after phylloquinone supplementation. They were on the view that phylloquinone triggers the transportation of chylomicron to the osteoblasts through circulatory system and utilizes for γ-glutamyl carboxylation of osteocalcin. Later, Schurgers et al. (2007) reported better performance of MK-7 supplementation (0.22 µmol/day) for the carboxylation process of osteocalcin owing to its prolonged residence time that elevates serum MK-7 concentration. During vitamin K deficiency, γ-carboxylated osteocalcin level is gradually suppressed due to unavailability of vitamin K consequently the levels of undercarboxylated osteocalcin (ucOC) and PIVKA-II are enhanced. Nonetheless, the phylloquinone administration @ 200µg/day significantly modulates the abnormal elevation of ucOC and PIVKA-II levels (Booth et al., 2001). In a similar study, Schaafsma et al. (2000) delineated that insufficiency of vitamin K is associated with higher level of circulating undercarboxylated osteocalcin. The intake of vitamin K improves the plasma osteocalcin concentration thereby reduces undercarboxylated osteocalcin content, bone loss and fractures (McKeown et al., 2002).

Earlier, Jie et al. (1995) noticed that vitamin K intake elevates the serum carboyxlated osteocalcin content in elderly volunteers. Moreover, they observed an inverse association between carboxylated osteocalcin level, age, waist to hip ratio & body mass index. Later, Martini et al. (2006) observed that vitamin K intake improves the concentration of plasma
Table 25. Effect of vitamin K enriched dietary sources on carboxylated osteocalcin (ng/mL)

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<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<tbody>
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<td>D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>5.08±0.22</td>
<td>5.21±0.26</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>5.15±0.24</td>
<td>5.26±0.27</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>3.66±0.11&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.13±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>3.89±0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.32±0.09&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D<sub>1</sub>: Control
D<sub>2</sub>: Spinach (phyloquinone)
D<sub>3</sub>: Natto/fermented soybean (menaquinone-7)
D<sub>4</sub>: Synthetic menadione (K<sub>3</sub>)
Figure 9. Percent increase of carboxylated osteocalcin compared to control
osteocalcin in women. Additionally, provision of vitamin K<sub>2</sub> imparts positive influence on the bone metabolism of healthy females. In this context, Koitaya <em>et al.</em> (2009) documented that low dose of vitamin K<sub>2</sub> (1.5 mg/day) for 4 weeks caused a substantial elevation (50%) in the level of γ-carboxylated osteocalcin in fifty four postmenopausal women. Earlier, Booth <em>et al.</em> (2003) expressed that after the ingestion of phylloquinone (450 µg), vitamin K dependent biochemical indicators are improved significantly.

Previously, Binkely <em>et al.</em> (2002) observed that maximum γ-carboxylation of osteocalcin is attained after supplementation of vitamin K @ 1000 µg to the vitamin K deficient postmenopausal women. In this connection, the optimum level of fully carboxylated osteocalcin is necessary for proper bone maturation. The osteocalcin is synthesized in the mature osteoblasts in the presence of 1,25- dihydroxyvitamin D. The calcium binding capacity of osteocalcin is primarily dependent upon the concentration of γ-carboxylatation of its Gla residue. In this reference, fully carboxylated osteocalcin is important for bone health (Truong and Booth 2011). Earlier, Tsukamoto <em>et al.</em> (2000) evaluated the effect of natto on circulating concentration of γ-carboxylated osteocalcin in healthy volunteers. They prepared different types of natto with varying concentrations of MK-7 and provided to healthy individuals for 14 days. The results indicated that γ-carboxylated osteocalcin level is affected non-significantly by low MK-7 natto however, its high level (1295 or 1730 µg) significantly enhances the concentration of this trait.

From the present results, it is assumed that vitamin K enriched dietary sources especially natto is valuable to attain higher plasma osteocalcin level due to higher retention time of MK-7 in the circulatory system and active participation in the carboxylation process.

4.4.4.2. Undercarboxylated osteocalcin (ucOC)

The statistical analysis in Table 26 showed significant effect of treatments on plasma undercarboxylated osteocalcin (ucOC) in study I & II. The means pertaining to ucOC (study I; trial 1 & 2) indicated the maximum value for D<sub>1</sub> group (3.21±0.14 & 2.87±0.12 ng/mL) that gradually declined in D<sub>2</sub> (3.15±0.13 & 2.82±0.12 ng/mL), D<sub>3</sub> (3.09±0.14 & 2.74±0.13 ng/mL) and D<sub>4</sub> (3.06±0.16 & 2.72±0.11 ng/mL). Likewise in study II (trial 1), the highest ucOC value was noticed in control (D<sub>1</sub>) i.e. 4.08±0.18 ng/mL however, D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> groups
showed a gradual decline for this trait as 3.79±0.14, 3.71±0.15 and 3.49±0.17 ng/mL, respectively. Nevertheless, 2\textsuperscript{nd} trial showed ucOC levels by 4.01±0.17, 3.75±0.15, 3.68±0.13 and 3.46±0.15 ng/mL for respective groups.

It is noticeable from Figure 10 that percent reduction of ucOC in study I (trial 1 & 2) was 1.87 & 1.74, 3.74 & 4.53 and 4.67 & 5.23\% for D\textsubscript{2}, D\textsubscript{3} and D\textsubscript{4}, respectively. However, vitamin K deficient rabbits showed higher decline by 7.11 & 6.17, 9.07 & 8.15, 14.46 & 13.83\% for respective groups.

The present results are in agreement with the findings of Yasui \textit{et al.} (2006), observed that circulating undercarboxylated osteocalcin percentage is high during vitamin K deficient state. They documented that serum vitamin K level is negatively correlated with the undercarboxylated osteocalcin concentration. Moreover, the ratio of undercarboxylated osteocalcin to total osteocalcin elevates during vitamin K deficiency (Truong and Booth 2011). Earlier, Schurgers \textit{et al.} (2004) noticed the plasma undercarboxylated osteocalcin (ucOC) levels in male and female as 3.4±1.7 and 3.9±1.6 ng/mL, respectively. However, the intake of anticoagulant enhanced the circulating ucOC level by 24.4±6.4 ng/mL that was substantially decreased to 6.1 ng/mL with the intake of vitamin K (300 to 500 µg/day) for seven days.

In Irish healthy subjects, the adequate phylloquinone intake substantially dampened the undercarboxylated osteocalcin (ucOC) level nevertheless, they showed higher circulating phylloquinone level (Martini \textit{et al.}, 2006). Additionally, increased concentration of plasma undercarboxylated osteocalcin is linked to reduced mineralization process of bones (Truong and Booth 2011). Earlier, Koitaya \textit{et al.} (2009) observed a significant decline for plasma undercarboxylated osteocalcin concentration in postmenopausal women by providing vitamin K\textsubscript{2} during 4 weeks.

The vitamin K is essential for carboxylation of osteocalcin, in case of serum vitamin K insufficiency, this process is hampered resulting higher amount of undercarboxylated osteocalcin in the blood. In this course, an inverse association has been observed between ucOC and serum phylloquinone & MK-7 concentrations (Truong and Booth 2011). Earlier, Tsukamoto \textit{et al.} (2000) noticed a momentous decline in the plasma undercarboxylated
Table 26. Effect of vitamin K enriched dietary sources on undercarboxylated osteocalcin (ng/mL)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>3.21±0.14ᵃ</td>
<td>3.15±0.13ᵇ</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>2.87±0.12ᵃ</td>
<td>2.82±0.12ᵇ</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>4.08±0.18ᵃ</td>
<td>3.79±0.14ᵇ</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>4.01±0.17ᵃ</td>
<td>3.75±0.12ᵇ</td>
</tr>
</tbody>
</table>

**= Highly significant
*= Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
Figure 10. Percent decrease of ucOC compared to control
osteocalcin level by the ingestion of menaquinone-7. One of the researchers groups, van Summeren et al. (2009) conducted a double blind randomized placebo controlled trial to evaluate the effect of menaquinone-7 on the circulating undercarboxylated osteocalcin. They found that inactive ucOC level was reduced after supplementation of MK-7 for 8 weeks. They further deduced that MK-7 ingestion did not impart any significant effect on blood coagulation parameters and circulating ucOc & cOC concentrations in placebo group. One of their peers, Kuwabara et al. (2009) elucidated that low intake of vitamin K is associated with elevated plasma undercarboxylated osteocalcin level due to less carboxylation of vitamin K depended proteins.

Earlier, Małyszko et al. (2002) reported that administration of vitamin K for 2-8 weeks reduces its deficiency biomarkers especially plasma undercarboxylated osteocalcin. They also observed that the ratio between carboxylated and undercarboxylated osteocalcin was improved after the supplementation of vitamin K. Previously, Binkley et al. (2000) also established that high intake of vitamin K either through food or supplements decreases the level of ucOC.

In a research study, Sokoll and Sadowski (1996) evaluated the variations in undercarboxylated osteocalcin level with age in both genders. They observed progressive increase in the plasma undercarboxylated osteocalcin concentration with age. They also noticed that plasma ucOC percentage was increased from third to the fifth decade of the life. The low plasma phylloquinone level due to less dietary intake of vitamin K (>10µg/day) is inversely correlated with ucOC concentration (McKeown et al., 2002).

Later, Bolton-Smith et al. (2009) conducted a two year double blind placebo controlled trial in healthy Scottish subjects to investigate the effect of vitamin K on its dependent proteins. They observed that vitamin K supplementation coupled with vitamin D decreased the ucOC level by 51%. They concluded that vitamin K intake through dietary source substantially increases the carboxylation of osteocalcin and has better potential to alleviate the threat of vitamin K deficiency.
From the above debate, it is inferred that among vitamin K enriched dietary sources menaquinone-7 is more efficient to curb the synthesis of ucOC due its active participation in vitamin K cycle thus regulates the process of $\gamma$-carboxylation.

4.4.4.3. Protein induced by vitamin K absence or antagonist-II (PIVKA-II)

The results in Table 27 showed significant effect of treatments on plasma PIVKA-II (protein induced by vitamin K absence or antagonist-II) during study I (normal rabbits) and study II (vitamin K deficient rabbits). The means for PIVKA-II in study I were compiled as 3.02±0.14, 2.98±0.12, 2.89±0.10 & 2.86±0.13 ng/mL (trial 1) and 2.94±0.13, 2.90±0.10, 2.81±0.09 & 2.78±0.11 ng/mL (trial 2) for D$_1$, D$_2$, D$_3$ & D$_4$ groups, respectively. Similarly in study II, D$_1$ showed the maximum PIVKA-II value (9.01±0.34 & 9.53±0.30 ng/mL) followed by D$_2$ (8.21±0.31 & 8.71±0.26 ng/mL) and D$_3$ (7.92±0.25 & 8.34±0.22 ng/mL) whilst minimum in D$_1$ (7.76±0.39 & 8.18±0.41 ng/mL) for consecutive trials.

It is obvious from Figure 11 that in study I vitamin K dietary sources showed decrease in PIVKA-II level for D$_2$ (cooked spinach), D$_3$ (natto A) and D$_4$ (menadione); 2.64, 4.63 and 5.29% (trial 1) whereas 1.36, 4.42 and 5.44% (trial 2), respectively. For vitamin K deficient rabbits (study II), the maximum decline was observed in D$_4$ (17.42 & 14.16%) followed by D$_3$ (13.89 & 12.48%) and D$_2$ (9.37 & 8.61%) during both trials.

