Bioavailability and Health Effects of Isoflavones from Bangladeshi Soybean and Lentils in Postmenopausal Women

This dissertation is submitted as a requirement for the fulfillment of the degree of Doctor of Philosophy (PhD) in the Department of Community Nutrition, Bangladesh Institute of Health Sciences (BIHS), under the faculty of Postgraduate Medical Science & Research of the University of Dhaka

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DECLARATION

The thesis titled '**Bioavailability and Health Effects of Isoflavones from Bangladeshi Soybean and Lentils in Postmenopausal Women**' is submitted as a requirement for the fulfillment of the Doctor of Philosophy (PhD) degree in the Department of Community Nutrition, Bangladesh Institute of Health Sciences (BIHS) under the faculty of Postgraduate Medical Science & Research of the University of Dhaka. This work had been carried out in the Department of Community Nutrition, BIHS, and Department of Chemistry, University of Dhaka during the period of August 2011 to June 2015. To the best of my knowledge no part of the work has been submitted for another degree or qualification in any other Institute.

(**Prof Mamunar Rashid**) Ex-Principal, Ibrahim Medical College, & Former Advisor, Dept of Community Nutrition, BIHS, Dhaka Supervisor	(**Prof M Mosihuzzaman**) Emeritus Professor, Dept of Chemistry, University of Dhaka Supervisor	(**Prof Nilufar Nahar**) Chairman, Dept of Chemistry, University of Dhaka Supervisor

Prof M Mosihuzzaman is now in abroad. On behalf of him, we have given consent for submitting Farzana Saleh revised PhD Thesis.

Dedicated

To

My Beloved Family

ABSTRACT

Menopause is the transitional event of female life creating a considerable degree of clinical, psychological as well as social problem. Hormone Replacement Therapy (HRT) was thought to be a cornerstone in the management of menopause, but evidences accumulated in the recent past have raised serious questions regarding its safety and usability.

Isoflavones are phytoestrogens present in natural sourcesand they resemble estradiol in structure and manner of action. Genistein and daidzein which has been reported to be the most biologically active dietary isoflavones attract great deal of interest in today's researches. These two isoflavones bind weakly to estrogen receptorα and more strongly to estrogen receptor β, and as this binding is tissue-specific, they possess organ-specific estrogenic and antiestrogeniceffects and they do not have such side effects. In this context, isoflavones are getting increasingly more attention for therapeutic (as an alternate of HRT) interventions. A high intake of dietary isoflavoneshas been suggested to account for the lower rates of climacteric complaints, cardiovascular diseases, breast and endometrial cancers, and osteoporosis-related fractures.

In this study it was planned to identify the isoflavones present in soybean[*Glycinemax*(L.) Merr.], mung dal [*Vignaradiata*(L.) R. Wilczek] and masoor dal [*Lensculinaris* (Medik.)]; to quantify theisoflavones in above three foods and to measure the bioavailability of isoflavones in Bangladeshi postmenopausal women.

In the analytical part of the study, soybean seeds were collected from Jessore, the Southern agricultural area of Bangladesh. Mung and masoordals were purchased from a local supermarket of Dhaka city.Dry soybean, mung andmasoordalswere dried again, ground into powder (200 mesh) by grinder machine and kept in air-tight containersin a refrigerator until analysis was carried out.Standard genistein and daidzein were purchased from Sigma-Aldrich and were preserved at 4ºC and at-20 ºC, respectively.

An amount of 350 mL soy-milk was prepared from the 100g powder bean following a standard procedure and kept in refrigerator. The milk was transferred into four

different flasks, frozen in a methanol freezer and dried into powderby a freeze-dryer.A definite amount of soy-milk powder (7 g) was weighed and transferred into a round bottomed flaskand refluxed with n-hexane in a boiling water bath to free oil from the soy-milk powder. The oil free soy-milk powder was extracted with EtOAc. The supernatants were pooled, filtered through filter paper and evaporated to dryness using rotary vacuum evaporator. The resulting dry extract was dissolved in 1 mL of LC grade acetonitrile (ACN). It was then filtered through milipore filter (0.22 μM) and taken in sample vial(2 mL)before analysis in LC-PDA.

Masoor and mungdals sample were dried in an oven at 105ºC and ground into powder.A definite amount (25 g) of masoorand mungdals powder was weighed and transferred into a round bottomed flaskand refluxed with n-hexane in a boiling water bath to remove oil/fatty materials from the powder. The fat free mung and masoordals powder were extracted with EtOAc. The supernatants were pooled, filtered through filter paper and evaporated to dryness using rotary vacuum evaporator. The resulting dry extract was dissolved in 1 mL of LC grade ACN. It was then filtered through milipore filter (0.22 μM) and taken in sample vial (2 mL) before analysis in LC-PDA.

The separation of isoflavones was performed by LC-PDA on C18 column using mobile phase, ACN: H_2O (75: 25) with a flow rate of 0.5 mL/min, wavelength: 268 nm, loop size 20 μL and running time was 10 minutes.

Genistein and daidzein were identified in the oil free soy-milk, mung and masoordals with respect to retention time of certified standard genistein and daidzein.

Quantification of the isoflavones was done using external calibration curve of the two certified samples which were linear (r^2 were 0.999 & 0.997 for genistein and daidzein, respectively). Limit of Detection (LOD) (S/N ratio; 3:1) and Limit of Quantification (LOQ) (S/N ratio; 10:1) were found to be 0.0045 & 0.0135 ppm and 0.25 & 0.75ppm for genistein and daidzein, respectively.

Amount of total isoflavones, genistein and daidzeinwere found to be in the soy-milk (80.05μg, 43.81μg and 36.25μg per 100g dry weight powder), in the masoor dal (74.99 μg, 37.33 μg, 37.66 μg per 100g dry weight powder) and in the mung dal (71.66 μg, 44.00 μg and 27.66 μgper 100g dry weight powder) respectively.

In the bioavailability part of the study, a total of 16 healthy postmenopausal women (age, mean ±SD, 52.5±5.8 years) were included in the study with the later defined by at least 2year from the time of last menses. The subjects were asked to abstain from foods containing isoflavones at least for 1 week before and during the study. After an overnight fast, each individual was given 350mL of soy-milk, masoor, and mungdal soupswas given to every individual woman which was made from 100g soybean seed, masoor, andmungdalspowder respectively, delivering amounts of daidzein (36.25, 37.66 and 27.66µg respectively) and genistein (43.81, 37.33 and 44.00 µg respectively)as a single bolus.

The study was carried out under the department of Gynecology and Obstetrics, Bangladesh Institute of Health Sciences (BIHS) hospital. For study purpose, a postmenopausal woman was admitted in the BIHS hospital for 2 days. Blood samples (5 mL) werecollected by venipuncture, before (baseline) and then 2, 4, 6, 8, 24, 36 and 48 hafter consuming the prepared foods (soups and milk). Blood was drawn via a catheter for the more frequent samplings.The blood samples were centrifuged at 1200 x *g*and the serum (~2 mL) separated and immediately frozen at -20°C.The study subjects were given mung dal, masoor dal soups and soy-milk subsequently at the interval of 2 week. This 2 week was chosen as washout period. The same batch of soybean,masoor dal, andmung dalwas used throughout the study.

Serum sample was extracted and cleaned up using solid-phase extraction (SPEC18Cartridge). The cartridge was conditioned with water (1 mL x 3) followed methanol (1 mL x 3) and then water again.

Serum sample was thaw and was passed through the conditioned SPE cartridge, then aqueous 5% methanol (800 µL).The isoflavones were eluted in ethyl acetate-acetonitrile mixture (1:1; 400µL x 2).The concentrations of daidzein and genistein in serum were measured by LC following the procedure which was applied in food analysis.

The mean T_{max} for peak serum genistein concentrations of soy-milk, masoor, and mungwere obtained 6.0±1.85, 6.0±1.51, and 6.8±1.78 h respectively after administration of the single-bolus oral dose, with a mean C_{max}1.62±0.87, 4.03±3.91, and 5.15±3.02 µg/mL respectively. The AUC of the serum concentration was

7.41±4.16, 16±15.12 [median (range), 8.76 (2.18-58.34)], and 17.81±9.94 µg/mL in soy-milk, masoor, and mungdal soups respectively.

The results from the study lead to the following conclusions:

a) Two isoflavonesdaidzein and genistein were identified in soybean [*Glycine max* (L.) Merr.], mung dal [*Vignaradiata*(L.) R. Wilczek] and masoor dal [*Lens culinaris* (Medik.)]; b) Daidzein, and genisteinwere found to be 36.25 µg/100 gdry weight, and 43.81 µg/100 gdry weight in soybean; 37.66 µg/100gdry weight, and 37.33 µg/100 g dry weightin masoor dal; 27.66 µg/100 gdry weight, and 44.00 µg/100 gdry weight in mung dal respectively; c) The estimated mean of total isoflavones in locally produced soybean, masoor and mungdals was found to be 80.05µg/100 gdry weight, 74.99 µg/100 gdry weightand 71.66 µg/100 g dry weightrespectively; d) The assessed mean of maximum concentration of isoflavonegenistein was found to be 1.62 µg/mL for soy-milk, 4.03 µg/mL for masoor dal soup and 5.15 µg/mL for mung dal soup; f) The calculated mean of area under the curve (AUC) of isoflavonegenistein was found to be 7.41 µg/mL for soy-milk, 15.77 µg/mL for masoor dal soup and 17.81 µg/mL for mung dal soup; g) The average time to maximum concentration (T_{max}) of soy-milk, masoor and mung dal soups was 6 hour.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEME

LIST OF ABBREVIATIONS

WHO	World Health Organization
FMP	Final Menstrual Period
USA	United State of America
BBS	Bangladesh Bureau of Statistics
%	Percentage
CVD	Cardiovascular Disease
IHD	Ischemic Heart disease
HDL	High Density Lipoprotein- cholesterol
AD	Alzheimer Disease
β	Beta
α	Alpha
ERs	Estrogen Receptors
FSH	Follicle-Stimulating Hormone
LH	Luteinizing Hormone
IGF-1	Insulin-like Growth Factor 1
IL-1	Interleukin-1
TNF	Tumor Necrosis Factor
IL-6	Interleukin-6
OPG	Osteoprotegrin
HRT	Hormone Replacement Therapy
SERMs	Selective estrogen receptor modulators
NO	Nitric Oxide
WHI	Women's Health Initiative

RR	Relative Risk
SSRIs	Serotonin Reuptake Inhibitors
O-DMA	*O*-Demethylangolensin
C6	Carbon 6
C3	Carbon 3
OH	Hydroxyl
µg/g	Microgram Per Gram
mg/g	Miligram Per Gram
mg/day	Miligram Per Day
ng/mL	Nanogram Per Miliiter
RC	Red Clover
mg	Miligram
BMD	Bone Mineral Density
SHBG	Sex hormone binding globulin
TC	Total cholesterol
TG	Triglycerides
FFA	Free Fatty Acid
LDL-cholesterol	Low Density Lipoprotein-cholesterol
HDL-cholesterol	High Density Lipoprotein-cholesterol
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
Daidzein	4',7-Dihydroxyisoflavone
Genistein	4',5,7-Trihydroxyisoflavone
EtOAc	Ethyl Acetate

ACN	Acetonitrile
Na_2SO_4	Sodium sulfate
H_2O	Water
HPLC	High Performance Liquid Chromotography
PDA	Photodiode Array
SPE	Solid-Phase Extraction
rpm	Rotation per minute
µL	Micro Liter
µm	Micro Meter
cm	Centi Meter
g	Gram
mL	Mili Liter
min	Minute
ºC	Degree Celsius
µM	Micro Mole
ppm	Parts Per Million
mL/min	Mili liter Per Minute
nm	Nanometer
LOD	Limit of Detection
LOQ	Limit of Quantification
BIHS	Bangladesh Institute of Health Sciences
h	Hour
AUC	Area Under the Curve
C_{max}	Concentration $_{maximum}$

ANOVA	Analysis of Variance
SD	Standard Deviation
SPSS	Statistical Package For Social Sciences
BADAS	Diabetic Association of Bangladesh
LC-MS/MS	Liquid chromatography mass spectrometry
BMI	Body Mass Index
SBP	Systolic Blood Pressure
DBP	Diastolic Blood Pressure
Kg/m^2	Kilogram per miter square
mmHg	millimeter mercury
T_{max}	Time $_{maximum}$
µg/mL	Microgram Per Mililiter
µmol	Micromole
µmol/L	Micromole/ Liter
PPAR	Peroxisome Proliferator Activated Receptors
PTFE	Polytetrafluoro Ethylene

1. Introduction

1.1. Menopause

Menopause is an important event in a women's life. With the cessation of menstruation, a woman realizes that she has entered the non-reproductive stage of life. This realization along with various physiological changes occurring around this time brings a range of new experiences for the woman (1).

The term menopause signifies the permanent cessation of menstruation. World Health Organization (WHO) scientific group (1981) recommended the definition and updated in 1994 by the WHO Technical Working Committee on Menopause. The recommended definition of menopause is 'the permanent cessation of menstruation resulting from the loss of ovarian follicular activity (2, 3). It is recognized to have occurred after 12 consecutive months of amenorrhea, for which there is no other obvious physiological or pathological cause. Thus the Final Menstrual Period (FMP) can be ascertained only retrospectively.

The menopause is the counterpart of the menarche and refers only to cessation of menstruation; it is merely one manifestation of the climacteric and precedes complete cessation of ovarian function by several months or years. The terms 'menopause' and 'climacteric' are often used synonymously but they refer to essentially different conditions.

The age of the menopause does not depend on the age of menarche, the type of menstrual cycle, and the number of pregnancies, which a woman has had, marriage, climate or environment (4). In the absence of general or pelvic diseases, and with the possible exception of economic and social status, the only known factors governing it are familial and racial.

The age at which menopause occurs varies from country to country and among individuals in the same country. The age at which menopause occurs is between 45 to 55 years. From the age at menopause and respective population data, the number of postmenopausal women living in various parts of the world can be estimated. According to one estimate, in 1990 there were around 467 million women aged 50 years and above, all over the world. With improved life expectancy, it is expected that

the number of post-menopausal women will increase in the near future and the rate of this increase will be substantially faster in the developing world than in industrialized world (2, 3). In developed countries the usual age for the onset of menopause is between 50 and 51 years. The average age, which used to be 47 years in Britain and the USA, is now said to be 51 years (4, 5). Reliable data are not available for developing countries but, it was projected that between 1990 to 2030 in the developing regions, average annual growth rate of the number of women aged over 50 will be 2-3.5% (2, 3). And in India, the number is still estimated at 48 years (4, 5). According to the Bangladesh Bureau of Statistics (BBS, 1999) data, women of menopausal age (>49 years of age) constitute around 12% of the female population of our country (6).

Menstrual function may cease suddenly without warning, but the menopause is most often heralded by a gradual decrease in the amount and frequency of blood loss during several months or years. Every women from her late reproductive period passes through different stages of menopause, known as premenopause, perimenopause and postmenopause; but, a lot of controversies are present regarding the perimenopausal status which is also known as 'menopausal transition'. In this menopausal transition some sort of menstrual irregularities and menopausal symptoms may be seen in women (7). Although it is an obligatory physiological phenomenon, it creates a number of biological, psychological and social problems imposing additional risk and challenge in the life.

1.2. Burden of Menopause

The health status of menopausal women in a specific society is in many ways predetermined by the conditions of life it provides for women of all ages. The middle-aged women's health will certainly continue to be affected by the outcomes of earlier reproductive health events as well as the prevalence and incidence of menopausal problems which vary according to ethnic groups. For instance, Asian women seem to have fewer problems after menopause than their western counterparts. Nevertheless, problems do exist and seem to be increasing due to longer life expectancy and lifestyle changes of people in the South-East Asian region (8). The sufferings from menopause in respect to frequency and severity of symptoms and the number of

women seeking medical help varies widely depending upon social, personal, economic and cultural background.