The present results are in harmony with the findings of Booth et al. (2001). According to them, vitamin K deficient diet significantly elevates the concentration of protein induced by vitamin K antagonist-II (PIVKA-II). They also found significant reduction in PIVKA-II level by the intake of phylloquinone @ 200 $\mu$g/day for 10 days. Similarly, low intake of vitamin K retards its turnover rate resultanty increases the PIVKA-II level by depleting the store vitamin K from liver and bone (Harrington et al., 2007). In this regard, amount of osteocalcin, uncarboxylated PIVKA-II and MGP are the sensitive indicators of deprived vitamin K status (Iwamoto et al., 2006; Booth and Rajabi, 2008).

The circulating level of PIVKA-II is an estimation of undercarboxylated prothrombin. During vitamin K deficiency, its level enhances up to 53% and becomes normal by the administration of dietary phyloquinone (Martini et al., 2006). Earlier, Cushman et al. (2001) investigated the role of biochemical markers of vitamin K status during warfarin
<table>
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<th>Studies</th>
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<tr>
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<td>(Trial 2)</td>
<td>2.94±0.13ᵃ</td>
<td>2.90±0.10ᵇ</td>
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<tr>
<td><strong>Study II</strong></td>
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<tr>
<td>(Trial 1)</td>
<td>9.01±0.34ᵃ</td>
<td>8.21±0.31ᵇ</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>9.53±0.30ᵃ</td>
<td>8.71±0.26ᵇ</td>
</tr>
</tbody>
</table>

** = Highly significant  
* = Significant

Study I: Normal rabbits  
Study II: Vitamin K deficient rabbits

D₁: Control  
D₂: Spinach (phyloquinone)  
D₃: Natto/fermented soybean (menaquinone-7)  
D₄: Synthetic menadione (K₃)
Figure 11. Percent decrease of PIVKA-II compared to control
They observed that diet containing 65-80 μg of phylloquinone is associated with decline in PIVKA-II concentration. The gathered information of various studies indicated that stability against warfarin is improved by the daily intake of vitamin K (Holmes et al., 2012b). The PIVKA-II is an abnormal des-carboxylated prothrombin that is elevated in the blood during vitamin K deficiency or using warfarin. It is an important vitamin K deficiency indicator before establishing clinical symptoms. It has been confirmed by numerous researches that PIVKA-II is an inactive precursor of coagulation factor II (Baek et al., 2009; Shearer 2009; Holmes et al., 2012). Earlier, Lee et al. (2005) established a correlation between PIVKA-II and INR level and observed changes in PIVKA-II concentration after the commencement of warfarin administration.

The prothrombin time is a classical evaluation assay for vitamin K deficiency however, recent investigations have shown that circulating concentration of under–γ-carboxylated prothrombin (PIVKA-II), undercarboxylated osteocalcin (ucOC) and urinary Gla excretion are the sensitive biomarkers that alter their response during vitamin K variations. In vitamin K deficiency phase, plasma phylloquinone and factor VII are diminished about 70 and 20%, respectively whereas, the circulatory PIVKA-II levels is exceed approximately 67% (Booth and Suttie 1998). Earlier, Ferland et al. (1993) assessed the effect of depletion and repletion of vitamin K on the plasma PIVKA-II. The observed subjects showed gradual increase in PIVKA-II value during depletion period (10 µg/day) however, shifted towards normal level by taking vitamin K (45 µg/day). Afterwards, Sato et al. (2001) reported the effect of various menaquinones on the PIVKA-II level during warfarin induced hypoprothrominaemia. They concluded that among different homologues of vitamin K2, MK-7 performs better against the higher level of PIVKA-II than MK-4.

Conclusively, the vitamin K enriched dietary sources exhibited higher carboxylation of vitamin K dependent proteins especially osteocalcin and prothrombin. In this context, elevated undercarboxylated osteocalcin and PIVKA-II levels were modulated through the administration of cooked spinach and natto.
4.4.5. Serum vitamin K contents

4.4.5.1. Serum phylloquinone

The F values in Table 28 explicated that serum phylloquinone level was affected momentously as a function of treatments in both studies. Data regarding serum phylloquinone concentration for study I (trial 1) indicated that control group (D₁) had minimum level 9.76±0.39 ng/mL for serum phylloquinone that significantly increased by 11.97±0.41, 11.29±0.31 and 10.70±0.16 ng/mL in groups relied on cooked spinach (D₂), natto A (D₃) and menadione (D₄), respectively. Likewise in trial 2, the serum phylloquinone levels were 9.72±0.34, 11.80±0.28, 11.04±0.21 and 10.81±0.37 ng/mL for D₁, D₂, D₃ and D₄, respectively. During 8 weeks efficacy trial (study II), the highest concentration was recorded for D₂ (9.36±0.30 & 9.19±0.27 ng/mL) followed by D₃ (8.65±0.22 & 8.83±0.19 ng/mL) and D₄ (8.10±0.11 & 8.14±0.16 ng/mL) while the lowest level was observed in D₁ (6.34±0.09 & 6.31±0.03 ng/mL) for trial 1 & 2, correspondingly.

The Figure 12 depicted the percent increase in serum phylloquinone concentrations of rabbits fed on various preparations of vitamin K enriched dietary sources. In study I (trial 1 & 2), substantial increase by 23.78 & 21.40, 16.71 & 13.56 and 10.64 & 11.26% was observed in D₂, D₃ and D₄ groups, respectively as compared to control. Nevertheless during vitamin K deficiency, serum phylloquinone level was momentously increased and the highest value was recorded in D₂ 47.58% followed by D₃ & D₄ as 36.42 & 27.71%, respectively. Besides in 2nd trial, 45.63, 39.94 and 29.01% increase for this trait was observed in D₂, D₃ and D₄ groups, respectively.

It is documented that vitamin K enriched dietary sources significantly enhance the serum vitamin K concentration depending upon the amount and type of source. The outcomes of various bioevaluation studies involving humans and animals (rats, mice and rabbits) illuminated the role of vitamin K against blood coagulation disorders owing to its unique structure and mode of action (Shirakawa et al., 2005; Booth and Rajabi 2008; Shearer, 2009; Altay et al., 2012).

The results regarding significant incline in serum phylloquinone level with vitamin K enriched dietary sources in normal rabbits (study I) are in corroboration with the findings of
### Table 28. Effect of vitamin K enriched dietary sources on serum phylloquinone level (ng/mL)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>9.76±0.39&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.97±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>9.72±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.80±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>6.34±0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.36±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>6.31±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.19±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

** = Highly significant

Study I : Normal rabbits  
Study II : Vitamin K deficient rabbits  
D₁: Control  
D₂: Spinach (phylloquinone)  
D₃: Natto/fermented soybean (menaquinone-7)  
D₄: Synthetic menadione (K₃)
Figure 12. Percent increase of serum phylloquinone compared to control
Garber et al. (1999). They found a momentous increase in serum phylloquinone level after administration of spinach (165mg/Kg), broccoli (184 mg/Kg) and romaine lettuce (179 mg/Kg). The concentration of phylloquinone varied from 0.15 to 1.0 µg/L during fasting in adults (Booth and Rajabi, 2008; Shearer, 2009).

One of the researchers groups, van Hasselt et al. (2009) observed significant rise in circulating phylloquinone level after gastric administration of polymeric micelles of vitamin K (1 mg). The bile duct restores the absorption of vitamin K after duodenal administration with the combination of bile acid. The absorption of phylloquinone is affected by taking 13C-labelled cosmopolitan & animal-oriented meals in healthy non-obese subjects (Jones et al., 2009). During lower dietary phylloquinone intake, rapid decline is observed in plasma & liver phylloquinone and menaquinone concentrations (Usui et al., 1990). The exchangeable body pool of phylloquinone is decreased after taking vitamin K restricted diet. Likewise, faecal excretion of phylloquinone and its metabolites are reduced by 32% in subjects relied on vitamin K deficient diet (1 µg/kg) for 6 days (Olson et al., 2002). Similarly, subjects fed on vitamin K @ 5µg/day for 13 days showed 70 and 20% decline in serum phylloquinone level and factor VII activity, respectively (Booth and Suttie 2004). After vitamin K supplementation, serum phylloquinone concentration is increased about 10 folds (Binkley et al., 2000). It has been documented that liver phylloquinone concentration is positively correlated with dietary phylloquinone intake. The serum phylloquinone concentration is enhanced with ample supply of vitamin K to the liver for optimal synthesis of vitamin K dependent proteins (Kindberg and Sãoettie, 1989). Earlier, Garber et al. (1999) observed that circulating phylloquinone level is raised momentously after the ingestion of its synthetic form coupled with spinach. Later, Kristensen et al. (2008) explicated that phylloquinone supplementation uplifts the serum phylloquinone level by 94%.

The warfarin and its derivatives generally act as vitamin K antagonists and hamper the vitamin K recycling by inhibiting vitamin K epoxide reductase activity that hinders the conversion of vitamin K epoxide into vitamin K (Schurgers and Vermeer 2000). In this context, Khan et al. (2004) carried out a trial to explore the effect of vitamin K ingestion on serum phylloquinone concentration. They observed substantial increase in serum phylloquinone level from 155 to 345 pg/mL in subjects relied on vitamin K (7 to 377 µg/day)
along with simultaneous intake of anticoagulant. They deduced that vitamin K intake caused substantial increase in serum phylloquinone level depending upon the source. In another study, Booth *et al.* (1999) observed that bioavailability of phylloquinone from oil or broccoli varied non-significantly. The important carriers of phylloquinone are triglycerides rich, low and high density lipoproteins. During intestinal absorption, phylloquinone is assimilated with chylomicrons that secrete through lymph lacteals and enter the blood via thoracic duct (Shearer and Newman 2008). It is concluded from the present study that vitamin K rich sources are effectual to improve the serum vitamin K level with special reference to phylloquinone concentration.

### 4.4.5.2. Serum menaquinone-4 (MK-4)

It is obvious from the F values (Table 29) that treatments imparted significant variations on serum menaquinone-4 (MK-4) concentration in study I & II. Means pertaining to serum MK-4 concentration in study I (normal rabbits) indicated the values 1.65±0.04, 1.73±0.05, 1.77±0.06 and 1.72±0.04 ng/mL for D₁, D₂, D₃ and D₄ groups, respectively (trial 1). Likewise trend was noticed in trial 2, the serum MK-4 level was uplifted momentously from 1.71±0.03 to 1.83±0.05 ng/mL in D₁ to D₃ groups, correspondingly. Besides, in study II (trial 1), the lowest MK-4 level 0.92±0.02 ng/mL was observed in D₁ that elevated significantly in D₂ 1.03±0.03 ng/mL, D₃ 1.04±0.01 ng/mL and D₄ 1.02±0.02 ng/mL. Similarly in the follow up trial, significant improvement in MK-4 level was recorded.

The Figure 13 depicted percent increase in serum MK-4; in study I (trial 1 & 2) the highest value was recorded for natto A (D₃) 7.14 & 7.02% followed by cooked spinach (D₂) and menadione (D₄) 4.84 & 4.67 and 4.27 & 3.50%, respectively. Similarly in study II (trial 1 & 2), the maximum enhancement was observed in D₃ (13.04 & 15.05%) trailed by D₄ (10.86 & 11.82%) and D₂ (11.95 & 12.90%).

The outcomes of various scientific explorations indicated that vitamin K is converted into menaquinone-4 in animals. It has been established that phylloquinone is cleaved by intestinal bacteria to menadione that converts to MK-4 by geranyl-geranyl pyrophosphate alkylation process (Guillaumont *et al.* 1992, Sakamoto *et al.* 1996). Later, Davidson *et al.* (1998)
Table 29. Effect of vitamin K enriched dietary sources on serum menaquinone-4 level (ng/mL)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>1.65±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.73±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>1.71±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.79±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>0.92±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.03±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>0.93±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.05±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

** = Highly significant
* = Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/fermented soybean (menaquinone-7)
D₄: Synthetic menadione (K₃)
Figure 13. Percent increase of serum menaquinone-4 compared to control
conducted animal trial and validate the findings of vitamin K conversion to MK-4. Conversely, Thijssen et al. (1996) observed that gnotobiotic and conventionally housed rats have equal amount of MK-4 in the tissues and noticed less impact of bacteria on this process. Additionally, cell lines (kidney-derived cell line 293, liver derived cell line H-35) investigations have also proven that MK-4 conversion is dependent on the concentration of vitamin K (phyloquinone) and incubation time. However, its conversion process is hampered during in vitro and in vivo models by anticoagulant warfarin (Thijssen et al. 1996).

The present findings are in agreement with the previous work of Okano (2008), explicated the conversion of vitamin K to MK-4 in various tissues. They observed plasma phylloquinone and MK-4 level as 0.6 and 1.2 pmol/mL, respectively in rats fed on normal diet. Furthermore, MK-4 concentration is increased in tissues and serum as dose dependent manner from 0.1 to 10 μmol/kg body weight. Later, Okano et al. (2009) observed deuterium labeled MK-4 in mice relied on deuterium labeled phylloquinone. They suggested that vitamin K is converted to MK-4 via integral side chain removal. In the intestine, vitamin K releases menadione that subsequently converts to MK-4 by prenylation. Likewise, ingestion of MK-7 also increases the serum MK-4 level considerably (Sato et al., 2007). Earlier, Thijssen et al. (2006) observed that menadione (K₃) in the form of glucuronides and sulfate conjugates increases in male individuals relied on phylloquinone. They deduced that MK-4 present in the tissues is a metabolic product of menadione from ingested phylloquinone.

It is worth mentioning that synthetic menadione is also converted to MK-4 prior to act as cofactor for γ-glutamyl carboxylase. There are a number of tissues i.e. vessel wall, pancreas and testis that are able to convert phylloquinone to MK-4 (Schurgers and Vermeer 2000). It is transported to various organs via triglycerides rich lipoprotein, LDL and HDL that have relatively high effect on extrahepatic vitamin K status (Schurgers and Vermeer 2002). In the nutshell, vitamin K enriched dietary sources have potential to convert phylloquinone, MK-7 and menadione to MK-4 through prenylation.

4.4.5.3. Serum menaquinone-7 (MK-7)

The F values showed that menaquinone-7 (MK-7) level in different groups of rabbits was significantly affected by treatments in all studies (Table 30). In study I (trial 1) mean MK-7
values for $D_1$, $D_2$, $D_3$ and $D_4$ groups were $2.70\pm0.12$, $2.77\pm0.11$, $3.18\pm0.19$ and $2.73\pm0.12$ ng/mL whereas in 2nd trial, $2.90\pm0.13$, $2.98\pm0.14$, $3.51\pm0.17$ and $2.97\pm0.13$ ng/mL, respectively. In study II (trial 1 & 2), $D_1$ group depicted the lowest MK-7 concentration $1.02\pm0.04$ & $1.04\pm0.03$ ng/mL that substantially increased to $1.15\pm0.06$ & $1.19\pm0.07$, $1.27\pm0.07$ & $1.29\pm0.08$ and $1.13\pm0.05$ & $1.18\pm0.02$ ng/mL in $D_2$ (cooked spinach containing phylloquinone), $D_3$ (natto containing menaquinone-7) and $D_4$ (menadione) groups, respectively (trial 1 & 2).

It is obvious from the Figure 14 that vitamin K dietary sources $i.e.$ $D_2$, $D_3$ and $D_4$ groups resulted 2.59, 17.77 & 1.11% incline in serum MK-7, respectively whilst in trial 2, selected treatments caused 2.75, 21.03 & 2.41% rise (study I) for this trait. Likewise in study II, $D_3$ showed maximum increase 24.50 & 24.03% trailed by $D_2$ 12.74 & 14.42% and $D_4$ 10.78 & 13.46%, correspondingly (trial 1 & 2). The explorations of Sato et al. (2012) supported the current data for momentous effect of vitamin K enriched dietary sources on serum MK-7 level in vitamin K deficient rabbits (study II); they noticed that MK-7 @ 429 µg increases its circulating level 6 hr after ingestion in healthy females. They deduced that among menaquinones, MK-7 is well absorbed through intestine owing to its better bioavailability and efficacy. Previously, Tsukamoto et al. (2000) also revealed an increase in serum MK-7 level after the consumption of natto. They found that serum MK-7 level was significantly raised from 0.94 to 11.01 and 24.51 ng/mL, respectively after the consumption of natto @ 50 g for 1 to 7 days.

Numerous investigations have indicated the positive impact of natto on circulating MK-7 level. Frequent use of natto in the Eastern Japanese, significantly enhanced the serum MK-7 concentration by 5.26 ng/mL as compared to restricted women of Western Japan and Britain as 1.22 and 0.37 ng/mL, respectively (Kaneki et al., 2001). Earlier, it was documented that plasma MK-7 level was increased in subjects consuming 2 to 100 g of natto in a dose dependent manner (Sumi, 1999). Later, Vermeer (2003) noticed serum MK-7 level as 16.2 and 16.5 ng/mL, 6th hr after the ingestion of natto and synthetic MK-7, respectively compared to baseline value 0.4 ng/mL. It was reported that phylloquinone and menaquinone-7 concentrations in normal subject are 0.71 and 2.8 ng/mL, respectively whereas, vertebral fractured patients indicated lesser amount of menaquinone-7 by 2.1 ng/mL.
<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Study I</td>
<td>2.70±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.77±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>2.90±0.13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.98±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>1.02±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.15±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Study II</td>
<td>1.04±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.19±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

** = Highly significant

Study I: Normal rabbits  
Study II: Vitamin K deficient rabbits

D₁: Control  
D₂: Spinach (phyloquinone)  
D₃: Natto/fermented soybean (menaquinone-7)  
D₄: Synthetic menadione (K₃)
Figure 14. Percent increase of serum menaquinone-7 compared to control
Additionally, hip fractured patients demonstrated lower serum MK-7 and phylloquinone concentrations by 1.9 and 0.47 ng/mL (Kawana et al., 2001). Likewise, Schurgers and Vermeer (2000) observed the elevation in serum MK-7 level after the provision of 200 g natto in healthy individuals. They also inferred that peak concentration of serum MK-7 is about 80 nmol/L after 6 hr. Moreover, MK-7 is detectable in serum after 72 hr, indicating its higher bioavailability.

The side chain lengths of menaquinones play an important role in their bioavailability. The medium chain menaquinones especially MK-7 are better absorbed as compared to long (MK-8 and MK-9) and short (MK-4) chain menaquinones (Schurgers and Vermeer, 2000; Schurgers 2002). Furthermore, menaquinones readily absorb and convert to shorter chain menaquinones in the gastrointestinal tract that transport by bile-salt mediated pathway to the blood (Groenen-Van Dooren et al., 1995; IARC, 2000). The possible reasons for higher concentration of serum phylloquinone and more effectiveness of menaquinones are discussed herein. The phylloquinone turnover time is shorter as about 1.5 day (Olson et al., 2002). Moreover, vitamin K₁ and MK-7 is well absorbed with peak serum concentrations at 4 hr after the intake (Schurgers et al., 2007). Furthermore, vitamin K₂ is more stable in serum owing to its long half-life and accumulated about 7 to 8 folds higher during prolonged intake. Moreover, orally administered vitamin K₁ is converted to MK-4 in several organs of the rats and mouse during 24 hr of administration (Shirakawa et al., 2005). Later, Booth et al. (2008) explicated the phylloquinone is converted to vitamin K₂ with the same dose of 2,3-dihydrophylloquinone in male Fischer 344 rats of different ages groups. Irrefutably, natto has potential to raise the level of serum MK-7 towards healthy limit.

4.4.6. Thiobarbituric acid reactive substances (TBARS)

It is evident from the statistical analysis that serum thiobarbituric acid reactive substances (TBARS) levels of rabbits were affected momentously by the treatments in all studies (Table 31). Means regarding TBARS (study I; trial 1 & 2) indicated the highest value 21.31±0.91 & 21.63±0.84 nmol/L for D₁ that significantly reduced to 19.87±0.85 & 20.21±0.61, 18.26±0.78 & 18.62±0.72 and 20.42±0.54 & 21.81±0.79 nmol/L in D₂, D₃ and D₄ groups, respectively. Similarly in study II, the maximum TBARS value was noticed in D₁ (23.24±0.99 nmol/L) that substantially diminished in D₂ (21.23±0.75 nmol/L), D₃ (20.53±0.38 nmol/L) and D₄
Table 31. Effect of vitamin K enriched dietary sources on serum TBARS (nmole of malenaldehyde/L)

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<th>Diets</th>
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<td>D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Study I</td>
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<td></td>
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<tr>
<td>(Trial 1)</td>
<td>21.31±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.87±0.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>21.63±0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.21±0.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Study II</td>
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<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>23.24±0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.23±0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>22.54±0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.53±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D<sub>1</sub>: Control
D<sub>2</sub>: Spinach (phyloquinone)
D<sub>3</sub>: Natto/fermented soybean (menaquinone-7)
D<sub>4</sub>: Synthetic menadione (K<sub>3</sub>)
(22.19±0.66 nmol/L). Likewise declining pattern was observed during follow up trial, the TBARS values decreased from 22.54±0.76 to 19.82±0.55 nmol/L for D₁ to D₃ groups.

Previous studies indicated that spinach and natto especially their polyphenols have ability to attenuate the process of lipid peroxidation. These compounds are responsible for free radicals quenching and metal ions chelation. In this reference, Hadipour et al. (2003) explicated that vitamin K provision to adults and weaning rats significantly suppressed the synthesis of malondialdehyde (MDA) an indicator of lipid peroxidation. The vitamin K administration @ 28 mg/day caused 14% decline in MDA level in weaning rats.

The anticoagulants produce abnormal level of reactive oxygen species (ROS) that are responsible for initiation of lipid peroxidation in tissues. Moreover, cell membrane integrity is lost due to the reaction of polyunsaturated fatty acids & free radicals that produces lipid hydroperoxides and other secondary products like MDA, 4-hydroxynonenal & acrolein. However, natto exhibits soothing effect on elevated TBARS level by hindering the production of superoxide and chelates metal ions. Earlier, Wang et al. (2008) reported high TBARS values in hypercholesterolemic rats that were momentously declined 9.59% by consuming fermented soybean (Rilantono et al., 2000; Iwai et al., 2002). These compounds are synthesized during bacterial fermentation in natto preparation (Wang et al., 2007).