The period of menopause and post-menopause of a women's life is a universal event, but the sufferings from menopause in respect to frequency and severity of symptoms vary widely. It is known that hypoestrogenic state causes menopausal symptoms. Symptoms include mood and behavioral changes, hot flushes, sleep disturbance, vaginal dryness and more. Lack of estrogen production is also associated with an increased risk of suffering from diseases like osteoporosis and cardiovascular disease (CVD). The risk of cardiovascular morbidity and mortality is lower in premenopausal women compared to men of the same age, but the risk increases as women reach menopause (9). Due to an increased rate of bone loss during menopause, postmenopausal women have an increased risk of developing osteoporosis in the long run (10).

1.2.1. Hot flushes and night sweats - Hot flushes and night sweats are the most common characteristics of the menopause. Hot flushes arise as sudden feeling of heat in the face, neck and chest. Most women indicate that hot flushes begin with a sensation of pressure in the head, much like a headache. This increases in intensity until the physiologic flush occurs. Palpitations may also be experienced. The actual flush is characterized as a feeling of heat or burning in the face, neck and chest, followed immediately by an outbreak of sweating that affects the entire body but is particularly prominent over the head, neck, upper chest, and back. The duration of the whole episode varies from momentary to as long as 10 minutes; the average length is 4 minutes. The frequency varies from 1-2 per hour to 1-2 per week. The exact mechanism responsible for hot flushes is not known. Hot flushes occur after the spontaneous cessation of ovarian function, it has been presumed that the underlying mechanism is endocrinologic, related either to reduction of ovarian estrogen secretion or to enhancement of pituitary gonadotropin secretion (5).

Different authors have presented evidences that prevalence of hot flushes associated with menopause varies in different culture. For example, the prevalence has been reported to be 0% in Mayan Indian women, 10-20% in Hong Kong women, around

70% in Japanese women, 23% in Thai women, 45% in North –American women and up to 80% in Dutch women (11, 12).

Night sweats are the night time manifestation of hot flushes. It has been observed in about 75% of women who go through the natural menopause. Of those having flushes, 82% experience the disturbance for more than 1 year and 25-50% complain of the symptom for more than 5 years (5).

1.2.2. Sleep disturbance - Insomnia is often cited as a menopausal complain, but it usually a secondary effect of sleep disruption caused by the night sweats (5).

1.2.3. Changes in reproductive tract - Alteration of menstrual function are a clinical symptom of the climacteric symptoms which causes decreased in cervix size, along with a reduction of secretion of cervical mucus. This may contribute to excessive vaginal dryness which may cause dyspareunia (5).

1.2.4. Changes in urinary tract- Estrogen plays an important role in maintaining the epithelium of the bladder and urethra. Due to menopause, atrophic cystitis occurs which is characterized by urinary urgency, incontinence, and frequency without pyuria or dysuria (5).

The urogenital atrophy symptoms (vaginal dryness, vaginal irritation, itching, dyspareunia, vaginal bleeding nocturia, urgency, stress urinary incontinence and urinary tract infections) are usually progressive in nature and deteriorate with the time from the menopausal transition due to estrogen loss. Atrophic changes of the vulva, vagina and lower urinary tract can have a large impact on the quality of life of the menopausal woman (13).

1.2.5. Changes in mammary glands - Regression of breast size during and after menopause is psychologically distressing to some women. To those who have been bothered by cyclic symptoms of breast pain and cystic formation, the disappearance of these symptoms is a great relief (5). To reduce postmenopausal breast cancer risk, important strategies are of women should maintain a healthy weight and avoid weight gain (14, 15).

1.2.6. Osteoporosis - Osteoporosis is a systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in fragility of bone and susceptibility to risk of fracture. Bone loss occurs from around menopause because estrogen deficiency results in increased bone remodeling within the skeleton. Postmenopausal bone loss disproportionately affects cancellous bone which is found in vertebral bodies and at the end of long bones (5). Postmenopausal women with osteoporosis have lost their quality of life and suffered from different physical and social function (16).

1.2.7. Cardiovascular system changes ischemic heart disease (IHD) - Estrogen deficiency is associated with a reduction in the plasma level of the protective cholesterol, High Density Lipoprotein (HDL). Postmenopausal women are two and four times more likely to suffer IHD than their premenopausal counterparts. Early surgical menopause increases the risk of IHD (17).

1.2.8. Alzheimer's disease (AD) - Dementia refers to the loss of cognitive or mental abilities. For postmenopausal women one such factor which influences AD risk may be the estrogen deficiency (17, 18).

A variety of symptoms, occurring either single or together, are frequently reported as being part of a menopausal syndrome. Some frequently mentioned symptoms are depression, nervous tension, palpitation, headache, insomnia, fluid retention, backache, difficulty in concentrating and dizzy spells.

1.3. Hormonal Change in Menopause

In the menopause, there are major changes in androgen, estrogen, progesterone, and gonadotropin secretion.

1.3.1. Estrogens - The naturally occurring estrogens are 17β-estradiol, estrone, and estriol. They are secreted by the granulosa and the thecal cells of the ovarian follicles, the corpus luteum, and the placenta. The biosynthetic pathway involves their formation from androgens and by aromatization of androstenedione in the circulation. Aromatase also catalyzes the conversion of testosterone to estradiol. Estrogens facilitate the growth of the ovarian follicles, increase uterine blood flow and have important effects on the smooth muscle of the uterus. Estrogen action is mostly

mediated through estrogen receptors (ERs). In the human body, there are two different types of ERs; ERα and ERβ which are different in structure and biological function, Endogenous estrogen binds mainly to ERα (19). The two receptors are expressed differently in different tissues, where ERα is found mainly in breast and ovarian tissue and ERβ is found in brain, bone and bladder tissues (19, 20). In breast tissue, estrogen stimulates growth of the ductal epithelium and connective tissue. Estrogen however can impact negatively on this tissue, as it stimulates the growth of breast cancer cells (20).

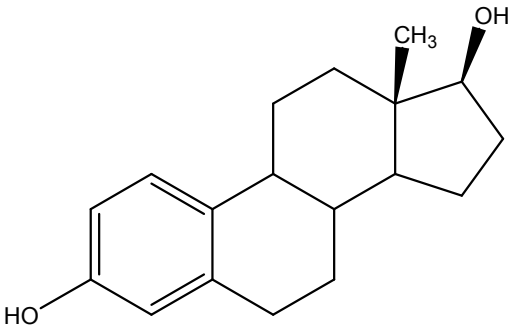


Figure 1: Structure of Estrogen

Estrogens increase the amount of uterine muscle and its content of contractile proteins. It decreases Follicle-stimulating hormone (FSH) secretion. Estrogen exerts both negative and positive feedback in Luteinizing hormone (LH) secretion. It lowers plasma cholesterol level. And prevents initiation of atherosclerosis. These actions may account for the low incidence of myocardial infraction and other complications of atherosclerotic-vascular disease in premenopausal women. There is considerable evidence that small doses of estrogen may reduce the incidence of cardiovascular disease after menopause (21) though few epidemiological and intervention studies show contradicting results on prevention of atherosclerosis in postmenopausal women (20). Estrogen also affects bones as osteoclasts and osteoblasts contain ERs. In osteoblasts, estrogen stimulates the secretion of the anabolic growth factor Insulin-like growth factor 1 (IGF-1), and inhibits the secretion of cytokines, Interleukin-1 (IL-1), Tumor necrosis factor (TNF) and Interleukin-6 (IL-6) that are involved in bone resorption. Estrogen also inhibits the function of osteoclasts by stimulating the synthesis and secretion of osteoprotegrin (OPG). Estrogen is thereby involved in reducing bone resorption and maintaining bone health (20, 22). After a woman has

passed the menopause, there is good clinical evidence of reduced endogenous estrogen production in most subjects. The greatest decrease is in estradiol. Its concentration is distinctly lower than that found in young women during any phase of their menstrual cycle (20).

1.3.2. Progesterone - Progesterone is secreted in large amounts by the corpus luteum and the placenta. It is an important intermediate in steroid biosynthesis in all tissues that secrete steroid hormones, and small amounts enter the circulation from the testes and adrenal cortex. Progesterone is responsible for the progestational changes in the endometrium and the cyclic changes in the cervix and vagina. It decreases the number of estrogen receptors in the endometrium and increases the rate of conversion of 17β-estradiol to less active estrogens. Large doses of progesterone inhibit LH secretion and potentiate the inhibitory effects of estrogens, preventing ovulation. During the follicular phase of the cycle, progesterone levels are low. With ovulation the levels rise greatly, reflecting the secretory activity of the corpus luteum.

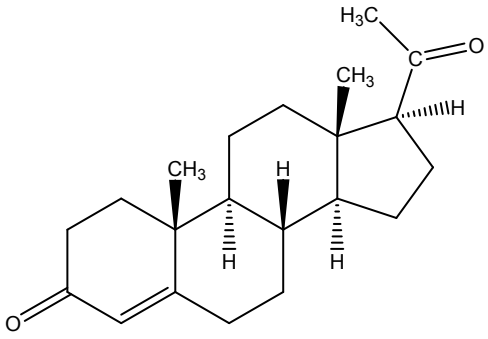


Figure 2: Structure of Progesterone

In postmenopausal women, the levels of progesterone are only 30% of the concentrations seen in young women during the follicular phase. Since postmenopausal ovaries do not contain functional follicles, ovulation does not occur and progesterone levels remain low (5).

1.3.3. Gonadotropins - The gonadotropins, FSH and LH act in concert to regulate the cyclic secretion of the ovarian hormones. FSH from the pituitary is responsible for early maturation of the ovarian follicles, FSH and LH together are responsible for final follicle maturation. A burst of LH secretion triggers ovulation and the initial formation of the corpus luteum. There is also a smaller midcycle burst of FSH

secretion, the significance of which is uncertain. LH stimulates the secretion of estrogen and progesterone from the corpus luteum (21). With the menopause, both LH and FSH levels rise substantially, with FSH usually higher than LH. This is thought to reflect the slower clearance of FSH from the circulation. Concentration of FSH are strikingly elevated during the early follicular phase and fall as estradiol increase during follicular maturation. FSH levels at the midcycle peak and late in the luteal phase are also consistently higher than those found in younger women and decrease during the midluteal phase. LH concentration are indistinguishable from those observed in younger women. The mechanism responsible for this early rise of FSH is probably related to inhibin. Inhibin is a polypeptide hormone that is synthesized and secreted by granulosa cells. It causes negative feedback on FSH release by the pituitary. As the oocyte number decreases, inhibin levels fall, resulting in a rise in FSH levels (5). Low estrogen levels alone do not appear to trigger hot flushes. Hot flushes appear to be related to gonadotropins. A close temporal association between the occurrence of flushes and the pulsatile release of LH has been demonstrated. The observation that flushes occur after hypophysectomy suggests that they are not due directly to LH release.

1.3.4. Androgens - During reproductive life, the primary ovarian androgen is androstenedione, the major secretory product of developing follicles. In postmenopausal women, there is a reduction of circulating androstenedioneto approximately 50% of the concentration found in young women, reflecting the absences of follicular activity. For testosterone, the level found in postmenopausal women is minimally lower than that found in premenopausal women before oophorectomy and is distinctly higher than the level observed in ovariectomized young women.

1.4. Management of Menopause

Management of menopause can be divided into three types such as: psychosocial, therapeutic and nutritional management.

1.4.1. Psychosocial

Anxiety, depression, postmenopausal symptoms, fatigue, sexual issues, relationship and family become important problem areas for the subjects. It is encouraging that the social awareness about menopause is gradually increasing. Menopause is now receiving more attention from researchers, physicians and media than ever before. Women today are much more open about menopause and more willing to take measures to relieve symptoms and prevent long-term problems. The postmenopausal women of our population is not getting enough support due to poor socio-economic condition, illiteracy, ignorance and inadequate health care system. During this period women are not well accepted in the society and family and they consider themselves as a burden. So, good pre-care is just as important as good aftercare to reduce problems and increase well-being. Addressing information and support in a timely fashion is important in providing success to psychological aftercare.

1.4.2. Therapeutic Management

1.4.2.1. Drug

Small doses of sedatives can be helped to control hot flushes. Hypotensive agents such as clonidine hydrochloride are sometimes advised. Tranquillizers may be helpful when nervous symptoms predominate. Osteoporosis can be corrected by bisphosphonates. Other agents are etidronate, paradronate and risedronate. Salmon calcitonin has the advantage of relieving the acute pain caused by subclinical vertebral fractures (4).

1.4.2.2. Hormone Replacement Therapy (HRT)

Selective estrogen receptor modulators (SERMs) are non-steroidal compounds that simulate the effect of estrogen in some tissues, but have an antagonistic effect in other tissues (20). This type of pharmaceuticals is used for treatment of diseases related to hypoestrogenic states including osteoporosis, CVD and cancer (23, 24). The combination of agonistic and antagonistic effects makes SERMs useful for treatment of symptoms of postmenopausal women. The estrogenic effect on bones and brain is desired to maintain bone strength and brain function, and agonistic effects on breast and endometrial tissue are avoided (23).

Hormone replacement therapy (HRT) is a treatment used for menopausal women to relieve menopausal symptoms and/or decrease the risk of hormone-dependent diseases. The treatment includes estrogen sometimes combined with a progestin, which is synthetic progesterone that ought to counteract the proliferative action of estrogen in the endometrium (25). Since menopausal symptoms are directly related to the result of deprivation of estrogen, the administration of this hormone should relieve these symptoms completely. About 1/3rd of a women's life is spent in the postmenopausal era and as a consequence the role of HRT is of relevance. For the symptomatic women, HRT with estrogen is clearly beneficial in improving quality of life in the menopausal years. The benefits are mainly the prevention or control of vasomotor symptoms like insomnia and genital atrophy. Hot flushes, sleep disturbances and atrophic urogenital symptoms, are most effectively treated by HRT (26, 27). Observational studies have indicated that HRT also reduces the risk of osteoporotic fractures and CVD (28, 29).

Estrogen has beneficial effects on the lipid profile and nitric oxide (NO) production. In The Nurses' Health study the risk of coronary events was reduced by 45% in users of HRT (25). Therefore, HRT has been recommended not only for treatment of incapacitating climacteric complaints, but also for the prevention of chronic diseases such as CVD (30). But there is also evidence of the opposite including the Women's Health Initiative (WHI) study which was finished prematurely because of an increased risk of coronary events (31). These contradictions could be attributable to biases in observational studies, and the timing of initiation of HRT (32).

Despite some positive effects of these synthetic hormones on postmenopausal women, they also have some negative effects. Usually, estrogen acts by inhibiting or reducing the output of gonadorophins by the hypothalamic-pituitary system. It is being prescribed for the relief of vasomotor symptoms. Its dose is gradually increased to achieve this effect. When the appropriate level is found, the same dose is maintained for one month and is then very slowly reduced. A disadvantage of this form of treatment is that many women fail to obey instructions to reduce the dose and, since it relieves symptoms, they continue to take them haphazardly over the course of many years. In such circumstance estrogen is likely to cause uterine haemorrhage,

endometrial hyperplasia and possible carcinoma (4). The use of unopposed estrogen by itself without progesterone in women with uterus, causes increased risk of endometrial hyperpalasia and cancer (17). It is now generally accepted that progesterone should always be given with estrogen therapy. Adding progestin to estrogen may prevent the development of endometrial cancer, this combination may cause some unwanted side effects, i.e. breast cancer, venous thromboembolism, stroke and coronary heart disease (33). There are contradicting results regarding the effect of combining estrogen with progestin. It is unclear whether progestin exerts proliferative or anti-proliferative effect on the human breast (34). In addition, patients receiving estrogen and progesterone therapy must have regular examination of the breasts and genital tract, blood pressure monitoring, blood sugar, liver function tests and lipid profile (4). The main reasons for discontinuing HRT are vaginal bleeding, breast tenderness, nausea, vomiting, and weight gain, fear of breast cancer and general belief of harmfulness of hormones. There are several large observational studies that indicate a further increase in cancer risk when using progestin, including The Nurses' Health study reporting a yearly increase in cancer risk of 3.3% when using estrogen alone compared to 9% for estrogen plus a progestin (25), parallel, it is accepted that short term HRT did not increase breast cancer risk significantly. In the Million Women Study it was found that the effects of specific types of HRT was associated with an increased risk of incidence and fetal breast cancer and the effect was substantially greater for estrogen-progesterone combinations than for other types of HRT (35). However, in another study, it was found that estrogen use in American Indian postmenopausal women was related to deterioration of glucose tolerance. Longer duration of estrogen users compared to current users were related to an increased risk of type 2 diabetes (36). Estrogen replacement therapy after menopause therefore improves the health and quality of life for women. The WHI randomized controlled trial has recently found that although estrogen-alone hormone therapy reduces the risk of hip and other fractures in healthy postmenopausal women with prior hysterectomy, it significantly increases the risk of stroke (but has no significant effect on the risk of coronary heart disease, breast or colorectal cancer) (31). Though, a number of randomized placebo-controlled trials have shown no benefit of HRT in primary or secondary prevention of CVD (37-42).