Earlier, Iwai et al. (2002) reported that copper sulfate (CuSO₄) induced TBARS is substantially suppressed by the provision of natto antioxidants after three weeks. The possible mechanism for MDA reduction is the scavenging ability of natto isoflavones (Ruiz-Larrea et al., 1997; Tikkanen et al., 1998). The antioxidant potential of natto is higher than that of steamed soybean however, isoflavone and tocopherol contents are at par (Esaki et al., 1990). Previously, Yokota et al. (1996) conferred that natto antioxidants significantly diminish the synthesis of serum TBARS in rabbits. For this phenomenon, 6''-O-succinyl-daidzin and 6''-O-succinyl-genistin contents of natto are gaining importance (Toda et al. 1999). During vitamin K deficiency, subjects relied on polyunsaturated fatty acids exhibit elevated serum TBARS level (Leray et al., 2001). Nevertheless, Kawashima et al. (1997) observed a significant reduction in TBARS concentration by administration of vitamin K₂ in hypercholesterolemic rabbits. They inferred that TBARS level is suppressed by the intake of
menaquinones in a dose dependent manner. Conclusively, ingestion of vitamin K enriched dietary sources reduces the elevated serum TBARS level owing to their antioxidant potential.

4.4.7. Serum lipid profile

The F values in Table 32 depicted that vitamin K enriched dietary sources imparted non-significant variations on total cholesterol, HDL, LDL and triglycerides levels in study I & II. In study I (trial 1), the maximum cholesterol level was observed in D₁ (67.85±2.26 mg/dL) followed by D₂ (65.20±2.16 mg/dL) and D₄ group (65.01±2.22 mg/dL) however, minimum value was noticed in D₃ (62.91±2.12 mg/dL). Likewise in trial 2, the highest cholesterol 64.91±2.05 mg/dL was recorded in D₁ group that reduced non-momentously to 59.02±1.93 mg/dL in D₃. Similar diminishing pattern regarding cholesterol was recorded in study II (trial 1 & 2) and the values were 70.26±2.84 & 72.59±2.01, 68.28±2.78 & 69.73±1.94, 67.11±2.96 & 66.46±2.32 and 69.61±1.81 & 71.33±2.35 mg/dL for D₁, D₂, D₃ and D₄ groups, respectively.

The means for HDL in study I (normal rabbits) were 27.15±1.22, 28.04±1.46, 28.16±1.10 and 27.82±0.94 mg/dL in D₁, D₂, D₃ and D₄ groups. Likewise in the follow up trial, non-significant elevation was recorded from 28.01±0.98 to 29.85±0.49 mg/dL in D₁ to D₃ groups. Similar trend was observed in study II (trial 1 & 2) and the values noticed for D₁, D₂, D₃ and D₄ groups were 29.12±1.23 & 30.16±1.95, 29.69±1.17 & 31.66±1.81, 30.12±1.71 & 31.96±0.75 and 29.13±1.27 & 30.27±1.78 mg/dL, respectively.

The serum LDL level was non-substantially decreased in normal rabbits (study I; trial 1 & 2) from 32.55±1.82 & 31.60±0.71 mg/dL in D₁ to 32.17±0.84 & 30.54±1.53, 29.86±1.23 & 28.92±0.99 and 32.94±1.12 & 30.99±0.78 mg/dL for D₂, D₃ and D₄ groups, respectively. Likewise, vitamin K deficient rabbits (study II; trial 1) indicated LDL value 34.76±1.93 mg/dL in D₁ that non-substantially diminished to 35.43±0.77, 34.93±1.18, 34.52±1.65 mg/dL in D₂, D₃ and D₄ groups, in corresponding manner. Similar decrease in serum LDL was observed in the proceeding trial.

Means compiled for triglycerides in study I (trial 1 & 2) were 60.78±2.33 & 62.91±2.42, 63.79±2.31 & 64.99±2.45, 64.45±2.32 & 65.01±2.38 and 62.80±2.29 & 63.10±2.33 mg/dL for D₁, D₂, D₃ and D₄ groups, respectively. During study II (trial 1 & 2), triglycerides levels
were noticed as 57.09±2.48 & 59.89±2.64 mg/dL in D₁, 59.63±2.63 & 62.02±2.30 in D₂, 61.13±2.55 & 60.82±2.61 in D₃ and 58.71±2.59 & 60.91±2.36 mg/dL in D₄ groups, correspondingly.

The present results are comparable with the findings of Kristensen et al. (2008), they noticed non-significant elevation in triglycerides level after the provision of phylloquinone @ 500 µg/day to the postmenopausal women for six weeks. They also inferred that HDL and LDL concentrations varied non-momentously by vitamin K supplementation. The variations in the HDL and LDL levels are too small and have no clinical significance. In contrary, some researchers have reported that different forms of vitamin K impart cholesterol lowering effect in animals and humans. The triglycerides level is declined after the provision of pharmacological dose of MK-4 in the dialysis patients (Nagasawa et al., 1998). Furthermore, they noticed that serum HDL and triglycerides levels are decreased after vitamin K₂ administration in hypercholesterolemic rabbits whilst, normal group showed non-momentous variations (Kawashima et al., 1997). Later, Beulens et al. (2010) performed multivariate analysis regarding the intake of vitamin K i.e. phylloquinone & menaquinone-7. They found that menaquinone-7 provision resulted a non-significant increase in HDL to total cholesterol ratio. Furthermore, phylloquinone did not impart any adverse effect on blood lipid profile.

Numerous studies indicated that chylomicrons facilitate the vitamin K transportation in the body after separating triglyceride part, by the action of lipoprotein lipase. In this reference, triglyceride-rich lipoproteins are the major transporter of vitamin K, constituted from triglyceride along with VLDL. However, vitamin K is also transported through LDL and HDL simultaneously (Kohlmeier et al., 1996; Lamon-Fava et al., 1998; Schurgers et al., 2002; Shearer and Newman, 2008). Some other researchers groups i.e. Schurgers and Vermeer (2000) & Schurgers and Vermeer (2002) carried out pharmacokinetics studies of vitamin K₁ and K₂. They observed that vitamin K imparts non-significant effect on lipid profile and the values were within normal range in the tested subjects.

One of their peers, McKeown et al. (2002) conducted a community based 4 years Framingham Heart Study to evaluate the role of vitamin K intake during cardiovascular complications. They expounded that higher intake of vitamin K enriched vegetables
<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D_1</td>
<td>D_2</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>67.85±2.26</td>
<td>65.20±2.16</td>
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<td>(Trial 2)</td>
<td>64.91±2.05</td>
<td>62.63±1.95</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>70.26±2.84</td>
<td>68.28±2.78</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>72.59±2.01</td>
<td>69.73±1.94</td>
</tr>
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<tr>
<td>Study I</td>
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<tr>
<td>(Trial 1)</td>
<td>27.15±1.22</td>
<td>28.04±1.46</td>
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<td>(Trial 2)</td>
<td>28.01±0.98</td>
<td>29.23±1.09</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>29.12±1.23</td>
<td>29.69±1.17</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>30.16±1.95</td>
<td>31.66±1.81</td>
</tr>
<tr>
<td>LDL</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>32.55±1.82</td>
<td>32.17±0.84</td>
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<tr>
<td>(Trial 2)</td>
<td>31.60±0.71</td>
<td>30.54±1.53</td>
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<tr>
<td>Study II</td>
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<tr>
<td>(Trial 1)</td>
<td>34.76±1.93</td>
<td>35.43±0.77</td>
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<tr>
<td>(Trial 2)</td>
<td>33.84±1.21</td>
<td>34.50±1.74</td>
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</tr>
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<td>(Trial 1)</td>
<td>60.78±2.33</td>
<td>63.79±2.31</td>
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<tr>
<td>(Trial 2)</td>
<td>62.91±2.42</td>
<td>64.99±2.45</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>57.09±2.48</td>
<td>59.63±2.63</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>59.89±2.64</td>
<td>62.02±2.30</td>
</tr>
</tbody>
</table>

NS = Non-significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D_1: Control
D_2: Spinach (phylloquinone)
D_3: Natto/Fermented soybean (menaquinones-7)
D_4: Synthetic menadione
including cooked and raw spinach exert positive influence on the plasma phylloquinone level. The higher plasma phylloquinone concentration is linearly correlated with serum triglyceride level. Likewise, some other investigations also delineated that serum triglycerides level is positively associated with the intake of vitamin K (Cham et al., 1999). Later, Booth et al. (2004) reported that lipid profile varied non-significantly in the volunteers by vitamin K supplementation.

4.4.8. Liver functioning tests

It is obvious from the F values that treatments substantially affected the serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels in study II whereas these parameters were affected non-momentously in study I (Table 33). The means (study I; trial 1) for D₁ (control), D₂ (cooked spinach), D₃ (natto A) and D₄ (menadione) were 65.43±3.22, 64.53±3.18, 62.86±3.11 and 63.77±3.15 IU/L, respectively. Similar pattern was observed in the proceeding trial. Nonetheless in study II (trial 1), serum ALT level was maximum in D₁ (79.55±3.83 IU/L) that significantly minimized in D₂ (72.11±3.25 IU/L), D₃ (67.51±3.18 IU/L) and D₄ (70.61±3.15 IU/L) groups. Similar response was noticed in trial 2; the highest value was observed in D₁ as 82.44±5.16 IU/L followed by 75.64±2.44 IU/L in D₂, 70.98±2.37 IU/L in D₃ and 73.04±2.33 IU/L in D₄ group.

The mean serum AST levels for D₁, D₂, D₃ and D₄ groups in study I (trial 1) were 58.67±2.51, 57.59±2.46, 56.92±2.33 and 57.56±2.29 IU/L, correspondingly. Likewise pattern was noticed in trial 2. However in study II (trial 1), means for AST indicated the maximum value in D₁ (85.85±3.67 IU/L) than that of D₂ (81.33±2.28 IU/L), D₃ (71.67±2.81 IU/L) and D₄ (76.82±3.07 IU/L). Similarly in trial 2, the recorded value for D₁ 78.76±3.98 IU/L was reduced in D₂ 77.01±3.64 IU/L followed by D₄ 69.91±3.28 IU/L however, D₃ group exhibited the minimum concentration as 65.24±3.08 IU/L (Table 33).

Liver performs numerous functions like synthesis and accumulation of various endogenous & exogenous substances, nutrients metabolism, clotting protein synthesis and detoxification. In this reference, ALT and AST are considered as important indicators of liver soundness. However during damaged or toxic conditions, these enzymes are elevated and leaked towards the blood stream that can be measured to assess liver injury (Pereira et al., 2005; Shirakawa et al., 2005).
Table 33. Effect of vitamin K enriched dietary sources on serum ALT and AST (IU/L)

<table>
<thead>
<tr>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
</thead>
<tbody>
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<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>65.43±3.22</td>
<td>64.53±3.18</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>64.41±2.94</td>
<td>63.51±2.91</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>79.55±3.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.11±3.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>82.44±5.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.64±2.44&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>58.67±2.51</td>
<td>57.59±2.46</td>
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<td>(Trial 2)</td>
<td>57.67±2.28</td>
<td>56.60±2.24</td>
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<tr>
<td>(Trial 1)</td>
<td>85.85±3.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.33±2.28&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>(Trial 2)</td>
<td>78.76±3.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.01±3.64&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant  
NS = Non-significant

Study I: Normal rabbits  
Study II: Vitamin K deficient rabbits

D<sub>1</sub>: Control  
D<sub>2</sub>: Spinach (phylloquinone)  
D<sub>3</sub>: Natto/Fermented soybean (menaquinones-7)  
D<sub>4</sub>: Synthetic menadione
Recently, Aljarallah et al. (2012) explored the safety aspects of vitamin K in human volunteers suffering from bone related disorders in a cross-sectional study. Liver functioning tests *i.e.* alanine aminotransferase (ALT) and aspartate transaminase (AST) were decreased in group relied on vitamin K. Nonetheless, these parameters are elevated during warfarin or heparin utilization. Previously, Konoplia et al. (1997) delineated that oral phylloquinone administration minimizes the biomarkers of liver damage *i.e.* ALT and AST caused by D-galactosamine and modulates the immune system. Likewise, Pereira et al. (2005) expounded that single dose of vitamin K (10 mg) enhances serum vitamin K level and reduces aspartate aminotransferase (AST) activity in adults with acute liver disease. Later, Yamada et al. (2007) investigated the role of vitamin K during cytomegalovirus hepatitis in cholestasis condition. They noticed that liver enzymes levels are mediated toward normal range after the supplementation of vitamin K.

Decisively, warfarin induced vitamin K deficiency causes elevation in the liver functioning enzymes that are ameliorated by consuming cooked spinach and natto. Hence, these dietary sources provide vitamin K along with antioxidants thereby modulate the liver enzymes concentrations.

**4.4.9. Kidney functioning tests**

The F values in Table 34 indicated that serum urea and creatinine levels were affected non-substantially in study I & II except for creatinine that affected momentously in study II. Mean serum urea values were 26.28±1.12, 26.04±0.81, 25.71±1.01 and 25.91±0.93 mg/dL for D₁, D₂, D₃ and D₄ groups correspondingly (study I; trial 1). However in study II (trial 1), means for serum urea, indicated the maximum value in D₁ (30.65±1.31 mg/dL) trailed by D₂ (29.39±1.26 mg/dL), D₃ (28.17±1.10 mg/dL) and D₄ (29.01±1.04 mg/dL). Similarly in trial 2, the recorded values for D₁ (study I & II) were 25.33±1.01 & 29.84±1.28 mg/dL, respectively that increased in D₂, D₃, and D₄ as 25.10±0.99 & 28.58±1.22, 24.96±1.03 & 27.37±1.17 and 24.76±0.98 & 28.19±1.20 mg/dL, correspondingly.

The means pertaining to creatinine indicated the values 1.32±0.06 & 1.30±0.05, 1.34±0.07 & 1.31±0.06, 1.31±0.09 & 1.29±0.04 and 1.33±0.06 & 1.31±0.07 mg/dL for D₁, D₂, D₃ and D₄ groups, respectively (study I; trial 1 & 2). Nevertheless in study II, a significant decline was
Table 34. Effect of vitamin K enriched dietary sources on serum urea and creatinine

<table>
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<th>Studies</th>
<th>Diets</th>
<th>F value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td><strong>Urea (mg/dL)</strong></td>
<td></td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td>(Trial 1)</td>
<td>26.28±1.12</td>
<td>26.04±0.81</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>25.33±1.01</td>
<td>25.10±0.99</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>30.65±1.31</td>
<td>29.39±1.26</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>29.84±1.28</td>
<td>28.58±1.22</td>
</tr>
<tr>
<td><strong>Creatinine (mg/dL)</strong></td>
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</tr>
<tr>
<td>Study I</td>
<td></td>
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</tr>
<tr>
<td>(Trial 1)</td>
<td>1.32±0.06</td>
<td>1.34±0.07</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>1.30±0.05</td>
<td>1.31±0.06</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>2.06±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.94±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>2.18±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.86±0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant  
NS = Non-significant

Study I: Normal rabbits  
Study II: Vitamin K deficient rabbits

D1: Control  
D2: Spinach (phyloquinone)  
D3: Natto/Fermented soybean (menaquinones-7)  
D4: Synthetic menadione
observed from 2.06±0.15 & 2.18±0.13 mg/dL (D₁) to 1.82±0.11 & 1.74±0.09 mg/dL (D₃) in trial 1 & 2 (Table 34).

The findings of Aljarallah et al. (2012) supported the present case that vitamin K supplementation non-substantially affected the serum urea level ranged from 9.39±0.64 to 10.38±2.91 nmol/L in human volunteers. The anticoagulant provision increased the serum urea in experimental animals (Sakaguchi et al., 2008). Earlier, National Toxicology Program published data regarding the enhancement of serum urea level in rats from 8.9 to 9.2 mg/dL after the ingestion of warfarin (NTP, 1993). Later, Rennenberg et al. (2010a) and Villines et al. (2009) reported that warfarin intake increases the serum creatinine concentration like in the present case. It has also been observed that vitamin K supplementation reduced the serum urea concentration in the renal failure patients (Małyszko et al., 2002).

Later, Thane et al. (2002) illuminated that plasma phylloquinone concentration was inversely correlated with the serum urea level of British nationals. In this context, Kulpa et al. (2011) observed that serum urea level increases during vitamin K deficiency in the chronic kidney patients. One of their peers, Nakano et al. (2011) delineated that during hypovitaminosis K, serum urea level is elevated. They also found that provision of vitamin K normalizes the serum urea level. Earlier, Hiroyuki et al. (2001) expounded that administration of fermented soybean rich in menaquinone-7 did not alter the kidney functioning indicators. Moreover, consumption of spinach, a source of vitamin K also maintained the normal functioning of kidneys (AESAN-2011).

The serum creatinine concentration varied non-significantly in phylloquinone supplemented groups. However, it is decreased by 79.5 mmol/L in the vitamin K deficiency conditions as compared to 85.0 mmol/L in the normal subjects (Aljarallah et al., 2012). The warfarin intake enhances serum creatinine concentration in normal and diseased individuals (Villines et al., 2009; Rennenberg et al., 2010a). The phylloquinone (vitamin K₁) or menaquinones (vitamin K₂) forms of vitamin K are generally considered as safe because they do not alter the normal physiological functioning of the body (Wang et al., 2008b).
4.4.10. Hematological analysis

4.4.10.1. Red blood cells indices

4.4.10.1.1. Red blood cells

The F values in Table 35 indicated non-significant differences in red blood cells (study I) whereas, in study II, this trait was affected significantly by vitamin K enriched dietary sources. Means regarding red blood cells (RBCs) for D₁, D₂, D₃ and D₄ groups in study I (trial 1) were 5.40±0.07, 5.64±0.06, 5.87±0.08 and 5.67±0.09×10¹²/L, respectively. Likewise pattern was observed in trial 2. However in study II (trial 1), the lowest value for this trait was observed in D₁ (3.84±0.06×10¹²/L) that significantly increased in D₂ (4.17±0.07×10¹²/L), D₃ (4.51±0.08×10¹²/L) and D₄ (4.99±0.09×10¹²/L). During trial 2, substantial rise in RBCs was observed and the values ranged from 4.04±0.07 to 5.08±0.10×10¹²/L in D₁ to D₄ groups, respectively. However, the values were shifted towards normal level due to vitamin K enriched dietary sources.

4.4.10.1.2. Hemoglobin (Hb)

It is inferred from the F values that treatments affected the hemoglobin (Hb) level non-significantly in study I however, this trait was affected momentously in study II (Table 35). In study I (trial 1), mean Hb values for D₁, D₂, D₃ and D₄ were 12.05±0.46, 12.52±0.53, 12.83±0.55 and 12.43±0.67 mg/dL, respectively. Likewise behavior was noticed in the subsequent trial. Nonetheless in study II (trial 1), the value for this trait in D₁ was 9.98±0.43 mg/dL that momentously enhanced to 11.90±0.28, 12.11±0.71 and 11.79±0.34 mg/dL in D₂, D₃ and D₄ groups, correspondingly. In 2ⁿᵈ trial, the lowest values was observed in D₁ (8.86±0.32 mg/dL) followed by D₂ (10.29±0.53 mg/dL), D₄ (10.62±0.49 mg/dL) and D₃ (11.43±0.66 mg/dL).

4.4.10.1.3. Hematocrit

The F values (Table 35) showed non-momentous effect of treatments on the hematocrit level of normal rabbits (study I) whereas significant variations were observed in vitamin K deficient rabbits (study II). In this connection, means for hematocrit in D₁, D₂, D₃ and D₄ were 32.35±1.52, 33.73±2.68, 34.57±1.55 and 34.04±1.19%, respectively (study I; trial 1).
Table 35. Effect of vitamin K enriched dietary sources on red blood indices

<table>
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<th>Studies</th>
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<th>F value</th>
</tr>
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<td>D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>Red blood cells (10&lt;sup&gt;12&lt;/sup&gt;/L)</td>
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<tr>
<td>(Trial 1)</td>
<td>5.40±0.07</td>
<td>5.64±0.06</td>
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<tr>
<td>(Trial 2)</td>
<td>6.08±0.09</td>
<td>6.21±0.09</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>3.84±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.17±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>4.04±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.39±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Hb (mg/dL)</td>
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<tr>
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<td>(Trial 1)</td>
<td>12.05±0.46</td>
<td>12.52±0.53</td>
</tr>
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<td>(Trial 2)</td>
<td>12.11±0.37</td>
<td>12.67±0.39</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>9.98±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.90±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>8.86±0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.29±0.53&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
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<tr>
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</tr>
<tr>
<td>(Trial 1)</td>
<td>32.35±1.52</td>
<td>33.73±2.68</td>
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<tr>
<td>(Trial 2)</td>
<td>30.39±1.75</td>
<td>31.69±1.33</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>34.61±1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.74±2.24&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trial 2)</td>
<td>36.91±1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.13±1.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV (fL)</td>
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<td>Study I</td>
<td></td>
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<tr>
<td>(Trial 1)</td>
<td>60.58±1.75</td>
<td>65.87±2.02</td>
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<td>(Trial 2)</td>
<td>59.47±1.54</td>
<td>64.59±2.91</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Trial 1)</td>
<td>62.90±2.65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64.99±1.70&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>(Trial 2)</td>
<td>64.14±2.78&lt;sup&gt;d&lt;/sup&gt;</td>
<td>65.89±1.72&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant  
NS = Non-significant

Study I : Normal rabbits  
Study II : Vitamin K deficient rabbits

D<sub>1</sub>: Control  
D<sub>2</sub>: Spinach (phylloquinone)  
D<sub>3</sub>: Natto/Fermented soybean (menaquinones-7)  
D<sub>4</sub>: Synthetic menadione
During the 2\textsuperscript{nd} trial a similar trend regarding hematocrit was observed. In study II (trail 1 & 2), this trait significantly elevated from 34.61±1.49 & 36.91±1.11\% (D\textsubscript{1}) to 35.74±2.24 & 37.13±1.51\% (D\textsubscript{2}), 37.35±2.31 & 39.24±1.65\% (D\textsubscript{3}) and 39.01±2.45 & 41.03±1.19\% (D\textsubscript{4}).

4.4.10.1.4. Mean corpuscular volume (MCV)

It is obvious from the F values (Table 35) that treatments depicted non-significant differences on MCV in study I however, study II behaved differently. Mean values for this attribute in study I (trial 1) were recorded in D\textsubscript{1}, D\textsubscript{2}, D\textsubscript{3} and D\textsubscript{4} groups i.e. 60.58±1.75, 65.87±2.02, 62.34±1.58 and 63.95±3.67 fL, respectively. Nevertheless in study II (trial 1), MCV values were 62.90±2.65, 64.99±1.70, 67.02±2.97 and 69.42±2.01 fL for D\textsubscript{1}, D\textsubscript{2}, D\textsubscript{3} and D\textsubscript{4} groups, respectively. Similarly in trial 2, dietary sources exhibited non-momentous increase for study I whereas a substantial rise was observed in study II thus validate the data.