A recent meta-analysis of 52 epidemiological studies revealed that current or recent users of HRT had a 37% increased risk of developing ovarian cancer (43). Long-term estrogen replacement therapy in postmenopausal women who have a uterus might has the disadvantage of being tissue agonists for endometrial tissue, which increases the incidence of endometrial cancer (33).

Few years ago, it was debatable regarding the risk of breast cancer due to usages of HRT. Now, it is well established that the HRT users are in increased risk of developing breast cancer than non-users (44-46). In The Million Women study including postmenopausal women they found a relative risk (RR) of 1.66 for developing breast cancer in current users of HRT compared with never users. The risk was greater for users of estrogen-progestin preparations than for estrogen-only preparations (RR: 2.0 and 1.3, respectively) (35). And there is doubt on the wisdom of using HRT for prevention of chronic diseases. HRT is currently recommended primarily for treatment of climacteric complaints (41, 47, 48).

1.4.3. Non-Hormonal Alternatives

The alarming reports briefly mentioned above have increased a search for effective alternatives for the management of menopause. There exist a variety of non-hormonal alternatives for treating menopausal complaints, including lifestyle modification such as keeping the body cool and exercising regularly. *Stadberg et al* (49) explored that 41.4% of Swedish postmenopausal women had used HRT at some time, while 45% had used a non-hormonal regimen. Clonidine, an α2-adrenergic receptor agonist, given either orally or trans-dermally, can alleviate hot flushes, but, because of adverse effects such as dry mouth, constipation and drowsiness in 10–50% of patients, it is not real alternative to HRT (50, 51). Beta-blockers such as propanol can alleviate palpitations, but their ability to improve hot flushes is modest and therefore they are not recommended for treatment of menopausal complaints (52). Anti-dopaminergic compounds, such as veralipride and methyldopa, may alleviate hot flushes, but because of a number of side effects they have no place in the treatment of menopausal symptoms (53).

Of selective serotonin reuptake inhibitors (SSRIs), fluoxetine and venlafaxine have been shown to reduce hot flushes in placebo-controlled randomized studies (54, 55).

They are often recommended for patients with severe symptoms and contraindications for HRT (56), but in clinical practice many women are reluctant to start using a psychotropic drug. In addition, SSRIs have moderate side effects, such as dry mouth, nausea, decreased appetite, constipation, sleeping difficulties, and withdrawal symptoms (56). Moreover, no long-term data exist on their final efficacy and safety. Hence they seldom are a real alternative to HRT in clinical practice. Vitamin E has been recommended for treating hot flushes, although its efficacy is poorly confirmed.

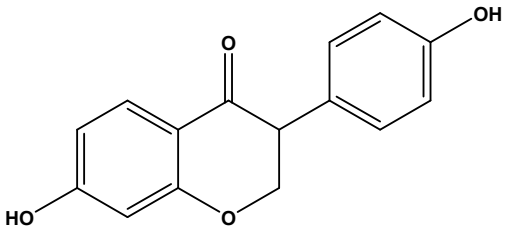
Herbs used for menopausal problems include black cohosh, chaste tree berry, dong quai, ginseng, evening primrose oil, motherwort and licorice (57). They are most often sold and used under the assumption that "natural" products are "good" for your health and cause no harm, although hardly any real research data exist on their efficacy and safety. Black cohosh is probably the most popular herb, and 40 mg of this herbal drug/day for 3 months has been shown to be equipotent to 0.6 mg of conjugated estrogens in relieving climacteric complaints and affecting bone metabolism without stimulating the endometrium (58).

It has been known for almost one century that some plant extracts exhibit estrogenic activities (59). The evidence was often anecdotal, but, for example, in the 1940s it was shown that sheep consuming high amounts of red clover became infertile because of the high intake of phytoestrogens (59). Research on phytoestrogens was greatly stimulated when it became possible to assess their concentrations in urine in the 1980s (60), and later on, they were also identified and measured in bile, blood, feces, semen, breast milk and saliva (59, 61).

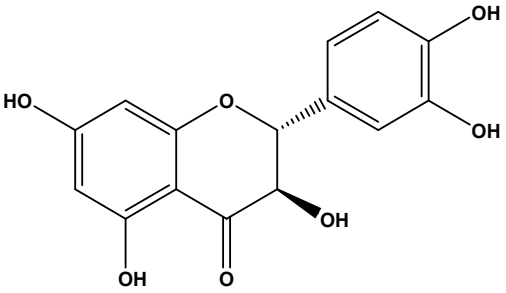
1.5. Phytoestrogens

Phytoestrogens are compounds derived from plants. These are structurally and functionally comparable to 17-β estradiol. Phytoestrogens can be classified in three groups, i.e., isoflavones, coumestans and lignans. The major isoflavones are genistein, daidzein, formononetin and biochanin A. Coumestrol is the most important coumestan. The major lignans are enterolactone and enterodiol, which are produced by colonic bacteria from their dietary precursors matairesinol and secoisolariciresinol (62). There are many classes of phytoestrogens but the most important are the

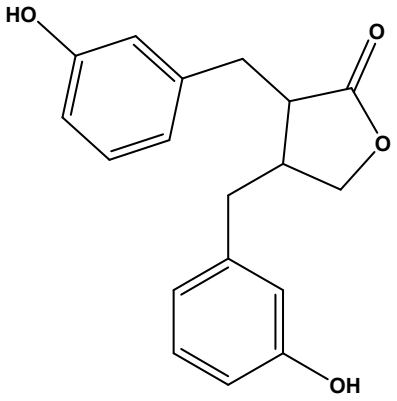
isoflavones which are found in legumes, especially soyabeans, lentils and chickpeas, and the lignans which are found in many fiber-rich foods, whole –grain cereals and nuts. However, small concentrations of phytoestrogens have been measured in several fruits and vegetables (63, 64).



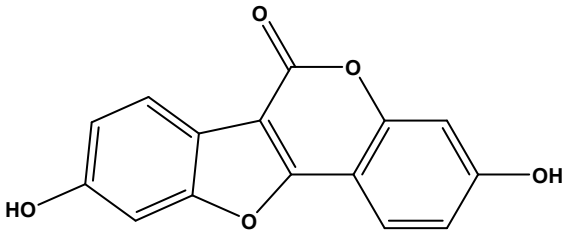
Isoflavonoid



Flavonoid



Lignan



Coumestrol

Figure 3: Structure of Phytoestrogens

Phytoestrogens have estrogen-like structure (59, 65) but they are not steroids. However, owing to the presence of phenolic rings in their molecules, they have the ability to bind to estrogen receptors (66). And they have a weak affinity for the estrogen receptors. They have estrogenic and anti-estrogenic activity. The food consumed by humans (67), soybeans contain the highest concentration of isoflavones.

Table 1: Classification and sources of phytoestrogens

Isoflavones	**Lignans**	**Coumestans**
Leguminous plants (soybean, lentils, beans, clover)	Flaxseeds, grains, cereals, fruits (guava), berries (lingonberry, bramble), vegetables (broccoli) Matairesinol →Enterolactone	Bean sprouts, fodder crops Coumesterol
BiochaninA →Genistein→ p-ethylphenol		
Formononetin →Daidzein→Equol, O-demethylangolensin (O-DMA)	Secoisolariciresinol →Enterodiol	

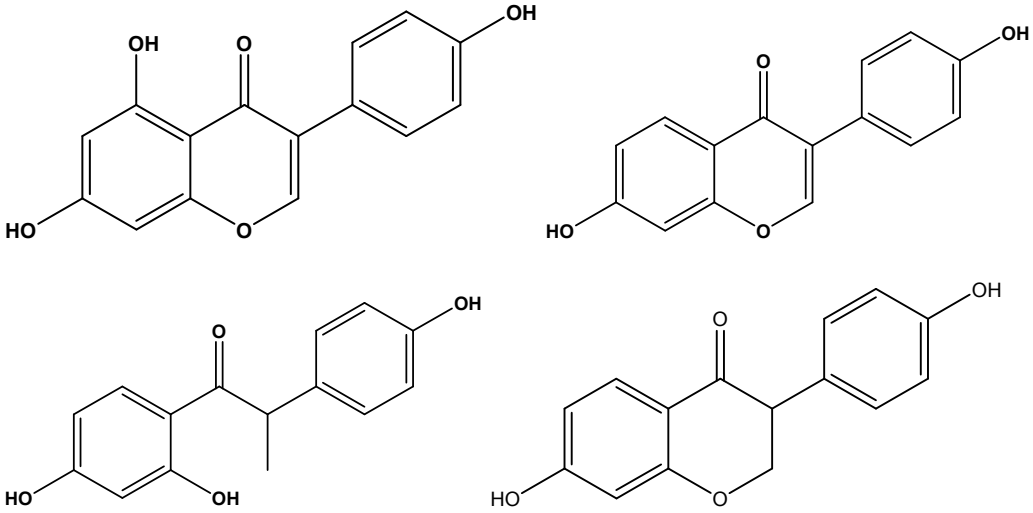


Figure 4: Chemical structure of genistein, daidzein and daidzein metabolites

1.6. Chemistry of Isoflavones

Isoflavones are polyphenolic compounds produced almost exclusively by the members of the Leguminosae (bean) family. These are produced from a branch of the general phenylpropanoid pathway which produces all flavonoid compounds in higher plants. Isoflavones have a chemical structure resembling that of estrodiol-17β, the

most potent mammalian estrogen. The major isoflavones, namely, genistein and daidzein, have several features in common with estradiol-17β (68).

Molecular mass of daidzein and genistein 254 and 270 and they are hydrophobic in nature (69). The aqueous solubility of these compounds are increased when they form glycosides with sugar molecules like glucose, or glucuronide (70). The isoflavone *i.e.* aglycones are stable under physiological conditions than the glycosides. Under acidic conditions, the glycosides can be hydrolyzed into corresponding sugar molecules and their aglycones by enzymes present in the gut and liver. In biological system hydrolyses during metabolism are much faster than chemical reactions (71).

Characteristic fetcher of Isoflavones are the presence of two phenol rings linked together by a three carbon bridge (72). The basic structure of flavonoids is based on the 15 carbon skeleton, *i.e.* C6 – C3 – C6.

The two C6 represents the number of carbon atoms of the two phenyl groups and the C3 represents the number of carbon atoms that bridge the two phenyl rings by a linear three carbon chain. Figure 5 explains the above description in most convenient way (72).

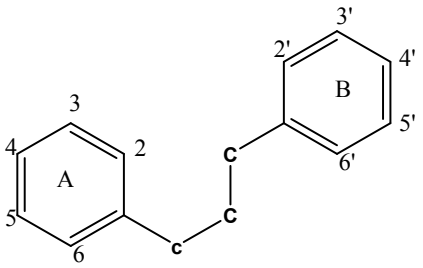
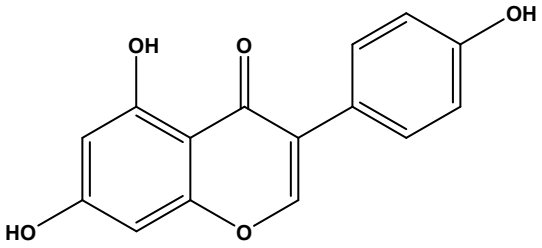
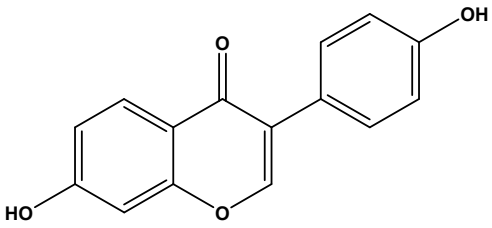


Figure 5: Basis Structure of flavonoid

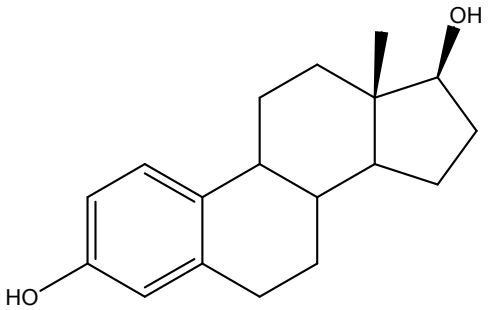
It has been already mentioned that flavonoids are widely distributed in terrestrial plants, and isoflavonoids are abundant in the seeds leguminous plants. It seems that nearly every plant is able to synthesize phytoestrogens, but their concentrations in most plants are negligible (73).



Genistein



Daidzein



Estradiol-17β

Figure 6: Structure of genistein, daidzein and estradiol

Isoflavones are found mainly in their glycoside form this means that part of the molecule is linked with different number of sugars. Isoflavones can exist as aglycones or in a conjugated form as glycosides. If the linkage of the sugar to the flavonoid aglycone is through an OH group then these are called as O-glycosylflavonoids and if the linkage is through C-C bond then these are called as C-glycosylflavonoids and this has an effect on their absorption and retention within the human boy (70). Concentrations aglycones are found less the glycosides (74). After ingestion, isoflavones glycosides are hydrolyzed by intestinal glycoside hydrolases, which release the aglycones, daidzein, genistein and glycitein. These may be absorbed or further metabolized to many specific metabolites including, equol and *p*-ethylphenol (70).

Although parent isoflavones has no physiological interest but their metabolites many have beneficial effect on human health like reduction of super oxide formation, anti-oxidants and anti-aging. It is considered that the phenol ring is a key structural element to be able to attach to the estrogen receptors and the flavonoid isomeric configuration increases their similarity to human estrogens (75).

1.7. Isoflavone contents in different vegetables and seeds

In nature isoflavones occur in more than 300 kinds of plants, mostly in the roots and seeds (76). The major dietary sources of isoflavonoids for humans are soybeans and soy-based foods (77, 78). Mitani *et al* (79) reported that daidzein and genistein were detected at high concentrations from dried soybeans. In other study it was stated that soybeans proved to be the richest source of genistein (63). Total isoflavone contents in Japanese varieties grown in two different years (1991 and 1992) were estimated to be from 2041 to 2343 μg/g and from 1261-1417 μg/g respectively (70). Daidzein and genistein content were found not as high as soybeans in Mung (green gram) and in Masoor dals (80).

Korean soybean seeds and Pungsannamulkong soy-sprouts have been analyzed for their isoflavones composition and the highest total isoflavones content was found in whole sprouts on day 7 (81). Quantification of daidzein and genistein, was determined by high performance liquid chromatography in 4 soybean cultivars and 26 soybean products. Genistein and daidzein content were varied on soybean cultivars and soy products (82).

A study was done in Iran to determine the daidzein and genistein in soy milk and the concentration of genistein in soy milk were higher than daidzein (83). Under the same cultivation conditions but the different maturity groups the content of isoflavones was determined and higher amount of isoflavones in soybean was obtained in early maturing groups (84). Dry soybeans contain 1.2-4.2 mg/g isoflavones (85). Their exact concentration depends on many factors, including the soil in which they are grown, climatic conditions, stage of their maturity or nature of cultivation. Generally, use of plant growth regulator and higher amount fertilizer are associated with lower content of isoflavones. Isoflavones concentration was also related to regional differences in food.

In plants, isoflavones are found as glycoconjugates, which are biologically inactive (86, 87). They are hydrolyzed to active forms – aglycones – by the action of intestinal bacteria, glycoside hydrolases enzyme and acidic condition (88). In humans, daidzein and genistein are considered the most important biologically active forms of isoflavones. These substances arise both by hydrolysis of biologically inactive forms

of glycoconjugates, as mentioned above and by metabolism from biochanin A and formononetin. Aglycone forms of isoflavones are transported from the intestine to the blood or they are further metabolized directly in the intestine. Degradation of isoflavones occurs in the liver, where they are conjugated with glucuronic acid and to a lesser degree with sulfates. They are excreted from the body in urine or bile. The major portion of daidzein and genistein is eliminated from the body within 24 hours (89, 90). In blood serum, the highest levels of isoflavones are reached within 2-8 hours after consumption.

It has been already known that isoflavones are found in a complex mixture of aglycones and the glycoside conjugates β, malonyl and acetyl glycosides ((91, 92). The malonyl and acetyl glycosides are less stable than the β-glycosides and readily convert into β-glycosides upon exposure to heat or other processing steps (66).The concentration and composition of the isoflavones in plants depend on factors like plant cultivar, environmental conditions and industrial processing (66). The pattern of conjugation of the isoflavones affects the hydrolysis or degradation process and thereby the bioavailability of the isoflavones (93). Processing of isoflavones containing foods can alter the total content of isoflavones as well as the ratio between glycosides and aglycones (92, 94).

The chemical composition of the dietary isoflavones may be a key determinant of its bioavailability and extent of biotransformation. Daidzein was reported to be more bioavailable than genistein (95), though this was not easy for drawing this conclusion, because few studies described opposite results (96, 97). It is difficult to determine pharmacokinetics when using a mixed matrix with multiple forms of the isoflavones administered together.

Methanol extract (98) of seven legumes [soybean (*Glycine max L.*), green bean (*Phaseolus vulgaris L.*), alfalfa sprout (*Medicago sativa L.*), mung bean sprout (*Vigna radiata L.*), kudzu root (*Pueraria lobata L.*), red clover blossom and red clover sprout (*Trifolium pratense L.*)] were analyzed for estrogenic activity human embryonic kidney (HEK 293) cells. All seven of the extracts exhibited preferential agonist activity toward ERbeta. The results showed that several legumes from above are a source of isoflavones with good levels of estrogenic activity and they are able to bind

to estrogen receptors (ERs) because of their structural similarity with 17-β-estradiol (99).

1.7. Bioavailability of Isoflavones

How varying dietary isoflavones intake influences the bioavailability is largely unknown in postmenopausal women, to our knowledge, there are few studies of the bioavailability of daidzein or genistein among this group (100-102).

These isoflavone glycoside are inactive and must be first hydrolyzed to become biologically active (60). Hydrolysis occurs in the gastrointestinal tract, where bioactive aglycones, daidzein and genistein are formed (60). The aglycones may be absorbed into the circulation or further metabolized, daidzein convert to equol and *O*-desmethylangolensin (*O*-DMA) and genistein transforms into estrogenically inactive *p*-ethylphenol (70, 103).

After absorption, isoflavones, like estrogens, are prone to enterohepatic circulation (60). They are entered into the bile, deconjugated by intestinal flora, reabsorbed, reconjugated by the liver, and excreted in the urine (59, 66). Peak plasma concentrations of isoflavones are attained 5–6 hours after oral ingestion and the half-life of systemic elimination is in the range of 6–8 hours (60, 96). Urinary excretion of isoflavones rises with increasing intake of soy isoflavones, but only to a certain extent; the absorption capacity becomes saturated at high doses (104).

In one study it was showed that the isoflavones of aglycone-rich soy food were absorbed faster and had higher bioavailability than those of glucoside-rich soy in postmenopausal Japanese women (100).

In postmenopausal Thai women study revealed that the bioavailability of genistein from soy beverage and soy extract capsules was similar though the bioavailability of daidzein in soy beverage was slightly lower than soy extract capsules. Therefore, their opinion was the total dose of isoflavones that benefits menopausal health is up to approximately 100 mg/day (101). Opposite findings was found from Stanford study and the investigator suggested that bioavailability might be higher from food sources of isoflavones than from tablets (105).

Another study showed that modest dosage of soya milk might provide the most bioavailable source of isoflavones, whether it was via an aglycone-rich fermented soya milk or a glucoside-rich soya milk (106). Bioavailability determined from the plasma appearance and disappearance curves after single-bolus oral administration of 50 mg of each of the isoflavones was similar for genistein and daidzein, and the glycosidic conjugates were more bioavailable (107).

The bioavailability of isoflavones in the gastrointestinal canal can be modified by changes in the gut bacterial flora (64, 108) and by other components such as diet (109-112), duration of soy consumption (112-114), antibiotic use, surgery, or bowel disease (59). The fate of dietary-ingested isoflavones varies from one person to another (115). There also appears to be a gender-dependent difference in metabolism, women metabolize diet-derived isoflavones more effectively (113) than men. The role of age and race in determining the ability to metabolize isoflavones is still unknown.

Some additional factors are also influenced the bioavailability of isoflavones and those are the doses of isoflavones and food matrix. One study was done on healthy women for assessing the bioavailability by consuming single-bolus soy nuts and optimum steady-state serum isoflavones concentration was found from modest intakes of soy foods consumed regularly throughout the day rather than from a single highly enriched product (104). In healthy adult women study, it was concluded that absorption, excretion and plasma concentration of isoflavones in humans depend upon the dose given (95).

Liquid matrix yielded a faster absorption rate and higher peak plasma concentrations than a solid matrix and aglycones in a fermented food were absorbed more rapidly than glycoside conjugates. These data were suggestive of an influence of gender, but no major influence of age (91). Isoflavones have weak estrogen and anti-estrogen effects and their overall influence depends upon the dose, frequency of use, individual metabolism, and the rest of the diet. Japanese women eat a good amount of soy foods do not seem to have a higher rate of breast cancer. Eating a small amount of soy, as part of a diet that has a wide range of foods, does not pose a risk or harm.

A randomized double-blind placebo-controlled study was done and only genistein was found to be significantly higher in serum of the soy group than in the placebo group,

and no significant differences were found in breast tissue homogenate concentrations of all analytes between the two groups (116).

1.8. Mechanism of Action

Isoflavones, such as genistein and daidzein, bind weakly to ERα and more strongly to ERβ (117). Estrogen α-receptors predominate in endometrium, ovarian stroma and breast cells, whereas estrogen β-receptors predominate in bone, endothelium and brain (20, 118). Because of the different tissue distribution of ERs, isoflavonoids possess organ-specific estrogenic and anti-estrogenic effects (117). Thus, they fulfill the criteria of SERMs. The final effect of isoflavonoids seems to depend, in addition to receptor binding, on the circulating levels of isoflavones and endogenous estrogen, so that a "certain amount" of estrogen may appear mandatory for isoflavonoid action (61, 117, 118).

In animal studies and *in vitro* studies using breast cancer cell lines, isoflavones have induced tumor suppression (66). This phenomenon can be explained by several mechanisms. First, genistein may specifically inhibit tyrosine kinases (119) and DNA topoisomerases I and II (120, 121). Second, genistein also arrests cell growth by interfering with signal transduction pathways (122). Third, isoflavonoids possess antioxidant (123), antiproliferative (124), anti-inflammatory (125), and antiangiogenetic properties (126), and they also inhibit the actions of cytokines and growth factors (127, 128). Finally, isoflavonoids can inhibit cytokine-stimulated nitric oxide formation (129) and aromatase activity (130), which may be of significance as regards tumor genesis. Thus, there exist several biologically reasonable explanations for an anticancer effect of isoflavonoids, but clinical evidence of these benefits is still unclear and awaits further evaluation.

1.9. Health effect of Isoflavones

Many plants consumed by humans contain dietary estrogen of which concentrations are generally low, several plants containing high concentration of isoflavones (131, 132), and if ingested in large quantities may evoke significant biological effects. Soy protein and other legumes are consumed in significant quantities by humans, the acute and chronic effects of exposure to these dietary estrogens may be biologically

important. The field of hormone or herbal therapy during or after menopause is very complicated and there is no consensus within the medical community regarding the best option for long term therapy. The most traditional doctors now prefer using low doses of hormones for a brief period of time to treat menopausal symptoms, but prefer not to continue hormone replacement therapy indefinitely as in the past.

Plant containing isoflavones are relatively weak estrogen, requiring much higher concentrations than estradiol to produce an equivalent biological response. Evidence is beginning to accrue that isoflavones may begin to offer protection against a wide range of human conditions, including breast, bowel, prostate, and other cancers; cardiovascular disease; brain function; alcohol abuse; osteoporosis; and menopausal symptoms (133). The basis for these effects has not been established, but the weak estrogenic activity of isoflavones may be a factor in conferring these properties (134).

1.9.1. Effect on Menopause

Isoflavones have estrogenic effect because they have structural similarities to estrogen and similar molecular mass. Estrogenic effects are reached when a steady-state plasma concentration of isoflavones of 50-800 ng/mL is reached (23). The 4'-hydroxyl group in the isoflavones structure is the binding site for ERs (96). There is also a difference in the binding affinities of genistein and daidzein. Genistein has a much higher binding affinity to ERβ than daidzein, which is due to a decrease in polarity of the ester functionality caused by interactions between the proximal hydroxyl group and the carbonyl group (135). The effect of isoflavones on ERs are similar to SERMs, and they show agonistic or antagonistic effect depending on the concentration of endogenous estrogen and the type of ER (136, 137). In case of a high level of endogenous estrogen, isoflavones bind to ERs and reduce the effect of endogenous estrogen, and thereby induce antagonistic effects. On the contrary, if there are low levels of endogenous estrogen in the body, isoflavones act as stand-ins for estrogen and exert agonistic effects (135).

It has been shown in several studies that equol producers have greater benefits of isoflavones containing diet compared to non-equol producers (104, 138). Equol binds ERβ receptors with 20% the affinity of 17β-estradiol, but is excreted in amounts 10 to 1000 times greater than endogenous estrogen depending on the diet (139). A larger

proportion of equol is found in serum in its free form compared to daidzein and estradiol (137). Only the unbound compounds can bind to ERs, and this difference means that the biological potency of equol is enhanced compared to estrogen (140). Furthermore, there are differences in the renal clearance of the different isoflavones and metabolites, and equol remains in plasma for a longer time than genistein and daidzein which further enhances its biological effect (138, 141).

The estrogenic effect of isoflavones is thought to help relieve menopausal symptoms including hot flushes. In a study 80 mg/d of Red Clover (RC) isoflavone aglycones administrated and resulted 74% reduction in menopausal symptoms (hot flush/night sweat frequency) in Austrian postmenopausal women (142). In one study sweat secretion over 24 h was measured in menopausal women suffering from menopausal symptoms. A mean reduction of 15% in hot flush frequency and 32% in intensity of hot flushes was found after daily intake of RC during 12 weeks (143).

There are also many examples of studies that either fail to show an effect or show similar affects in intervention and placebo groups. In a study where similar reductions in menopausal symptoms were seen in groups receiving 40 mg isoflavones and placebo (144). In another study the effect of 82 mg and 57 mg isoflavones supplements was investigated on the frequency of hot flushes in postmenopausal women. They found similar reductions in the intervention and placebo groups (145).

1.9.2. Effect on Osteoporosis

When menopause started in women estrogen production significantly reduced results in an imbalance in bone metabolism where the rate of bone resorption exceeds the rate of bone formation. This leads to a decrease in bone mineral density (BMD) and an increased risk of fractures and development of osteoporosis. Osteoporosis is a considerable complication in postmenopausal women.

Short-term administration of estrogens enhances bone density. Nevertheless, in light of relatively frequent vascular complications and increased risk of estrogen-dependent cancers, long-term hormonal replacement therapy is not recommended (25, 146).

Isoflavones might be able to improve the imbalance due to their estrogenic effects. *In vitro* and *in vivo* studies on rodents have shown that isoflavones reduce bone

resorption and therefore potentially could have antiosteoporotic effect (147). ERs have been found in osteoblast and osteoclast cells, and it has been shown that the action of daidzein is mediated through ERs in these cells, resulting in stimulation of osteoblasts and inhibition of osteoclasts (148).

Long-term administration of isoflavones was also found to positively affect bone metabolism (149, 150). Six-month genistein administration to postmenopausal women led to a significant increase in bone density and concurrent reduction in the concentration of biochemical markers of bone resorption (151). After twelve-month genistein administration, the increase in bone density was comparable to the effects of estrogen hormonal replacement therapy (152, 153).

Although the antiosteoporotic effect of isoflavones has some contradicting results, epidemiological and cross-sectional studies have shown a reduction in fractures and increases in BMD with increasing intake of soy products (154, 155). Some intervention studies have shown an improvement in BMD after administration of isoflavones, while others have failed to show a beneficial effect of isoflavones on BMD (156). The reason for conflicting results in the intervention studies might be differences in study designs including dosage, product form and study duration.

1.9.3. Effect on Cancers

It is known that some types of tumors, such as breast, prostate and colon cancers have a lower incidence in Asian countries compared to the population of western countries (157-160). Environmental factors appear to contribute largely to the development of these tumors (161).

In postmenopausal women, consumption of isoflavones was found to be associated with reduction of breast cancer incidence (162), and mammary gland density (163). These effects have been associated with the ability of isoflavones to increase serum sex hormone binding globulin (SHBG) concentration, thereby reducing the bioavailability of sexual hormones in hormone-dependent tissues (164).

In recent years the relationship between soy foods and breast cancer has become controversial. One study reported that the administration of isoflavones in the dose of 100 mg/day over a period of 12 months had no effect on cell proliferation of the

contra lateral unaffected breast in women who had been treated for breast cancer in the past (165). Similar result was found by Fabian *et al.* (2005) study, that isoflavones did not affect the number of a typical cells in the breast tissue (166). Due to these uncertain and contradictory findings, high isoflavones intake is not recommended in patients with known diagnosis of estrogen-dependent breast cancer (167). Isoflavones may stimulate the growth of existing estrogen-sensitive breast tumors. This controversy carries considerable public health significance because of the increasing popularity of soy foods and the commercial availability of isoflavones supplement. Overall, there is little clinical evidence to suggest that isoflavones will increase breast cancer risk in healthy women or worsen the prognosis of breast cancer patients. There is no evidence that isoflavones intake increases breast tissue density in pre- or postmenopausal women or increases breast cell proliferation in postmenopausal women with or without a history of breast cancer.

1.9.4. Effect on Diabetes

The prevalence of diabetes and metabolic syndrome is rapidly increasing worldwide. Isoflavones could potentially have a beneficial effect on these conditions by affecting lipid metabolism and glucose homeostasis. Several animal studies had shown promising effects of isoflavones on plasma lipid profiles and glucose levels. In a study with Zucker diabetic fatty rats lower plasma concentrations of total cholesterol (TC), triglycerides (TG) and free fatty acids (FFA) were seen after administration of isoflavones rich soy protein for ten weeks (168) and lower levels of fasting blood glucose were also detected throughout the treatment period (168). A meta-analysis of 38 studies had shown that soy proteins reduced serum concentrations of TC (9%), Low-density lipoprotein (LDL) cholesterol (13%) and TG (11%) in humans. The effect was most pronounced in hypercholesterolemic subjects (169). Another study was conducted with soy germ-enriched pasta which was rich in isoflavone aglycones and intake of this pasta was compared with conventional pasta in hypercholesterolemic adults and showed reductions in LDL cholesterol (170). It is known that estrogen has an impact on lipid profiles, and the positive effects of isoflavones might be due to their estrogenic properties. There are however also studies that failed to show an effect of isoflavones on the lipid profile (171, 172).

Contradictions were also found in the effect of isoflavones on glucose metabolism. A meta-analysis of 10 intervention studies showed that daily ingestion of isoflavones mixture did not reduce serum levels of glucose in non-Asian women (173). In another it was showed that soy protein failed to control the glycemic status after 8 weeks intervention in type 2 diabetics who did not manage their disease with medication (174).

1.9.5. Effect on Cardiovascular disease

The metabolic syndrome is associated with an increased risk of coronary heart disease and cardiovascular mortality and additionally increasing age and menopause is also associated with an increased risk of cardiovascular diseases (CVD) (175). Estrogen deficiency leads to unfavorable changes in lipid metabolism and an increase in serum cholesterol levels, which are risk factors for developing CVD.

CVD represent the main cause of death in western countries. In Asia, the incidence of the diseases is about eight times lower than in western countries (176). Besides hereditary factors, the disproportion in the incidence of cardiovascular diseases is assumed to be caused by nutritional factors. Soy is an important component of food in eastern countries so that a long-term intake of isoflavones by this population is high. This suggests that isoflavones may exert a protective effect on the cardiovascular system (160, 177-179).