4.4.10.2. White blood cells indices

4.4.10.2.1. White blood cells (WBCs)

The statistical data interpreted that treatments imparted non-significant effects on WBCs in all the studies (Table 36). In study I, mean WBCs in D\textsubscript{1} (6.40±0.21×10\textsuperscript{9}/L) increased non-substantially in D\textsubscript{2} (6.49±0.12×10\textsuperscript{9}/L) D\textsubscript{4} (7.20±0.25×10\textsuperscript{9}/L) and D\textsubscript{3} (7.67±0.15×10\textsuperscript{9}/L) groups. Similarly, in study II, this trait was higher in D\textsubscript{4} (6.44±0.27×10\textsuperscript{9}/L) as compared to D\textsubscript{3} (6.42±0.18×10\textsuperscript{9}/L), D\textsubscript{2} (6.13±0.45×10\textsuperscript{9}/L) and D\textsubscript{1} (5.80±0.31×10\textsuperscript{9}/L). Likewise pattern for this trait was observed in the subsequent trials.

4.4.10.2.2. Lymphocytes

It is obvious from the statistical analysis that lymphocytes were non-significantly affected by vitamin K enriched dietary sources in the entire experiment (Table 36). Mean values for this attribute in D\textsubscript{1}, D\textsubscript{2}, D\textsubscript{3} and D\textsubscript{4} (study I; trial 1) were 65.28±2.10, 64.94±3.09, 64.71±2.45 and 64.61±1.98\%, respectively. Likewise in study II (trial 1), lymphocytes were 58.98±2.50\% in D\textsubscript{1} that non-substantially increased in groups D\textsubscript{2} (61.39±2.54\%), D\textsubscript{3} (62.59±2.55\%) and D\textsubscript{4} (59.76±2.24\%). Likewise pattern for this trait was noticed during the next trials.
4.4.10.2.3. Monocytes

It is evident from F values (Table 36) that vitamin K enriched dietary sources imparted non-momentous differences on the monocytes in normal and vitamin K deficient rabbits (study I & II). In preliminary study D₁, D₂, D₃ & D₄ exhibited a non-momentous rise; 2.48±0.10 & 2.59±0.09, 2.51±0.12 & 2.56±0.14%, and 2.60±0.11, 2.82±0.15, 2.65±0.08 & 2.74±0.13%, respectively during trial 1 & 2. Additionally, study II (trial 1) behaved alike during successive trials and showed monocytes as 3.89±0.15 & 3.93±0.12, 3.82±0.16 & 4.01±0.11, 3.90±0.17 & 4.09±0.13 and 4.01±0.14 & 4.12±0.19% for D₁, D₂, D₃ and D₄, respectively.

4.4.10.2.4. Neutrophils

The F values in Table 36 presented that treatments non-significantly affected neutrophils level in the entire studies. Mean values for neutrophils in study I (trial 1 & 2) were 61.97±2.61 & 57.16±2.55 (D₁) and 63.04±2.67 & 58.22±2.66% (D₄). Likewise, the values for D₁, D₂, D₃ & D₄ groups in study II (trial 1 & 2) were 59.50±2.54, 61.29±2.57, 61.86±2.43 & 62.98±2.69% and 62.48±2.67, 64.35±2.75, 64.95±2.18 & 66.13±2.83%, respectively.

The results of present study are in harmony with the previous finding of the Brodsky et al. (2009) that warfarin intake reduces the red blood cells in patients. Later, Chen et al. (2012) expounded that bleeding symptoms of the subjects are minimized by the provision of vitamin K that improved the abnormal hematological parameters. Moreover, during vitamin K deficiency, cholestasis disease caused excessive destruction of the red blood cells (Harrington et al., 2008). Later, Malyszko et al. (2002) reported that in vitamin K deficient condition of renal failure subjects, vitamin K is linearly associated with red blood cells count, hemoglobin and serum calcium levels. Afterwards, Pucaj et al. (2011) delineated that menaquinone-7 @ 2.5, 5.0 and 10 mg/kg/day did not impart any significant variations on the hematological parameters including red blood cells, hemoglobin, hematocrit and mean corpuscular volume (MCV) as well as white blood cells indices.

There is no evidence of adverse effects of phylloquinone consumption @ 10 mg/day (Scientific Committee on Food, 2003). Phylloquinone and menaquinone forms of vitamin K are generally considered as safe. Moreover, the Expert Group on Vitamins and Minerals has also established the safe range for vitamin K i.e. 1µg/kg body weight (EVM, 2003).
Table 36. Effect of vitamin K enriched dietary sources on white blood cells indices

<table>
<thead>
<tr>
<th></th>
<th>Studies</th>
<th>Diets</th>
<th>F value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>White blood cells (10⁹/L)</td>
<td>Study I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Trial 1)</td>
<td>6.40±0.21</td>
<td>6.49±0.12</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>6.44±0.17</td>
<td>6.58±0.23</td>
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<td></td>
<td>Study II</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>5.80±0.31</td>
<td>6.13±0.45</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>6.02±0.16</td>
<td>6.36±0.33</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>Study I</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>65.28±2.10</td>
<td>64.94±3.09</td>
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<td></td>
<td>(Trial 2)</td>
<td>63.37±2.65</td>
<td>62.12±1.99</td>
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<td></td>
<td>Study II</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>58.98±2.50</td>
<td>61.39±2.54</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>57.67±1.96</td>
<td>58.58±3.10</td>
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<tr>
<td>Monocytes (%)</td>
<td>Study I</td>
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<tr>
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<td>(Trial 1)</td>
<td>2.48±0.10</td>
<td>2.59±0.09</td>
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<td>(Trial 2)</td>
<td>2.60±0.11</td>
<td>2.82±0.15</td>
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<td></td>
<td>Study II</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>3.89±0.15</td>
<td>3.82±0.16</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>3.93±0.12</td>
<td>4.01±0.11</td>
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<tr>
<td>Neutrophils (%)</td>
<td>Study I</td>
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<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>61.97±2.61</td>
<td>62.98±2.59</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>57.16±2.55</td>
<td>58.35±2.66</td>
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<tr>
<td></td>
<td>Study II</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>59.50±2.54</td>
<td>61.29±2.57</td>
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<tr>
<td></td>
<td>(Trial 2)</td>
<td>62.48±2.67</td>
<td>64.35±2.75</td>
</tr>
</tbody>
</table>

* = Significant; NS = Non-significant

Study I : Normal rabbits
Study II : Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/Fermented soybean (menaquinones-7)
D₄: Synthetic menadione
Moreover, synthetic menadione may interfere in the function of glutathione resulting oxidative damage to the endothelial cells (Wang et al., 2008). Likewise, no specific adverse effects are documented for administration of phylloquinone and MK-4 in humans (Binkley et al., 2009). Earlier, Goldsmith et al. (1995) narrated that the continuous intake of vitamin K enhanced the platelet count however, haemoglobin and hematocrit concentrations behaved non-momentously.

4.4.11. Serum electrolytes

The statistical analysis revealed non-significant effect of vitamin K enriched dietary sources on serum sodium, potassium and calcium levels in study I and II, except for serum calcium content that varied momentously in study II (Table 37). In preliminary study, means for serum sodium (Na) in D1 (control), D2 (cooked spinach), D3 (natto) and D4 (menadione) groups were 141.30±5.04, 140.03±5.38, 138.61±5.21 and 140.30±5.09 µg/dL, respectively. Likewise during study II, serum Na levels were 141.36±6.30 µg/dL (D1), 142.07±4.77 µg/dL (D2), 140.79±5.98 µg/dL (D3) and 139.90±5.67 µg/dL (D4). Likewise pattern regarding serum Na level was observed in the subsequent trials of study I & II, respectively.

In study I (trial 1), mean serum potassium (K) levels for D1, D2, D3 and D4 were 4.92±0.14, 4.86±0.17, 4.96±0.21 and 5.02±0.12 mmol/L, respectively. Similarly, serum K content (study II) in D1 was 4.87±0.09 mmol/L that differed non-substantially from D2, D3 and D4 as 4.82±0.13, 4.81±0.07 and 4.87±0.15 mmol/L, correspondingly. In trial 2 (study I & II), likewise behavior was recorded for serum potassium content (Table 37).

The serum calcium levels in D1, D2, D3 and D4 groups were 5.12±0.25, 4.96±0.14, 4.95±0.13 and 5.02±0.09 mg/dL, correspondingly (study I; trial 1). Similar pattern for this trait was noticed in trial 2 (Table 37). However, vitamin K enriched dietary sources imparted significant variations regarding serum calcium level in the vitamin K deficient rabbits (study II; trial 1) as 5.70±0.26, 5.32±0.22, 5.28±0.18 and 5.14±0.12 mg/dL in D1, D2, D3 and D4, respectively. During trial 2, maximum serum Ca content was noticed in D1 (5.87±0.29 mg/dL) followed by D2 (5.70±0.19 mg/dL) and D3 (5.47±0.15 mg/dL) whereas minimum level for this trait was observed in D4 group (5.37±0.10 mg/dL).
Table 37. Effect of vitamin K enriched dietary sources on serum electrolytes

<table>
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<th>Diets</th>
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<td></td>
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<td>D₁</td>
<td>D₂</td>
</tr>
<tr>
<td>Sodium (µg/dL)</td>
<td>Study I</td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>141.30±5.04</td>
<td>140.03±5.38</td>
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<td></td>
<td>(Trial 2)</td>
<td>143.80±6.03</td>
<td>142.51±5.98</td>
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<td>Study II</td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>141.36±6.30</td>
<td>142.07±4.77</td>
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<td>(Trial 2)</td>
<td>139.55±4.26</td>
<td>137.00±6.11</td>
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<td>Potassium (mmol/L)</td>
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<td></td>
<td>(Trial 1)</td>
<td>4.92±0.14</td>
<td>4.86±0.17</td>
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<td>(Trial 2)</td>
<td>5.05±0.19</td>
<td>5.00±0.12</td>
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<td></td>
<td>Study II</td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>4.87±0.09</td>
<td>4.82±0.13</td>
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<tr>
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<td>(Trial 2)</td>
<td>5.01±0.13</td>
<td>4.98±0.09</td>
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<tr>
<td>Calcium (mg/dL)</td>
<td>Study I</td>
<td></td>
<td></td>
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<tr>
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<td>4.96±0.14</td>
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<td>(Trial 2)</td>
<td>5.17±0.28</td>
<td>5.01±0.19</td>
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<tr>
<td></td>
<td>Study II</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Trial 1)</td>
<td>5.70±0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.32±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Trial 2)</td>
<td>5.87±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.70±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* = Significant;  NS = Non-significant

Study I: Normal rabbits
Study II: Vitamin K deficient rabbits

D₁: Control
D₂: Spinach (phyloquinone)
D₃: Natto/Fermented soybean (menaquinones-7)
D₄: Synthetic menadione
The present results are comparable with the earlier work of Pucaj et al. (2011), they observed non-momentous effect of MK-7 supplementation @ 2.5, 5.0 and 10 mg/kg on serum sodium (Na), potassium (K) and calcium (Ca) levels. Earlier, Koitaya et al. (2009) elucidated that pharmacological dose of vitamin K₂ (MK-4) decreased the serum calcium level. The reduced level of serum calcium is possibly due to its utilization in carboxylation process of vitamin K dependent proteins. The biochemical action of vitamin K as cofactor for vitamin K dependent protein carboxylase, catalyzes the glutamic acid (Glu) to γ-carboxyglutamic acid (Gla) thus improves the calcium binding activity of these proteins (Shearer and Newman, 2008). Furthermore, serum osteocalcin concentration is positively associated with calcium kinetics and rate of bone formation (Lee et al., 2007; Brown et al., 2009).