Soy isoflavones or their metabolites were found to improve endothelial function as well as to decrease blood pressure and arterial stiffness (180-183). Reduction of blood pressure was reported in normotensive and hypertensive women in association with soybean consumption (184, 185). A diet with high intake of soy was reported to decrease plasma triglyceride levels, total cholesterol, LDL cholesterol and increase HDL cholesterol (180, 186).

1.9.6. Effect on Central Nervous System

The beneficial effects of the isoflavones have been reported on cognitive functions in human (187, 188). Long-term administration of soy or preparations of isolated isoflavones had found to improve in learning, logical thinking and planning ability in postmenopausal women (189-191). Regular consumption of soy in both sexes

reported compromised cognition and accelerated brain atrophy (192). Epidemiological studies point to lower rates of dementia in Asian population (193, 194) who consumed soy regularly.

Thus, in terms of both health promotion and chronic disease prevention, the potential public health impact of daily soy consumption could be important, especially in postmenopausal women.

The consumption of isoflavones vary around the world; in Japan, Taiwan and Korea, where various products made of soy are a typical part of the everyday diet, people are estimated to consume 20–80 mg of isoflavones per day (61, 195) and Europe/USA has been estimated to be 0.5–3 mg/day (61). Soybean are the richest source of isoflavones and the dietary intake of soy isoflavones in South-East Asia is not so popular. In this region, people mainly get isoflavones from mung and masoor dals. Although, dietary intakes of isoflavones from soy foods [range from 15-50 mg/d] (62, 63, 196) in Asian people were significantly lower than doses being used in clinical studies (101, 197, 198).

A baseline survey was conducted in the BIRDEM (199) for determining the quantitative and qualitative intake of phytoestrogens in Bangladeshi menopausal women and its relation to clinical outcome. In that study, the phytoestrogens content of food (like wheat, rice, fruits, beans, cabbage, onion, garlic, potato, tomato etc) were based on only literature. Another study (200) was carried out for assessing the association of phytoestrogens and risk markers of CVD in Bangladeshi postmenopausal women and the dietary intake of phytoestrogens by menopausal women was calculated by a specific food frequency questionnaire which was made using literature. In risk markers of CVD study (200) recommended that phytoestrogens containing food intake should be encouraged for reducing risk markers in postmenopausal women. In our country, a short period intervention study (201) was ran where soy-milk isoflavones (which contained 30 mg isoflavones and the contents were calculated from literature) impact was observed on homocysteine and C-reactive protein in menopause women but the result was not significant.

In the above studies, the amount of phytoestrogens and isoflavones was calculated from literature which was not so authentic due to variation in type and quantity of

phytoestrogens rich foods in different countries. In the above studies it was not possible to do the chemical analysis of phytoestrogens in our local foods because of limited resources, financial and technical support. However, no studies have been under taken to clarify effective types and forms of isoflavones, optimal intake, and individual variation in isoflavones bioavailability in the postmenopausal women of our population.

2. Objectives

The Bangladeshi post-menopausal women are not getting enough support due to poor socio-economic condition, illiteracy, ignorance and inadequate health care system. During this period many women are not well accepted in the society and family and they consider themselves as a burden. It is paradoxical that HRT is more focused in poorer countries where economic consideration itself is a great obstacle to achieve the goal. It is also difficult for menopausal women to bear the extra burden of expensive HRT therapy. Therefore, it is necessary to evaluate easily accessible food materials which contain high amounts of isoflavones. Soybean and lentils, particularly mung and masoor dals contain isoflavones. In Bangladesh, a considerable amount of soybean is being produced though the practice of soybean intake is not yet widespread among our population. However, lentils are commonly consumed by our people. In the present study, the initiative has been taken to find out the isoflavones content in locally produced soybean, mung and masoor dals and the effective usage of these inexpensive plant products as replacement of conventional HRT.

The objectives of the study are-

i. To identify the isoflavones present in soybean [*Glycine max* (L.) Merr.], mung [*Vigna radiata* (L.) R. Wilczek] and masoor dals [*Lens culinaris* (Medik.)]

ii. To quantify the isoflavones in soybean [*Glycine max* (L.) Merr], mung [*Vigna radiata* (L.) R. Wilczek] and masoor dals [*Lens culinaris* (Medik.)]

iii. To measure the bioavailability of isoflavones in Bangladeshi postmenopausal women

3. Experimental Study

3.1. Standards

Daidzein (4′,7-Dihydroxyisoflavone, $C_{15}H_{10}O_4$, MW 254.2, purity 98%), and Genistein (4′,5,7-Trihydroxyisoflavone, $C_{15}H_{10}O_5$, MW 270.2, purity 98%) were purchased from Sigma-Aldrich and were preserved at 4ºC and at - 20 ºC, respectively.

3.2. Solvents and reagents

Liquid Chromatography (LC) grade acetonitrile (ACN) and acetone were purchased from Merck, Germany. Extra pure analytical grade ethyl acetate (EtOAc), n-hexane and anhydrous sodium sulfate (Na_2SO_4) were purchased from Merck KGaA (Darmstadt, Germany). Deionized and hydrocarbon free water was used for analysis, was made from a Water purification System (BOECO, Germany).

3.3. Filtration and degassing of mobile phase

Water and ACN were filtered in a Sartorius vacuum pump device (pre-cut membrane with 0.45 µm pore size) and degassed for about half an hour by the same system before use. The sample filtration was done through PTFE (polytetrafluoro ethylene; pore side 0.22 µm) syringe filter cartridge.

3.4. Evaporation

All the evaporations were carried out under reduced pressure using rotary vacuum evaporator (Büchi; No. 517-6100-00-0) at water bath temperature not exceeding 40^0C.

3.5. Instruments

Grinder machine (locally made), freeze-dryer (Hetosicc CD 52 Heto Lab Equipment, Danmark) were used for grinding and drying small amount of water and trace amount of organic solvents. All ground joint glass apparatus were purchased from Pyrex, UK.

Balance (Adam Equipment), power sonic 610 (Hwashin Technology Company, Korea), Vaccmaster (Alltech, Brazil), micropipette (100-1000 µL; Tharmo scientific, Sweden) and cannula (Romsons, India) were used in different phase of the studies.

C18 solid-phase extraction (SPE) cartridge (Phenomenex, Macclesfield, UK) was used for sample clean-up.

3.6. Liquid Chromatography (LC)

For all Chromatographic analysis, a Shimadzu SCL 10A *vp* LC system (Shimadzu, Kyoto, Japan) equipped with PDA detector (SPD-10A *vp*) having high pressure binary pump were attached. A Rheodyne injector, (loop size 20 µL), column was at ambient temperature was used for analysis.

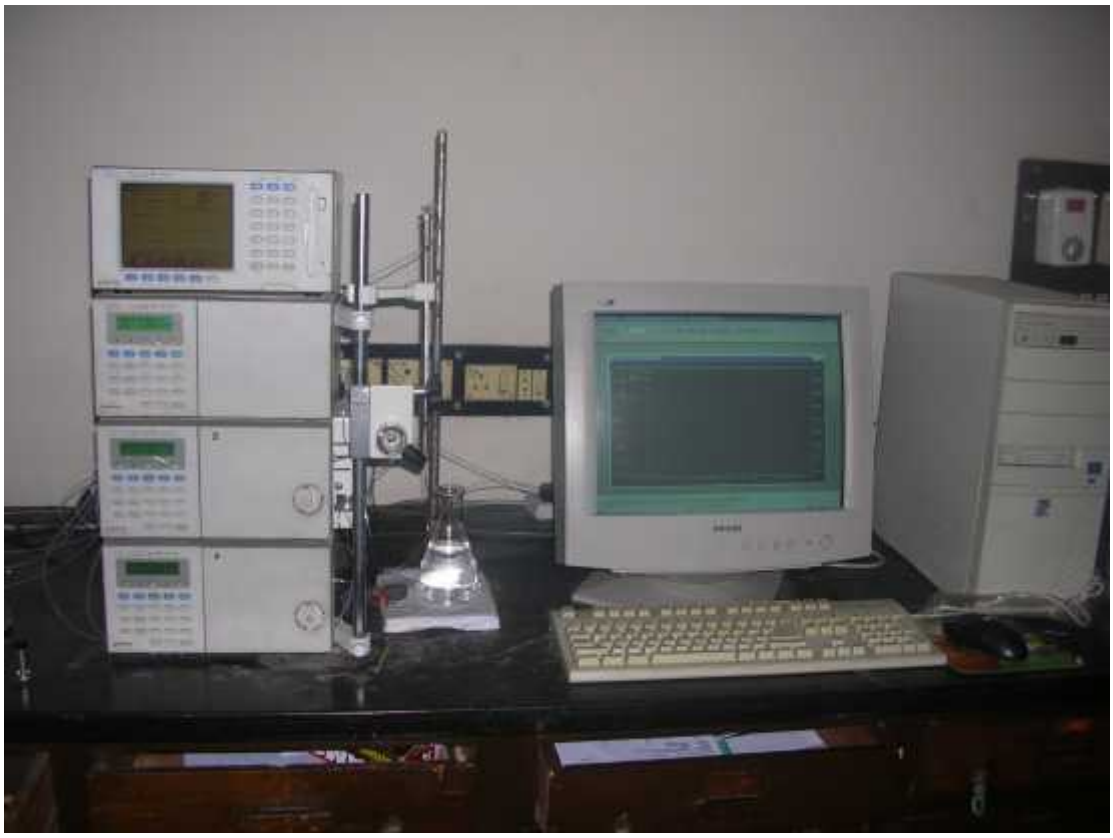


Figure 7: LC Shimadzu *Class* 10 *vp*

3.7. Analysis by LC-PDA

Liquid Chromatographic (LC) analyses were performed on a Supelco discovery reversed phase C18 column (25 cm × 4.6 mm *i.d.* particle size: 5 µm, 20 µL loop size sample). Separations were performed at ambient temperature. Standards and cleaned extracts (100 µL) were injected through a Rheodyne injector. Separations were carried at 268 nm using an isocratic mobile phase of acetonitrile and water (ACN: H_2O) (75: 25) with a flow rate of 0.5 mL/min and running time was 10 minutes.

3.8. Collection of samples

Soybean seeds were collected from Jessore, the Southern agricultural area of Bangladesh. Mung and masoor dals were purchased from a local supermarket of Dhaka city. Dry soybean, mung and masoor dals were dried again, ground into powder (200 mesh) by grinder machine and kept in air-tight containers in a refrigerator until analysis was carried out.

3.9. Extraction

3.9.1. Extraction and cleaned up of soybean sample

Soybean sample was dried in an oven at 105ºC and ground into powder. The dried powder (15 g) was extracted with n-hexane (150 mL x 3; 30 min in each) in refluxing condition. The sample was filtered by decantation followed by filtration under vacuum. The organic phase was discarded and the residue was dried at room temperature.

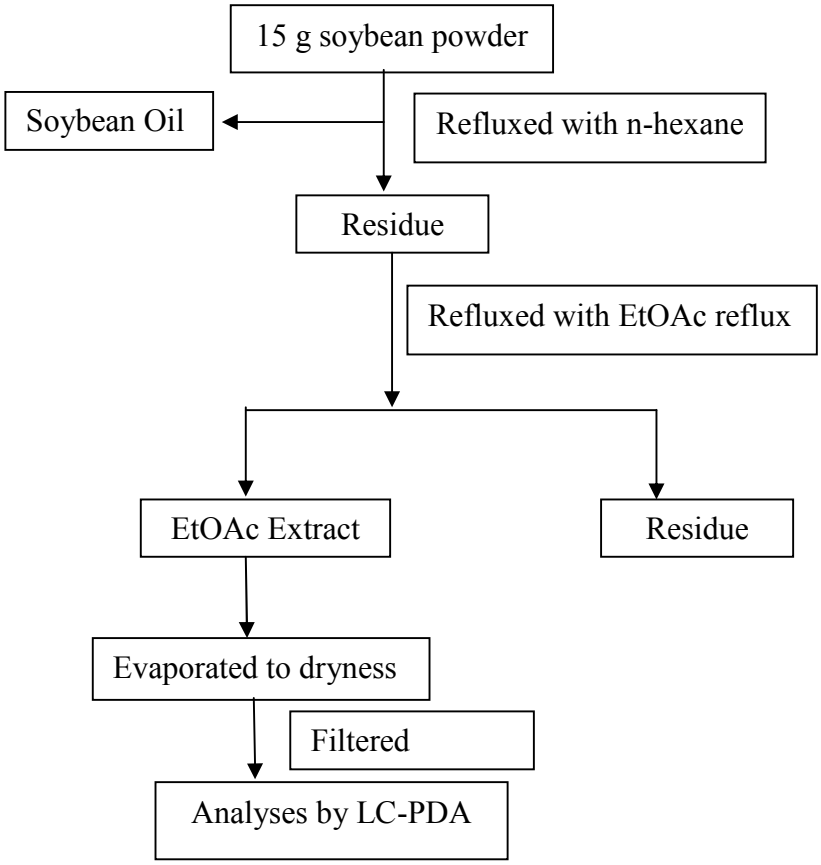
The hexane extract free residue was further extracted with ethyl acetate (100 mL x 3; boiling water bath; 30 min) and filtered. The ethyl acetate extract part was treated with anhydrous magnesium sulphate, evaporated to dry mass and re-dissolved in LC grade ACN (Scheme 1). The residue was discarded.

The extract in ACN was filtered through LC sample filter having pore size 0.22 µM, transferred into a sample vial of 2 mL size and analysis by LC-PDA.

3.9.2. Analysis of cleaned extract by LC-PDA

LC system was conditioned by passing mobile phase until smooth base line was obtained. Certified Standards of daidzein and genstein of 10 µg/mL level were

injected separately and retention time of the two isoflavones were found to be 5.52 and 6.03 min, respectively. Chromatogram of daidzein and genistein are given in Fig No. 8 and 9.



Scheme 1: Extraction of isoflavones from soybean

3.9.3. Identification of isoflavones in soybean extract

Clean extract of soybean was injected, two peaks were found to be at 5.5 and 5.9 min, respectively.

3.9.4. Preparation of soy-milk

Whole soybean (100 g) was immersed in drinking water in a pot for 4-5 hours. The soft beans (water socked) were washed with water, blended into mould by a kitchen blender, 500 mL water was added to the mould, boiled for 3 min with stirring by a wooden kitchen stirrer. The milk was collected by squeezing through pre-cleaned cloth filter. The prepared milk was boiled again for 20 min with stirring to reduce the volume ~350 mL. The milk was cooled and kept in a refrigerator before use.

The prepared milk was transferred into four different volumetric ground joint flasks (500 mL), frozen in a methanol freezer and dried into powder by a freeze-dryer.