In this continuation, vitamin K dependent carboxylation of the osteocalcin enhances calcium binding resultantly improves the bone mineral density (Hara et al., 1995). There are numerous calcium binding proteins *i.e.* osteocalcin and calbindin that contain carboxyglutamate. During vitamin K deficiency, carboxylation process is hampered and these proteins are not bind with hydroxyapatite, resulting in less utilization of serum calcium. Moreover during bariatric surgery, calcium metabolism is altered due to malabsorption of fat soluble vitamins especially vitamin K (Slater et al., 2004). Earlier, it has been documented that higher intakes of menaquinones activate the vitamin K dependent proteins involved in the regulation of calcium deposition in the bones and thus hinder the arterial calcification (Luo et al., 1997; Vermeer and Braam, 2001).

The serum and urinary excretion of calcium is affected by the administration of vitamin K (1 mg/day) after 2 weeks in healthy postmenopausal women. It was observed that calcium excretion is decreased after the ingestion of vitamin K whilst, osteocalcin level was improved (Feskanich et al., 1999). One of their peers, Knapen et al. (1999) reported that vitamin K ingestion significantly affects the serum creatinine and urinary calcium concentrations. For carboxylation process of vitamin K dependent proteins, calcium is one of the important cofactors. For the reasons being, serum calcium level is momentously decreased due to vitamin K enriched dietary sources in the vitamin K deficient rabbits.

For the present exploration, it is concluded that the tested vitamin K enriched dietary sources *i.e.* cooked spinach and fermented soybean are effectual for the proper regulation of blood
coagulation related mechanisms especially vitamin K dependent proteins. The fermented soybean (natto) containing menaquinone-7 was more effective to minimize the symptoms of the vitamin K deficiency than that of spinach containing phylloquinone. The serum vitamin K level including serum phylloquinone, menaquinone-4 and menaquinone-7 concentrations were momentously increased by the intake of spinach and fermented soybean. Moreover, vitamin K dependent proteins were effectively regulated by the ingestion of cooked spinach and natto. From the present study results, it is suggested that fermented soybean and cooked spinach should be included in the diet based therapy for the improvement of the vitamin K level in the vulnerable segment.
Micronutrients have gained immense attention in novel dietary strategies owing to their affirmative influence on normal body functioning. Presently in the developing economies like Pakistan, deficiencies of various vitamins especially vitamin K is prevailing due to lack of awareness, poor nutritional practices and drugs interaction. The current project is an effort to investigate the nutritional and biochemical behavior of locally grown vitamin K enriched dietary sources. Purposely, soybean (Faisal Soybean) and spinach (Desi Palak) were analyzed for their nutritional profile, vitamin K contents and antioxidant potential. Afterwards, four different vitamin K enriched food products were developed and subjected to characterization & sensory profiling. During the bioevaluation trial, vitamin K enriched dietary sources were tested against blood coagulation disorders, bone mineral density, vitamin K dependent proteins and serum vitamin K contents in normal and vitamin K deficient rabbits.

Chemical profiling of spinach and soybean showed moisture, crude protein, crude fat, crude fiber, ash and nitrogen free extract (NFE) as 90.71±4.14 & 8.96±0.45, 2.03±0.95 & 32.28±1.99, 0.32±0.007 & 18.64±1.02, 0.58±0.02 & 2.93±0.16, 1.24±0.06 & 3.38±0.19 and 5.01±0.11 & 33.97±1.15%, respectively. Moreover, tested raw materials contain appreciable amount of potassium (K), zinc (Zn), magnesium (Mg), calcium (Ca), iron (Fe), sodium (Na) and copper (Cu).

The HPLC quantification of vitamin K (phyloquinone) showed that spinach is rich source of phylloquinone (378.09±13.89 µg/100g) compared to soybean (29.79±1.23 µg/100g). However, extract from selected raw materials (methanolic and ethanolic extract) exhibited good antioxidant potential as estimated by various antioxidant assays i.e. total phenolic content (TPC), DPPH, β-carotene antioxidant activity and FRAP. Mean squares revealed that antioxidant indices were momentously affected by raw materials and solvents.

During product development module, four types of vitamin K enriched dietary products were prepared i.e. T1 (cooked spinach), T2 (reconstituted spinach), T3 (natto A) and T4 (natto B).
The spinach based vitamin K enriched dietary products contained higher phylloquinone (vitamin K₁) whereas natto prepared by varying fermentation conditions had maximum menaquinone-7 (vitamin K₂). The prepared dietary sources of vitamin K exhibited significant variations in proximate composition except for crude fiber. In this context, cooked spinach (T₁) showed highest moisture 75.64±3.51% followed by reconstituted spinach (T₂) 71.86±2.96%, natto B (T₄) 62.72±3.11% and natto A (T₃) 56.47±2.81%. However, protein content was maximum 15.39±0.65% in T₃ trailed by 13.19±0.54% in T₄ and 6.11±0.21% in T₂ whilst lowest value 5.29±0.18% for this trait was recorded in T₁. The crude fat contents were 0.81±0.03, 0.92±0.02, 7.31±0.31 and 7.61±0.32% in T₁, T₂, T₃ and T₄, respectively. Nonetheless, developed vitamin K products showed non-substantial differences in crude fiber content. The minerals profile of vitamin K products showed non-substantial differences in crude fiber content. The minerals profile of vitamin K products showed non-substantial differences due to treatments.

Mean squares showed substantial variations for phylloquinone and menaquinone-7 contents of prepared vitamin K enriched dietary products. The maximum phylloquinone concentration was recorded in cooked spinach (T₁) 368.81±13.96 µg/100g followed by reconstituted spinach (T₂) 270.07±9.45 µg/100g whilst natto A (T₃) showed the minimum value for this trait (26.90±0.94µg/100g). Furthermore, maximum menaquinone-7 (MK-7) was observed in natto A (T₃) 803.82±21.14 µg/100g followed by natto B (T₄) 681.35±16.85 µg/100g. Conversely, MK-7 was not detected in the cooked and reconstituted spinach.

The statistical analysis showed that treatments and solvent imparted significant differences on the antioxidant indices of prepared vitamin K enriched products. The formulated products exhibited the maximum TPC as 714.94±32.10 mg GAE/100g for T₁ followed by 700.21±24.01 mg GAE/100g in T₂ and 405.83±17.80 mg GAE/ 100g in T₃ while lowest value 395.59±14.5 mg GAE/ 100g was observed for T₄. Likewise, DPPH and antioxidant activities were highest in cooked spinach (59.02±2.19 & 52.70±2.35%) trailed by reconstituted spinach (57.42±2.42 & 51.00±1.99%) and natto A (34.05±1.37 & 25.09±1.11%) whereas, minimum values were noticed in natto B (32.35±1.08 & 23.75±0.92%). The recorded values for ferric reducing antioxidant power (FRAP) of vitamin K enriched dietary products were 2.04±0.10, 1.99±0.09, 1.27±0.04 and 1.23±0.06 µmol trox1 Eq/100g in T₁, T₂, T₃ and T₄, respectively.
The sensory response of vitamin K enriched dietary sources were assessed using 9-point hedonic scale system for color, flavor, taste, texture and overall acceptability. Mean squares for sensory response exhibited significant variations in tested traits. In this reference, the maximum color scores were attained by T1 followed by T2, T3 and T4 as 7.86±0.34, 6.94±0.26, 6.84±0.30, 6.04±0.25, respectively. The recorded scores for flavor were 7.96±0.35 (T1), 7.34±0.32 (T2), 7.02±0.30 (T3) and 6.14±0.26 (T4). Likewise, the taste scores for T1, T2, T3 and T4 were 7.78±0.24, 7.18±0.27, 7.08±0.25 and 6.98±0.29, respectively. Means for texture in various treatments expounded variations from 7.56±0.33 to 6.16±0.26 for T1 to T4, correspondingly. Moreover, vitamin K enriched products i.e. T1, T2, T3 and T4 got overall acceptability scores as 7.84±0.34, 7.74±0.34, 7.14±0.31 and 6.74±0.29, correspondingly. On the basis of nutritional profile, vitamin K content, antioxidant potential and hedonic response, two best products, one from each raw material i.e. cooked spinach (D2) and natto A (D3) were selected for bioevaluation study along with synthetic menadione (D4) and control (D1).

To explore the effect of the vitamin K against vitamin K deficiency related discrepancies, efficacy trial was carried out. Purposely, bioevaluation trial was divided into two phases; study I (normal rabbits) and study II (vitamin K deficient rabbits). For vitamin K deficiency induction, warfarin and ciprofloxacin were given to the rabbits for 15 days prior to the provision of vitamin K enriched dietary sources (D2, D3 and D4) along with control.

Mean squares explicated that treatments imparted non-significant differences on feed, water intakes and body weight however, time intervals affected these traits momentously in both studies. The recorded values for body weight for D1 at 1st and 8th week were 1564.95±57.87 & 1884.38±69.71, 1559.23±57.69 & 1878.47±69.49, 1556.25±57.58 & 1892.54±69.52 and 1549.72±56.95 & 1850.33±68.82 g/rabbit for D2, D3 and D4, respectively. Likewise, in study II, the body weights were 1536.32±54.18 to 1693.71±41.82, 1523.63±54.80 to 1709.53±59.52, 1527.99±51.95 to 1746.32±45.33 and 1516.89±55.10 to 1783.33±69.71 g/rabbit in D1, D2, D3 and D4 groups, respectively from the initiation till termination of the study.

Vitamin K enriched dietary sources imparted significant variations regarding bleeding and clotting time. In this context, bleeding time in study I (normal rabbits), were 1.81±0.071,
1.77±0.060, 1.75±0.073 and 1.70±0.049 min for D1, D2, D3 and D4 groups, respectively. During study II, the highest bleeding time (2.42±0.088 min) was observed in D1 that declined momentously in D2 (2.31±0.078 min), D3 (2.17±0.071 min) and D4 (2.08±0.069 min). The clotting time showed the maximum value 12.42±0.43 min for D1 group that significantly reduced in D2 as 12.19±0.51 min, D3 11.98±0.56 min and D4 group 11.78±0.61 min, respectively. Likewise, in study II the observed values were 14.34±0.68, 13.89±0.59, 13.46±0.53 and 12.99±0.48 min in D1, D2, D3 and D4 groups, respectively.

The statistical analysis revealed that treatments imparted significant differences on prothrombin time (PT) in study I & II. The PT values were recorded as 12.41±0.63, 12.07±0.53, 11.93±0.68 and 11.78±0.57 sec in D1, D2, D3 and D4, respectively. During study II, the highest value was observed for D1 (15.01±0.80) that suppressed momentously to 14.14±0.96, 13.43±0.77 and 13.14±0.88 sec in D2, D3 and D4 groups, respectively. In study I & II (trial 1), the percent decline was observed as 2.73 & 5.79, 3.86 & 10.52 and 5.07 & 12.45% in D2, D3 and D4 groups, respectively compared to control. Similar pattern was noticed in the follow up trial.

The activated partial thromboplastin time (APTT) decreased significantly as a function of vitamin K enriched dietary sources in study I & II. The highest APTT value 17.43±0.66 sec was observed in D1 that momentously diminished to 17.17±0.44, 17.04±0.56 and 16.85±0.41 sec in D2, D3 and D4 groups, correspondingly. Likewise in study II (trial I), the APTT values differed momentously as 22.17±0.81, 19.64±0.55, 19.15±0.69 and 18.18±0.45 sec in D1, D2, D3 and D4 groups, respectively. Regarding percent reduction, D4 (menadione) was proved more effective against elevated level of APTT with 17.98% decrease in study II. However, percent decline for D3 and D2 was recorded as 13.60 and 11.40%. Likewise, decreasing trend was noticed in subsequent trial.

The plasma fibrinogen level was uplifted non-significantly due to treatments in study I whereas substantial increase was observed during study II. Vitamin K deficient rabbits (study II) showed increasing trend for plasma fibrinogen concentration in D2 (324.67±15.01 mg/dL), D3 (329.52±15.18 mg/dL) and D4 (334.24±15.73 mg/dL) as compared to control (307.15±15.98 mg/dL). The means regarding INR showed non-momentous variations in study I however, this trait depicted significant effect due to treatments in study II. In the
study II (trial 1), vitamin K deficient rabbits indicated maximum INR value 2.45±0.10 for D<sub>1</sub> that momentously suppressed to 2.37±0.14, 2.33±0.13 and 2.28±0.11 in D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> groups, respectively. The subsequent experiment also showed similar declining trend for these parameters.

Mean squares indicated significant variations in bone mineral density (BMD) due to treatments throughout the studies. The lowest level of BMD was recorded in D<sub>1</sub> as 0.242±0.07 g/cm<sup>2</sup> that increased non-substantially in D<sub>2</sub> 0.244±0.005 g/cm<sup>2</sup>, D<sub>3</sub> 0.246±0.006 g/cm<sup>2</sup> and D<sub>4</sub> 0.245±0.005 g/cm<sup>2</sup>. Likewise in study II, the lowest BMD level was observed D<sub>1</sub> 0.222±0.009 g/cm<sup>2</sup> however, vitamin K enriched dietary sources including D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> caused non-momentous enhancement in this attribute as 0.227±0.010, 0.231±0.012 and 0.226±0.011 g/cm<sup>2</sup>, correspondingly.

The statistical analysis revealed that plasma carboxylated osteocalcin (cOC) concentration was substantially increased as a function of treatment in both studies. The plasma cOC level was significantly enhanced in groups relied on vitamin K enriched dietary sources i.e. D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> as 5.21±0.26, 5.32±0.28, 5.33±0.29 ng/mL as compared to control (D<sub>1</sub>) 5.08±0.22 ng/mL. In study II trial 1, the lowest plasma cOC 3.66±0.11 ng/mL was recorded in D<sub>1</sub> that increased to 4.13±0.13, 4.26±0.13 and 4.30±0.14 ng/mL in respective groups. The graphic representation illustrated plasma cOC level was momentously enhanced in D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> as 2.58, 4.72 and 5.70%, whereas, 12.98, 16.57 and 17.68 % was observed in study II trial 1. The subsequent trial also revealed increase for this trait.

The plasma undercarboyxylated osteocalcin (ucOC) level was affected substantially as a function of treatment in study I & II. Means pertaining to ucOC showed the highest value for D<sub>1</sub> group as 3.21±0.14 ng/mL that progressively suppressed in D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> as 3.15±0.13, 3.09±0.14, 3.06±0.16 ng/mL (study I, trial 1). Similarly, the maximum ucOC values were recorded in D<sub>1</sub> group 4.08±0.18 ng/mL that significantly declined in D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> 3.79±0.14, 3.71±0.15 and 3.49±0.17 ng/mL. The percent reduction in ucOC due to vitamin K enriched sources in study I, trial 1, was 1.87, 3.74 and 4.67%. Nevertheless, menadione (D<sub>4</sub>) provision resulted highest ucOC reduction in study II (14.46 %) followed by natto (D<sub>3</sub>) and cooked spinach (D<sub>2</sub>) as 9.07% and 7.11%. Likewise, diminution in ucOC level was recorded in the second trial.
Means for plasma protein induced by vitamin K antagonist-II (PIVKA-II) depicted significant differences due to treatments in study I & II. In study I (trial 1) maximum decline 17.42% was recorded in D₄ followed by 13.89% in D₃ and 9.37% in D₂. However, 2.64, 4.63 and 5.29% reduction was observed in study I as a function of treatments i.e. D₁, D₂, D₃ and D₄, respectively. The PIVKA-II levels were noticed as 3.02±0.14, 2.98±0.12, 2.89±0.10 & 2.86±0.13 ng/mL for D₁, D₂, D₃ & D₄, respectively. Likewise in study II, D₁ revealed the maximum PIVKA-II concentration 9.01±0.34 ng/mL, trailed by D₂ 8.21±0.31 ng/mL and D₃ 7.92±0.25 ng/mL whereas lowest level was observed in D₄ as 7.76±0.39 ng/mL. Similar declining tendency was observed consecutive trial.

The data regarding serum phylloquinone level explicated that treatments contributed momentous differences on different rabbits groups. Means for normal rabbits (study I, trial 1) presented lowest level of serum phylloquinone in D₁ 9.76±0.39 ng/mL that gradually enhanced by 11.97±0.41, 11.29±0.31 and 10.70±0.16 ng/mL in groups relied on cooked spinach (D₂), natto (D₃) and menadione (D₄), respectively. In study II, the vitamin K deficient rabbits showed an improvement from 6.34±0.09 (D₁) to 9.36±0.30 ng/mL (D₂).

Regarding percent increase in serum phylloquinone concentration of rabbits in study I (trial 1), substantial rise 23.78, 16.71 and 10.64% was observed in D₂, D₃, and D₄ groups, respectively. Moreover during vitamin K deficiency, maximum increase for serum phylloquinone level was recorded in D₂ 47.58% followed by D₃ and D₄ 36.42 & 27.71%, respectively. Similarly, trial 2 behaved alike. It is exhibited from the F values that treatments imparted significant variations on serum menaquinone-4 (MK-4) concentration in study I & II. Means pertaining to this trait in study I (normal rabbits) indicated the values 1.65±0.048, 1.73±0.054, 1.77±0.056 and 1.72±0.044 ng/mL for D₁, D₂, D₃ and D₄ groups, respectively (trial 1). Likewise, in study II (trial 1), the lowest MK-4 level 0.92±0.018 ng/mL was observed in D₁ that elevated significantly in D₂ 1.03±0.032 ng/mL, D₃ 1.04±0.010 ng/mL and D₄ 1.02±0.21 ng/mL. Regarding percent increase in serum MK-4; in study I (trial 1) the highest value was recorded for natto A (D₃) 7.27 followed by cooked spinach (D₂) and menadione (D₄) 4.84 and 4.24%, respectively. Similarly in study II (trial 1), the maximum enhancement was observed in D₃ (13.04%) trailed by D₂ (11.95%) and D₄ (10.86%).
Similarly in the follow up trial, significant improvement in MK-4 levels were recorded in respective groups.

The F values showed that menaquinone-7 (MK-7) level in different groups of rabbits was significantly affected by treatments in all studies. In study I (trial 1) mean MK-7 values in D₁, D₂, D₃ and D₄ groups were 2.70±0.12, 2.77±0.11, 3.18±0.19 and 2.73±0.12 ng/mL. In study II (trial 1), D₁ group depicted the lowest MK-4 concentration 1.02±0.04 ng/mL that substantially increased to 1.15±0.06, 1.27±0.07 and 1.13±0.05 ng/mL in D₂, D₃ and D₄ groups, respectively. The vitamin K dietary sources i.e. D₂, D₃ and D₄ groups resulted 2.59, 17.77 & 1.11% incline in serum MK-7, respectively (study I, trial 1). Likewise in study II, D₃ showed the maximum increase 24.50% trailed by D₂ 12.74% and D₄ 10.78%, correspondingly. Similar trend was observed in the next trial.

It is evident from the statistical analysis that serum thiobarbituric acid reactive substances (TBARS) levels of rabbits were affected momentously by the treatments in all studies. Means regarding TBARS (study I; trial 1) indicated the highest value 21.31±0.91 nmol/L in D₁ that significantly reduced to 19.87±0.85, 18.26±0.78 and 20.42±0.87 nmol/L in D₂, D₃ and D₄ groups, respectively. Similarly in study II, the maximum TBARS value was noticed in D₁ (23.24±0.99 nmol/L) that substantially diminished in D₂ (21.23±0.91 nmol/L) D₃ (20.53±0.38 nmol/L) and D₄ (22.19±0.90 nmol/L). Similar declining pattern was observed during follow up trial. The F values showed that vitamin K enriched dietary sources imparted non-significant variations on total cholesterol, HDL, LDL and triglycerides levels in study I & II.

In the nutshell, the vitamin K enriched dietary sources are proved effective to manage blood coagulation related threats with special reference to abnormally elevated blood coagulation and vitamin K dependent proteins concentrations. The natto based menaquinone-7 performed better to alleviate vitamin K deficiency biomarkers as compared to spinach based phylloquinone. The serum vitamin K level especially serum phylloquinone, menaquinone-7 and menaquinone-4 concentrations were significantly improved by the consumption of selected vitamin K dietary sources. Likewise, vitamin K dependent proteins were effectively managed with the provision of vitamin K enriched dietary sources. It is suggested that natto and spinach should be introduced in diet based therapy to up lift the vitamin K status of the vulnerable segment.
RECOMMENDATIONS

- Micronutrients supplementation should be encouraged at mass level to curtail various nutritional deficiencies with special reference to under nourished and elderly population.
- Indigenously available cost effective vital ingredients must be promoted among the public to improve their dietary status and tackle the threat of hidden hunger.
- Dietitians should advice spinach and natto (fermented soybean) to the vulnerable segments for supplying ample amount of vitamin K.
- Spinach consumption should be promoted as a folk food to escalate vitamin K level for normal physiological functioning.
- Equitable soybean cultivation should be supported at national level to provide economical substitute of vitamin K and edible oil.
- Integrated efforts must be dedicated for the development of micronutrient enriched therapeutic edibles in the developing economies experiencing nutrition transitions.
- Health professionals should motivate the target population for the consumption of vitamin K rich traditional foods to improve bone health and clotting factors.
- Awareness campaigns regarding vitamin K based dietary sources ought to be launched by establishing health and therapeutic diet synergy among the malnourished communities.
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APPENDICES

APPENDIX I

Performa for sensory evaluation of vitamin K enriched dietary products

Name of the judge……………………………… Date……………..

<table>
<thead>
<tr>
<th>Character</th>
<th>T₀</th>
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<td>Color</td>
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<td>Overall acceptability</td>
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Signature……………………..

INSTRUCTIONS

Chew sample and record the score for color, flavor, taste, texture and overall acceptability using the following 9-point Hedonic Scale:

- Extremely poor 1
- Very poor 2
- Poor 3
- Below fair above poor 4
- Fair 5
- Below good above fair 6
- Good 7
- Very good 8
- Excellent 9

Note:

1. Chew a sample and assign the score for color, flavor etc.
2. Before proceeding to the next sample, rinse mouth with water.
3. Make inter comparison of the sample and record the score.
4. Don't disturb the order of samples.