3.9.5. Extraction and cleaned up of soy-milk sample

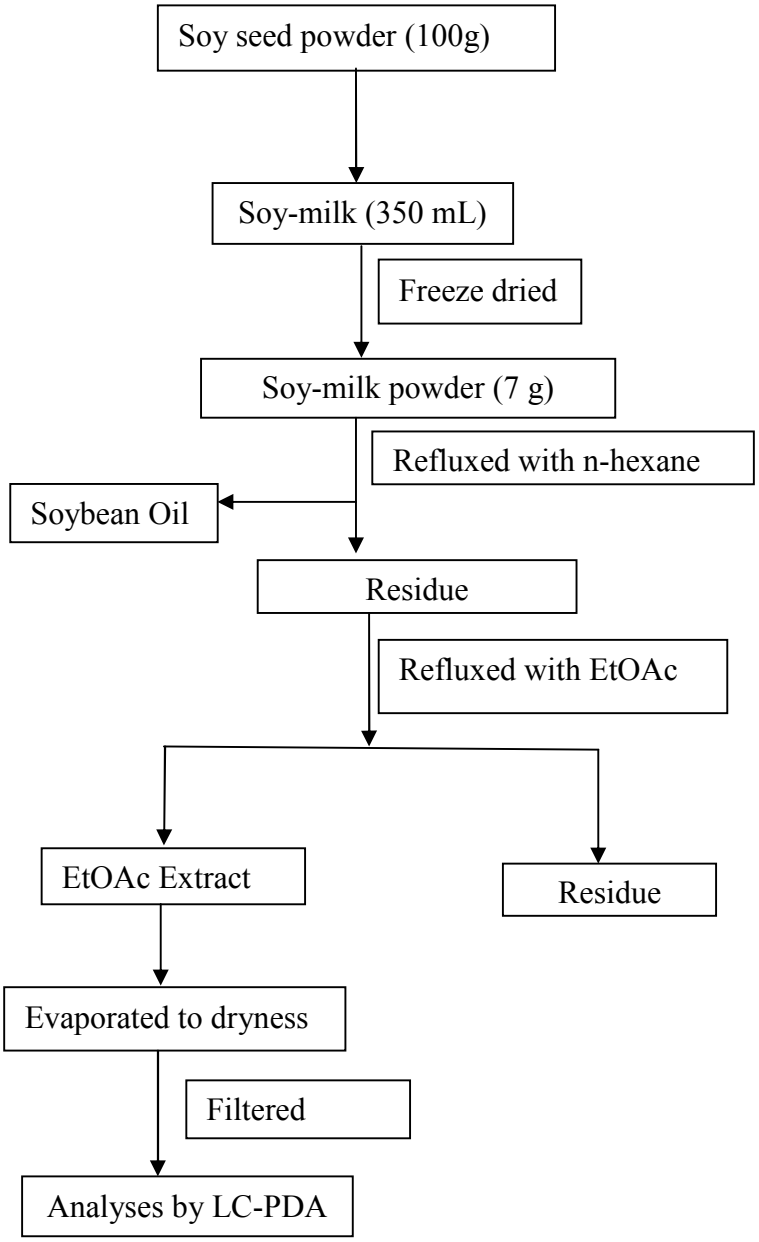
Soy-milk powder (7 g) was transferred into a round bottomed flask and refluxed with n-hexane (200 mL x 3; 30 min) in a boiling water bath. The extract was filtered and discarded. The oil free soy-milk powder was air dried.

The hexane extract free residue was further extracted with ethyl acetate (100 mL x 3; boiling water bath; 30 min) and filtered. The ethyl acetate extract was treated with anhydrous magnesium sulphate, filtered, the filtrated was evaporated into dry mass and re-dissolved in LC grade ACN (Scheme 2). The residue was discarded.

The extract in ACN was filtered through LC sample filter having pore size 0.22 μM, transferred into a sample vial of 2 mL size and analyzed by LC-PDA.

3.9.6. Analysis of cleaned extract by LC-PDA

LC system was conditioned by passing mobile phase until smooth base line was obtained. Certified Standards of daidzein and genistein (10 μg/mL) were injected separately and retention time of the two isoflavones were found to be 5.52 and 6.03 min, respectively.



Scheme 2: Extraction of isoflavones from soy-milk

3.9.7. Identification of isoflavones in soy-milk powder

Clean extract of soy-milk was injected, two peaks were found to be at 5.5 and 5.9 min, respectively. Presence of daidzein and genistein were found in the soy milk powder. Chromatogram of the cleaned extract of soy-milk powder in given in Fig No. 10.

3.9.8. Extraction and cleaned up of masoor dal powder sample

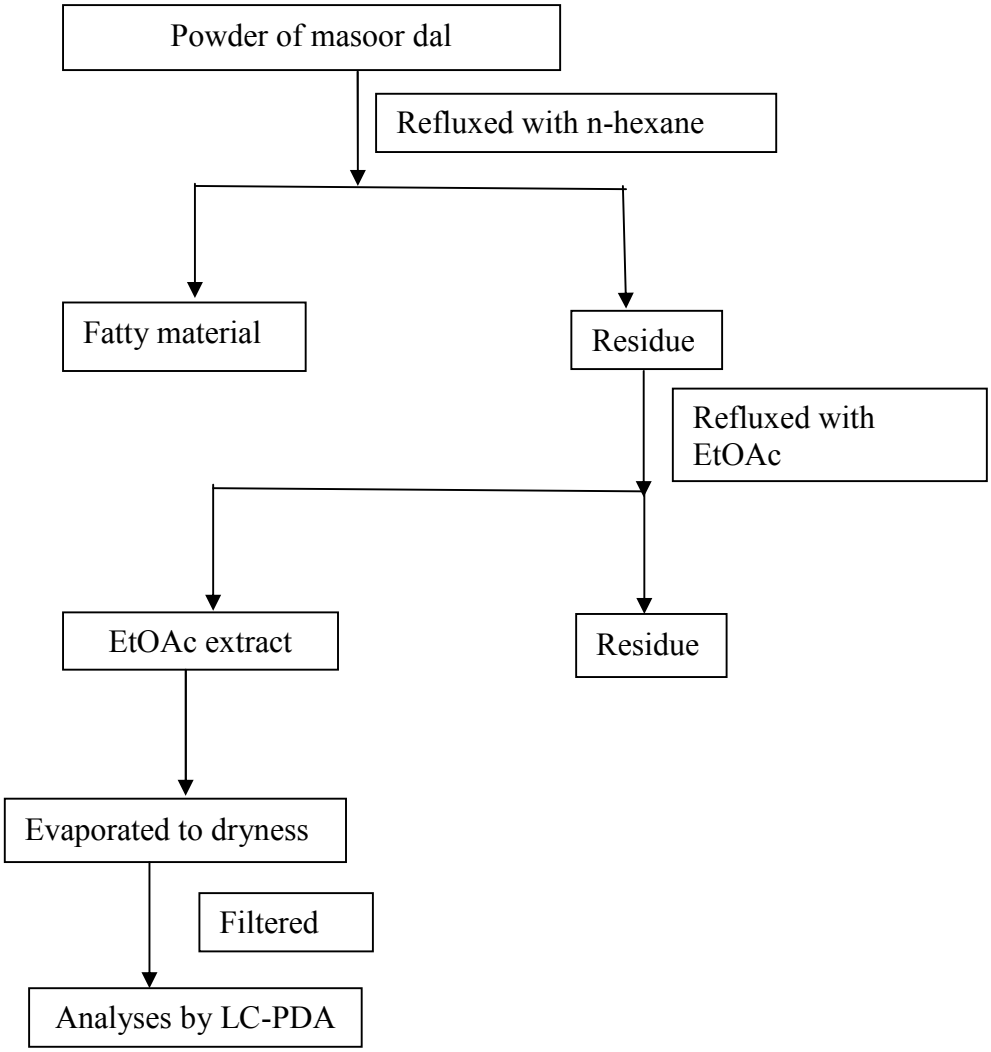
Masoor dal sample was dried in an oven at 105ºC and ground into powder. The dried powder (25 g) was extracted with n-hexane (200 mL x 1; 30 min) in refluxing condition. The sample was filtered by decantation followed by filtration under vacuum. The organic phase was discarded and the residue was dried at room temperature.

The hexane extract free residue was further extracted ethyl acetate (100 mL x 3; boiling water bath; 30 min) and filtered. The ethyl acetate extract was treated with anhydrous magnesium sulphate, filtered, the filtrated was evaporated to dry mass and re-dissolved in LC grade ACN (Scheme 3). The residue was discarded.

The extract in acetonitrile was filtered through LC sample filter having pore size 0.22 μM, transferred into a sample vial of 2 mL size and analyzed by LC-PDA.

3.9.9. Analysis of cleaned extract by LC-PDA

LC system was conditioned by passing mobile phase until smooth base line was obtained. Certified Standards of daidzein and genistein (10 μg/mL) level were injected separately and retention time of the two isoflavones were found to be 5.52 and 6.03 min, respectively.



Scheme 3: Extraction of isoflavones from masoor dal powder

3.9.10. Identification of isoflavones in masoor dal extract

Clean extract of masoor dal was injected, two peaks were found to be at 5.5 and 5.9 min, respectively. Daidzein and genistein were found to be present in masoor dal. Chromatogram of masoor dal extract is given in Fig No. 11.

3.9.11. Extraction and cleaned up of mung dal powder sample

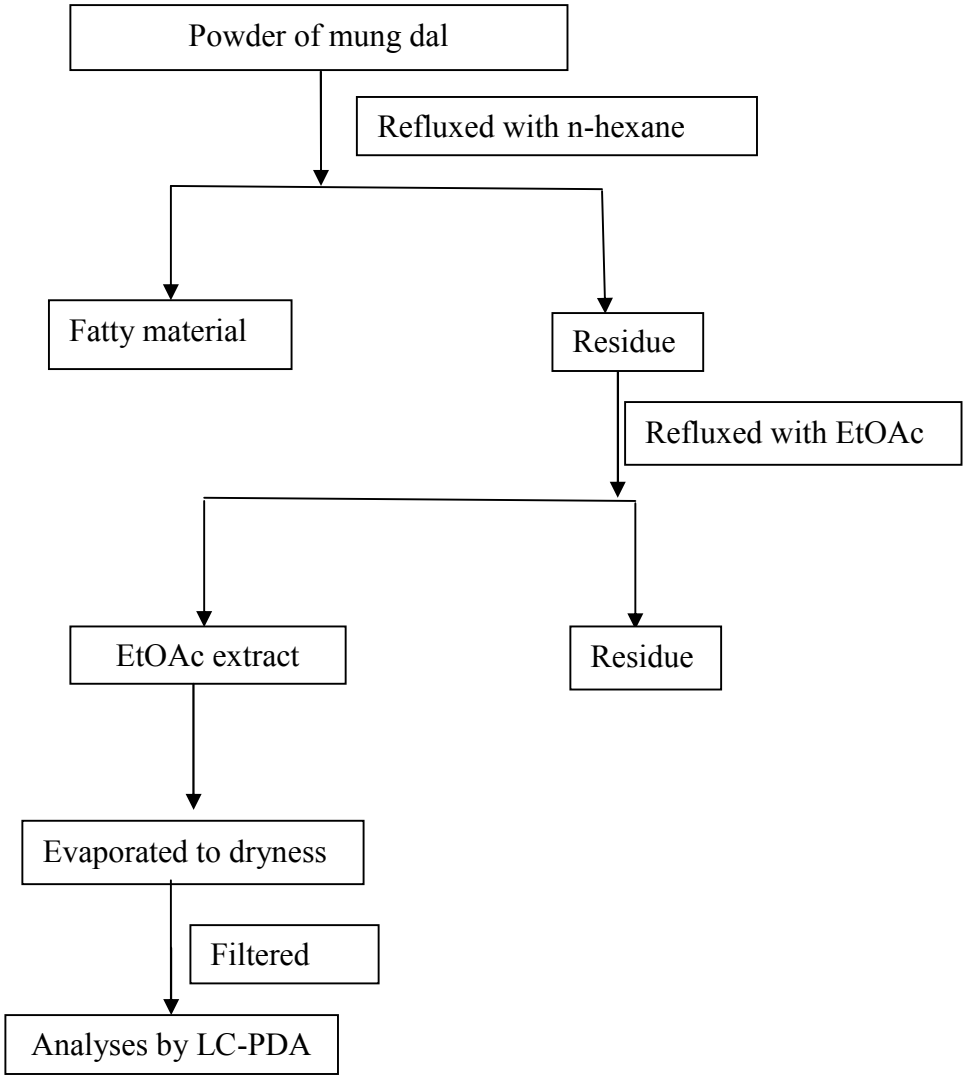
Mung dal sample was dried in an oven at 105ºC and ground into powder. The dried powder (25 g) was extracted with n-hexane (200 mL x 1; 30 min) in refluxing condition. The sample was filtered by decantation followed by filtration under vacuum. The organic phase was discarded and the residue was dried at room temperature.

The hexane extract free residue was further extracted ethyl acetate (100 mL x 3; boiling water bath; 30 min) and filtered. The ethyl acetate extract was treated with anhydrous magnesium sulphate, filtered, the filtered was evaporated to dry mass and re-dissolved in LC grade ACN (Scheme 4). The residue was discarded.

The extract in acetonitrile was filtered through LC sample filter having pore size 0.22 μM, transferred into a sample vial of 2 mL size and analysis by LC-PDA.

3.9.12. Analysis of cleaned extract by LC-PDA

LC system was conditioned by passing mobile phase until smooth base line was obtained. Certified Standards of daidzein and genistein (10 μg/mL) level were injected separately and retention time of the two isoflavones were found to be 5.52 and 6.03 min, respectively.



Scheme 4: Extraction of isoflavones from mung dal powder

3.9.13.Identification of isoflavones in mung dal extract

Clean extract of masoor dal was injected, two peaks were found to be at 5.6 and 5.9 min, respectively. Daidzein and genistein were found to be present in mung dal. Chromatogram of mung dal extract is given in Fig No. 12.

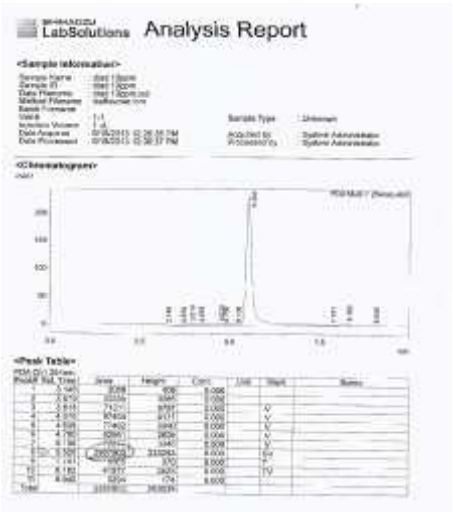


Figure 8: Chromatogram of daidzein

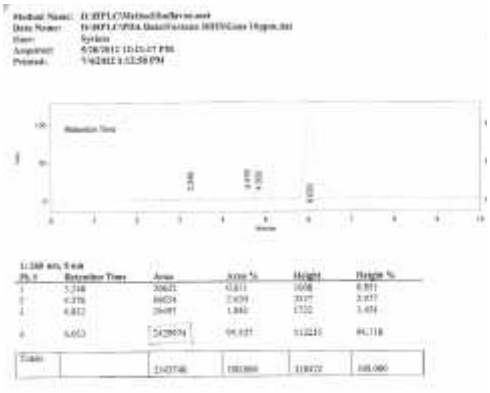


Figure 9: Chromatogram of genstein

Figure 10: Chromatogram of soy-milk

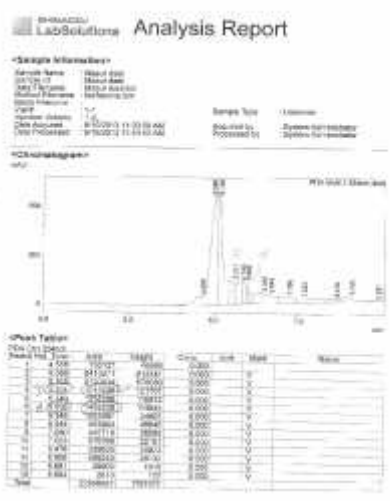


Figure 11: Chromatogram of masoor dal

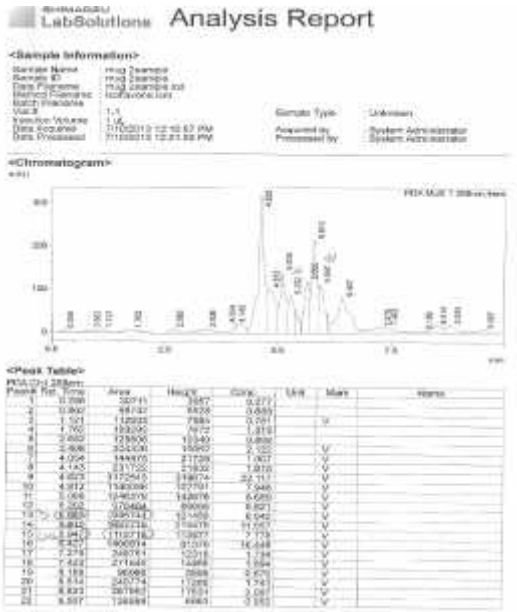


Figure 11: Chromatogram of mung dal

3.9.14.Preparation of primary standard solution

The primary standard solutions (200 µg/g) were prepared by dissolving separately certified daidzein (0.002 g) and genstein (0.002 g) in LC grade ACN (10 mL). The prepared solutions were labeled indicating the name of the standard, concentration, solvent and the date of preparation. The meniscuses of the solutions were marked with permanent ink. These primary standard solutions were stored in a freezer at -20°C.

3.9.15.Preparation of secondary standard and working standard solutions

The primary standard solutions were taken from the freezer to reach room temperature and checked the meniscus of the layer. Then 1.0 mL of solutions were diluted with 9.0 mL of respective solvent in 10 mL volumetric flask to make 20 µg/mL secondary standard solutions. These solutions were labeled indicating substance, concentration and the date of preparation and finally the meniscuses of the solutions were marked with permanent ink and saved in the freezer. The secondary standard solutions were diluted with appropriate amount of respective solvent to get working standard solutions of respective standard to make calibration curve.

3.9.16.Limit of Detection (LOD) and Limit of Quantification (LOQ)

Serially diluted standard solution of 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 µg/mL for daidzein and serially diluted standard solution of 0.00225, 0.0045, 0.009, 0.018, 0.036 and 0.072 µg/mL for genistein were injected gradually and LOD was found to be 0.25 and 0.0045 for daidzein and genistein respectively.

LOQ (Signal/Noise ratio; 10:1) was found to be 0.75 and 0.0135 for daidzein and genistein, respectively.

3.9.17.Preparation of calibration curve

The standard solution of 0, 5, 10, 15, 20 and 25 µg/mL were injected to LC-PDA. From the six chromatograms of the solutions of daidzein and genistein two calibration curve were made by plotting area vs. concentration (µg/g). The calibration curve were given below in Fig No. 13 and 14.

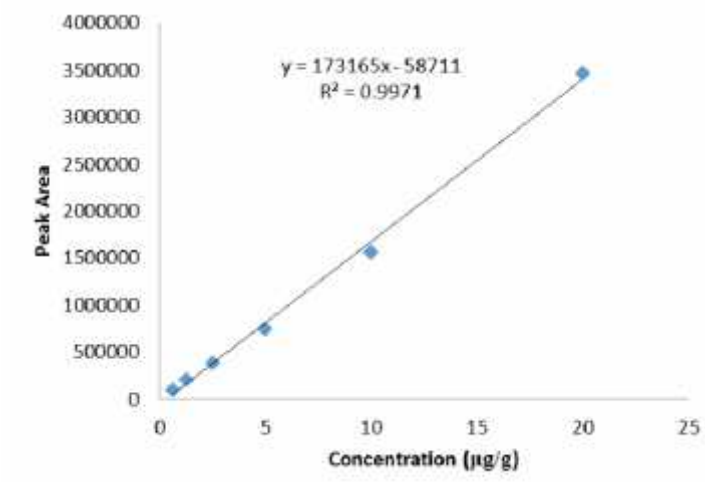


Figure 13: Calibration curve of standard Daidzein

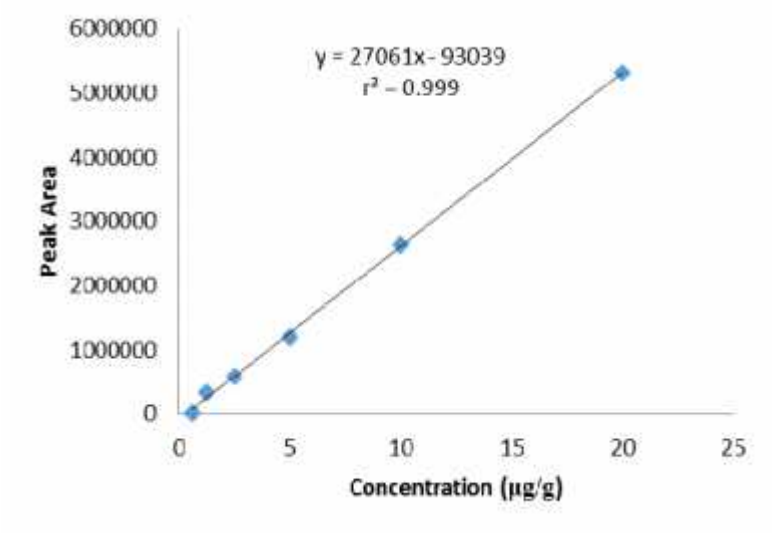


Figure 14: Calibration curve of standard Genistein

3.9.18. Quantitation of isoflavones in soybean extract

Quantitation of daidzein and genistein were done with respect to external calibration curve of daidzein and genistein (Figure 13 & 14). Amount of daidzein and genistein were found to be 1.58 µg/100 g and 1.75 µg/100 g in the soybean.

3.9.19. Quantification of isoflavones in soy-milk powder

Quantitation of daidzein and genistein were done with respect to external calibration curve of daidzein and genistein (Figure 13 & 14). Amount of daidzein and genistein was given in the Table 3.

3.9.20. Analysis of cleaned soy-milk extract by LC/MS-MS

The cleaned of soy-milk powder extract was analysed by liquid chromatography-mass spectrometry using Shimadzu LCMS-8050 with electrospray ionization (ESI), a triple quadrupole (QQQ) mass analyzer. Nebulizing and collision gas was N_2. Separations were performed on a Shim-pack GISS C_{18} column (250 x 4.6 mm i.d.; particle size 5 µm). The carrier gas pipe was 5 m. The molecular mass of diadzein is 254.2 g mol^{-1}. Multiple–reaction monitoring (MRM) measurement was conducted by ESI using positive mode. The major fragment ions were observed at 186, 253, and 339, respectively.

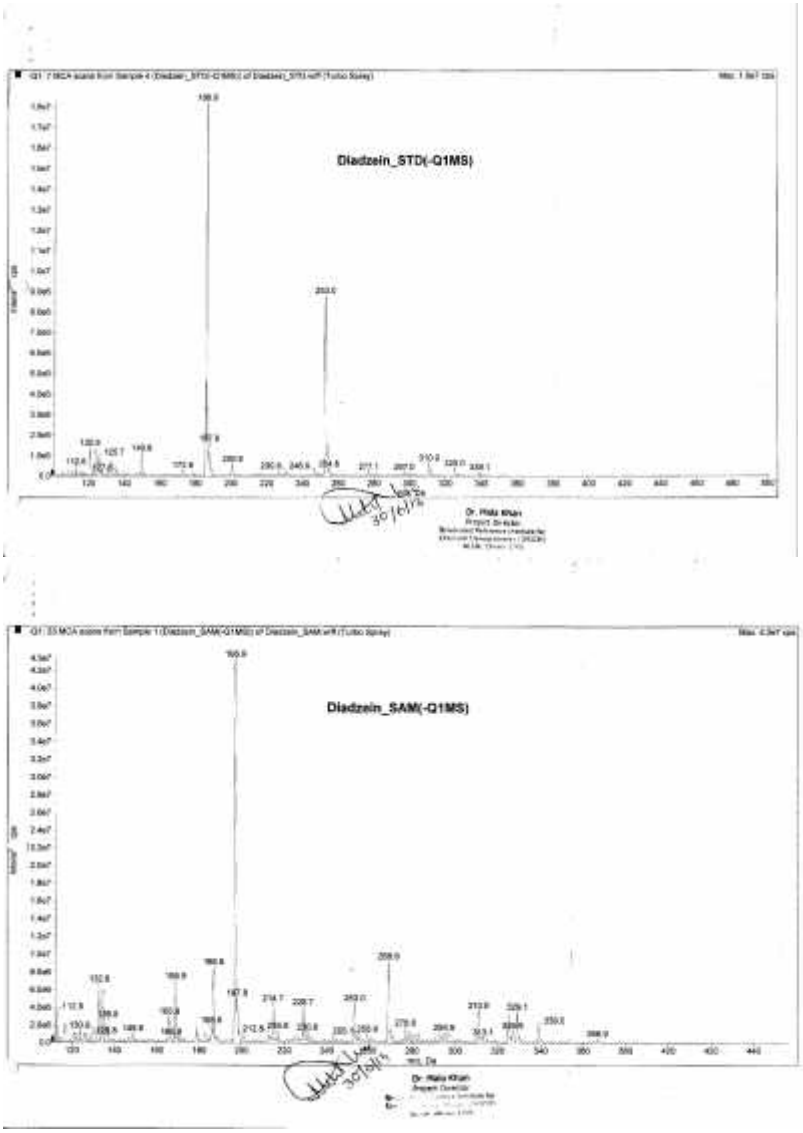


Figure 15: LC-MS/MS Spectra of standard samples and analytes

3.9.21. Quantitation of isoflavones in masoor dal extract

Quantitation of daidzein and genistein were done with respect to external calibration curve of daidzein and genistein (Figure 13 & 14). Amount of daidzein and genistein were given in the Table 3.

3.9.22. Quantitation of isoflavones in mung dal extract

Quantitation of daidzein and genistein were done with respect to external calibration curve of daidzein and genistein (Figure 13 & 14). Amount of daidzein and genistein found were given in the Table 3.

For quantification, concentration of the corresponding analyte was found out from standard calibration curve taking into consideration that the peak area was in the midpoint of the curve (considering linearity of the curve). Amount of unknown analytes in the respective samples were found out using the following formula:

$$\text{Amount of unknown sample} = \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Area}_{\text{Std}} \times \text{Conc.}_{\text{Matrix}}}$$

3.9.23. Recovery Experiments

The soy-milk powder (3 g) was taken inn a Teflon tube, daidzein (10 µg/mL) was added to the powder, shaken for 30 sec, kept at room temperature for 2 hour. The spiked sample and un-spiked (control) were extracted following the same extraction procedure as of soy-milk powder (Scheme 2). The following formula was used for recovery experiments.

$$R = \frac{A_m \times C_{st}}{A_{st} \times C_m} \times \frac{100}{M_{st}}$$

Where R is the recovery (%), A_m is the peak area of the analyte in the matrix, A_{st} is the peak area of the analyte in the standard, C_m is the concentration of the analyte in the matrix, C_{st} is the concentration of the analyte in the standard, and M_{st} is the mass of the analyte in the standard.

3.10. Selection of postmenopausal women

A total of 18 healthy postmenopausal women were selected for nearly 45 days study including 3 times hospitalization 2 days in each time. Among them two women dropped out due to their difficulties to be involved for 45 days study. Postmenopausal women were chosen who has natural menopause or due to surgery for last 2 years and aged between 45 to 60 years. The women who had pre-existing chronic renal, liver, pulmonary or cardiovascular disease were not within the study group. They should not have antibiotics within the preceding 3 months periods and were taking oral contraceptives or hormone replacement therapy.

3.10.1. Serving of soy-milk

Food chart was given to every woman and advised to avoid foods containing isoflavones (such as mung, masoor dals, soybean, raw garlic, green bean, potatoes, sweet potatoes, nuts, chickpeas, wheat flour, grapefruit, dates, egg and nut) at least for 1 week before and during the study. After an overnight fast, 350 mL of soy-milk was given to every individual woman as a single bolus. Soy-milk was made from 100 g of soybean. The same batch of soybean was used throughout the study.

3.10.2. Bioavailability of daidzein and genistein in soy-milk

Sixteen postmenopausal women were admitted to Bangladesh Institute of Health Sciences (BIHS) hospital for 2 days and 3 nights. Blood sample was collected from each woman in the morning before breakfast. Then freshly prepared soy-milk (~350 mL) was served to each woman. Blood samples were collected from each woman at 2, 4, 6, 8, 24, 36 and 48 hr (8 times including 0 time) and total one hundred twenty eight (8x16=128) blood samples were collected from 16 postmenopausal women. The blood samples were centrifuged at 1200 rpm and the serum (~2 mL) separated and immediately frozen at -20°C. After 2 days and 3 nights the women were discharged from BIHS hospital for home.

3.10.3. Preparation of mung and masoor dals soup

Whole mung and masoor dals (100 g) were washed with clean water, 500 mL water and small amount of salt (NaCl) turmeric powder were added to it, boiled for 25 min, with stirring by a wooden kitchen stirrer to reduce the volume ~350 mL.

3.10.4.Serving of masoor dal soup

Food chart was given to every woman and advised to avoid foods containing isoflavones (such as mung, masoor dals, soybean, raw garlic, green bean, potatoes, sweet potatoes, nuts, chickpeas, wheat flour, grapefruit, dates, egg and nut) at least for 1 week before and during the study. After an overnight fast, 350 mL of masoor dal soup was given to every individual woman as a single bolus. Masoor dal soup was made from 100 g each of masoor dal powder. The same batch of masoor dal was used throughout the study.

3.10.5.Bioavailability of daidzein and genistein in masoor dal soup

Sixteen postmenopausal women were admitted to Bangladesh Institute of Health Sciences (BIHS) hospital for 2 days and 3 nights. Blood sample was collected from each woman in the morning before breakfast. Then freshly prepared masoor dal soup (~350 mL) was served to each woman. Blood samples were collected from each woman at 2, 4, 6, 8, 24, 36 and 48 hr (8 times including 0 time) and total one hundred twenty eight (8x16=128) blood samples were collected from 16 postmenopausal women. The blood samples were centrifuged at 1200 rpm and the serum (~2 mL) separated and immediately frozen at -20°C. After 2 days and 3 nights the women were discharged from BIHS hospital for home.

3.10.6.Serving of mung dal soup

Food chart was given to every woman and advised to avoid foods containing isoflavones (such as mung, masoor dals, soybean, raw garlic, green bean, potatoes, sweet potatoes, nuts, chickpeas, wheat flour, grapefruit, dates, egg and nut) at least for 1 week before and during the study. After an overnight fast, 350 mL of mung dal soup was given to every individual woman as a single bolus. Mung, dal soups was made

from 100 g each of mung dal powder. The same batch of mung dal was used throughout the study.

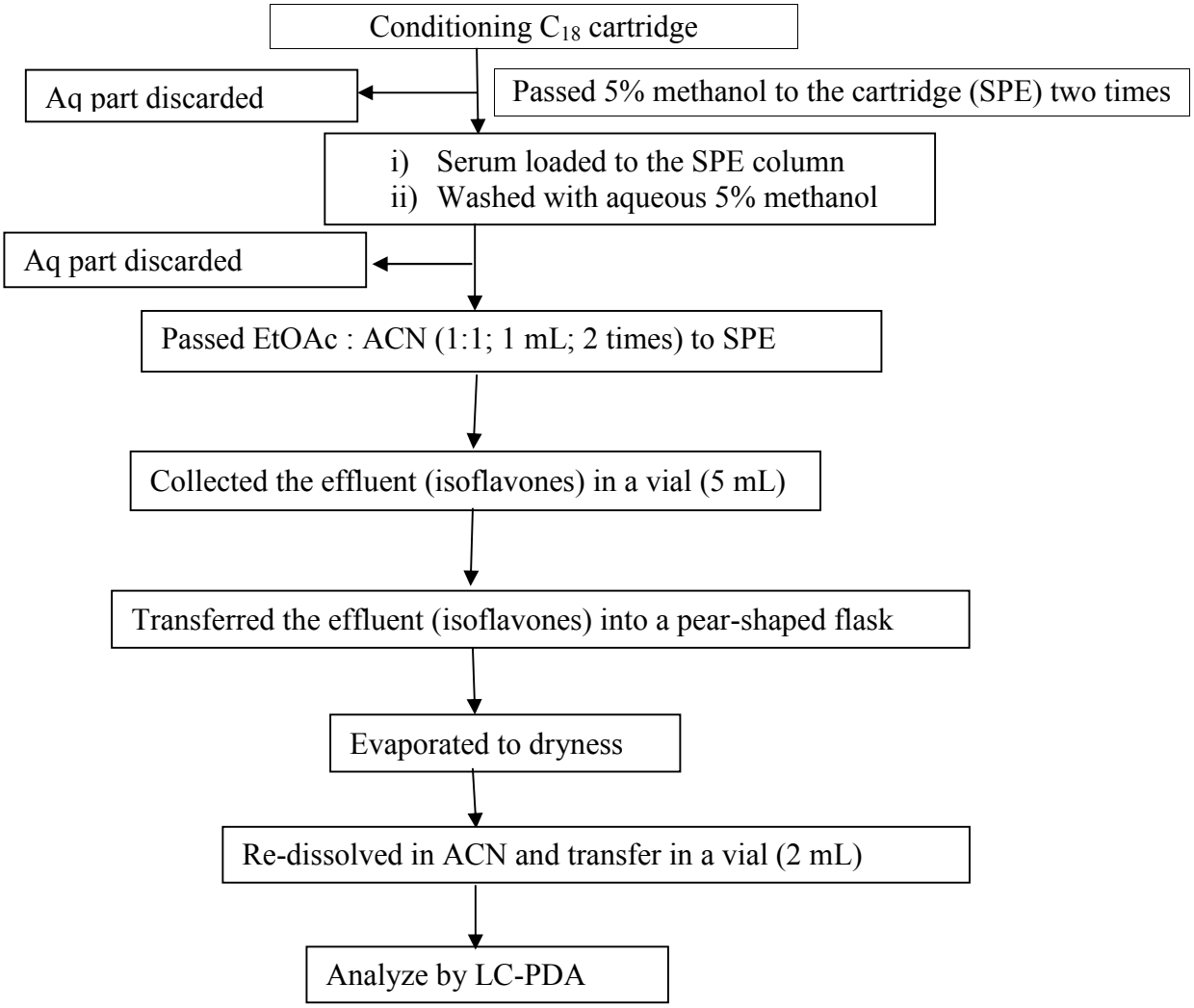
3.10.7.Bioavailability of daidzein and genistein in mung dal soup

Sixteen postmenopausal women were admitted to Bangladesh Institute of Health Sciences (BIHS) hospital for 2 days and 3 nights. Blood sample was collected from each woman in the morning before breakfast. Then freshly prepared mung dal soup (~350 mL) was served to each woman. Blood samples were collected from each woman at 0, 2, 4, 6, 8, 24, 36 and 48 hr (8 times including 0 time) and total one hundred twenty eight (8x16=128) blood samples were collected from 16 postmenopausal women. The blood samples were centrifuged at 1200 rpm and the serum (~2 mL) separated and immediately frozen at -20°C. After 2 days and 3 nights the women were discharged from BIHS hospital for home.

3.10.8.Isoflavones extraction from human serum

Isoflavones were extracted from the defrosted serum and sample was cleaned up using solid-phase extraction (SPE C18 Cartridge). The cartridge was conditioned with water (1 mL x 3) followed methanol (1 mL x 3) and then water again.

Serum sample was thawed and was passed through the conditioned SPE cartridge, then aqueous 5% methanol (800 µL).The isoflavones were eluted in ethyl acetate-acetonitrile mixture (1:1; 400 µL x 2) (Scheme 5).



Scheme 5: Extraction of biological sample (serum)

3.10.9.Quantitation of daidzein and genistein in human serum samples

Sixty four serum samples were cleaned from 16 women fed soy milk, similarly 64 for mung and 64 for masoor dal soups. Total numbers of serum sample became one hundred ninety two (64 x 3 =192).

The cleaned serum samples were filtered through LC samples filter (0.22 µm pore size), and analyzed by LC-PDA. Chromatograms of the samples are given below (Fig No. 16-24**).**

Amount of isoflavones were calculated using external calibration curve of certified standard of daidzein and genistein. Quantitation formula is given below.

$$\text{Amount of unknown sample} = \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Area}_{\text{Std.}} \times \text{Conc.}_{\text{Matrix}}}$$

Figure 16: Chromatogram of the serum sample of one subject at 4 h after consuming soy-milk

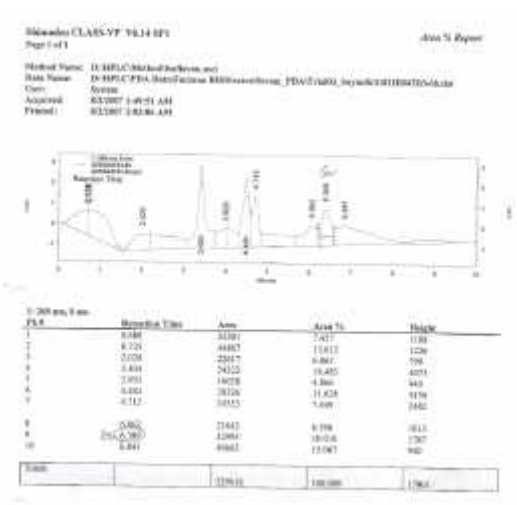


Figure 17: Chromatogram of the serum sample of one subject at 6 h after consuming soy-milk

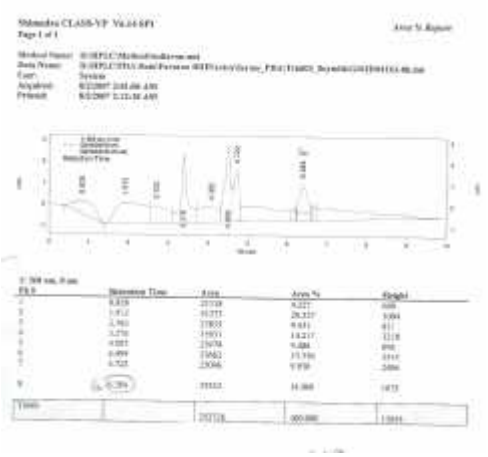


Figure 18: Chromatogram of the serum sample of one subject at 8 h after consuming soy-milk

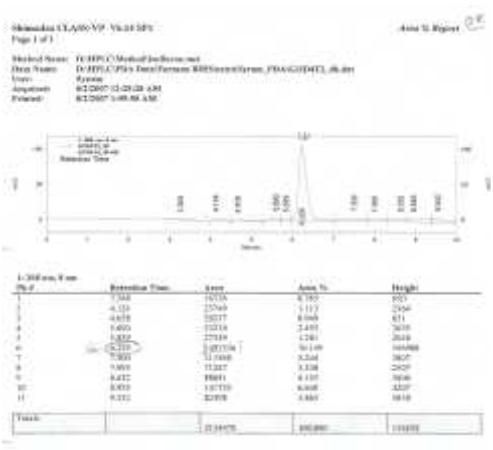


Figure 19: Chromatogram of the serum sample of one subject at 4 h after consuming masoor dal soup

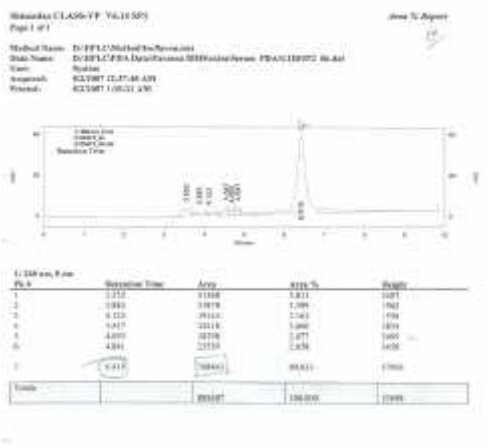


Figure 20: Chromatogram of the serum sample of one subject at 6 h after consuming masoor dal soup

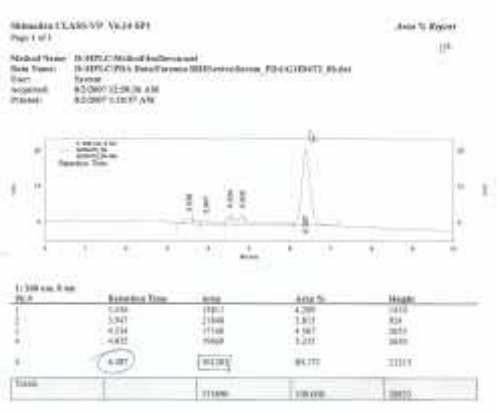


Figure 21: Chromatogram of the serum sample of one subject at 8 h after consuming masoor dal soup

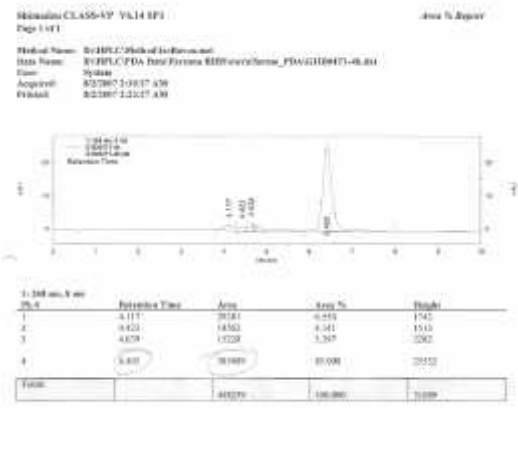


Figure 22: Chromatogram of the serum sample of one subject at 4 h after consuming mung dal soup

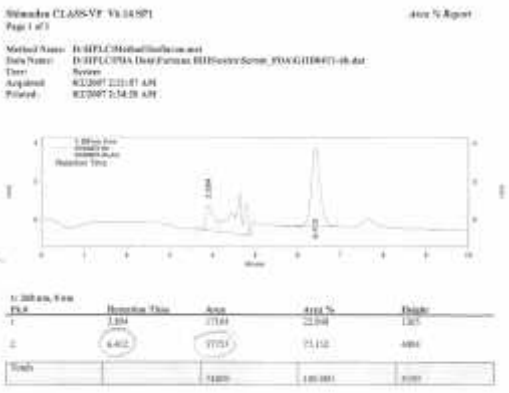


Figure 23: Chromatogram of the serum sample of one subject at 6 h after consuming mung dal soup

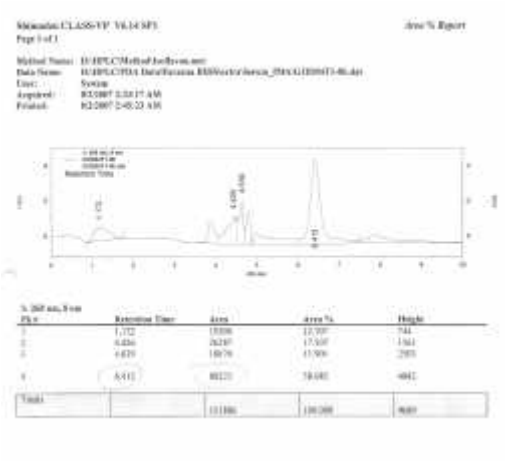


Figure 24: Chromatogram of the serum sample of one subject at 8 h after consuming mung dal soup

3.10.10. Determination of pharmacokinetics of daidzein and genistein serum samples

The daidzein and genistein serum concentration-time profiles for each individual and mean concentrations at each dose were determined employing a non-parametric estimation of AUC and C_{max}.

4. Results and Discussion

Soybean is one of the good sources of edible oil and several soy products are used as food like soy biscuits, tofu, etc. The soy sample was taken to identify and quantify the presence of isoflavones. In this study, reported method (202) was followed for the extraction of isoflavones with slight modification and validation. Soybean oils was removed before extraction of isoflavones for avoiding possible binding the oil with non-polar stationary phase of C18 LC and also get back less matrix effect. Isoflavones were identified and quantified in oil free soybean for method verification in LC-PDA system with respect to certified standard isoflavones. Later the same method was followed for soy-milk, masoor, mung dals as well as for blood samples.

Soy-milk is easy to serve and milk is very popular in the Western part of world, soy-milk was prepared from soybeans to identify and quantify isoflavones present in the soybean samples. Quantitation of flavonoids were done by LC-PDA which is more advance method than conventional methods of identification and quantitation by UV-VIS Spectrophometer which gives quantitation of total flavonoids instead of individuals (203, 204).

4.1. Limit of Detection (LOD), Limit of Quantification (LOQ) and Linearity

Limit of Detection (LOD) was determined three times of peak than background noise (S/N ratio; 3:1) and Limit of Quantification (LOQ) ten times higher than the noise (S/N ratio; 10:1). Limit of Detection (LOD), Limit of Quantification (LOQ) were found to be 0.0045 & 0.0135 μg/mL and 0.25 & 0.75 μg/mL for genistein and daidzein respectively (Table 2). The results showed that at very low level the two isoflavonoids are possible to identify and quantify by LC-PDA. Linearity was found to be satisfactory [(r^2: 0.999 and 0.997 for genistein and daidzein, respectively] which were found from the calibration curves of the two certified reference samples.

Table 2: Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Isoflavones analyzed	ƛ (nm)	r^2	LOQ (µg/mL)	LOD (µg/mL)
Genistein	268	0.999	0.0135	0.0045
Daidzein	268	0.997	0.75	0.25

Efficiency of methods was determined by recovery experiment. Recovery of daidzein and genistein were found to be 118% and 116% respectively which are within the acceptable value according to the Codex Alimentary Commission (205).

Identification of genistein and daidzein were done by comparing retention times of the certified samples and the experimental samples *i.e.* oil free soybean, soymilk and blood samples. All the analytical conditions of certified isoflavones and in three different matrix were kept same. The reference standard solutions were injected into the LC- PDA under the same condition of cleaned extract of food samples. The retention time of standard and unknown samples supposed to be same under the same analytical conditions; two peaks of the sample were same with the retention times of the standard compounds. Thus isoflavones present in the samples were identified.

4.2. Quantitation of daidzein and genistein in soy-milk

Three replicate analyses were carried out for soy-milk following same procedure as was done for identification oil free soybean. Mean and standard deviation of the samples were calculated and results are presented in Table 3. Standard deviation of the sample (2.67-4.37) was within the acceptable limit. Amount was expressed as µg per 100 g dried soy-milk. Amount of daidzein and genistein in soy-milk was found to be 36.25 µg/100 g and 43.81µg/100 g respectively. Total isoflavones was found to be 80.05 µg/100 g in soy-milk.

Soybean is being cultivated in some places of Bangladesh, still not available in market; in India stands fifth in soybean production in the world and sixth in terms of leading soybean consuming countries. The total isoflavones content in Indian soy-milk (82) were much higher than Bangladeshi produced soybean, in Korean (206), Turkey (207, 208) and in America (209), total soy-milk was also found to be higher.

Variations in contents and composition of the isoflavones occur as a consequence of different factors among which the most examined factors were the genotype of the seed, the year and location of seeding (210). Amount in cultivar's varieties is also dependent on the climatic conditions of the countries as well.

4.3. Quantitation of daidzein and genistein in masoor dal

Masoor dal and mung (green gram) belong to the family Leguminosae and the most common food stuff consumed by the Bangladeshi community. Dal is called poor mens` protein. They were also selected for the analysis of isoflavones in this study. Same method of extraction, cleanup and analysis were followed for the two matrices like soybean.

Three replicate analyses were carried out for masoor and mung dal. Mean and standard deviation of the sample was calculated and results are presented in Table 3. Standard deviation of the sample (17.89-10.68) was within the acceptable limit. Amount was expressed as μg per 100 g dried masoor dal. Amount of daidzein and genistein in masoor dal was found to be 37.66 μg/100 g and 37.33 μg/100 g respectively. Total isoflavones was found to be 74.99 μg/100 g in masoor dal. The total amount of isoflavones in masoor dal was found to be close to the total amount of isoflavones (80.05 μg/100 g) in soy-milk.

4.4. Quantitation of daidzein and genistein in mung dal

Three replicate analyses were carried out for mung dal. Mean and standard deviation of the sample was calculated and results are presented in Table 3. Standard deviation of the sample (8.51-22.87) was within the acceptable limit. Amount was expressed as μg per 100 g dried mung dal. Amount of daidzein and genistein in mung dal was found to be 27.66 μg/100 g and 44 μg/100 g respectively. Total isoflavones was found to be 71.66 μg/100 g in mung dal. The total amount of isoflavones in mung dal was also found to be close to the total amount of isoflavones (80.05 μg/100 g) in soy-milk.

Table 3: Amount of daidzein, genistein and total isoflavones in soy-milk, masoor and mung dals

Food Items	**Mean ± SD**
Soy-milk	μg/ 100 g powder
Daidzein	36.25 ± 2.67
Genistein	43.81 ± 4.37
Total isoflavones	**80.05 ± 6.87**
Masoor dal	
Daidzein	37.66 ± 17.89
Genistein	37.33 ± 10.68
Total isoflavones	**74.99 ± 28.57**
Mung dal	
Daidzein	27.66 ± 8.51
Genistein	44.00 ± 22.87
Total isoflavones	**71.66 ± 17.21**

Soybean and soymilk are not popular in Bangladesh community; it is not being cultivated in the country, nor is being imported except small amount is produced in southern part of Jessore district. Lentils are common food items among the people of Bangladesh and these are growing in the country from long time. In case of shortage, these have to be imported to meet up the need of protein source. Lentils are not well studied for their isoflavones contents except a report of *Mazur et al* (80). As masoor and mung are consumed by cooking, to follow the similar food habit condition, soup of the two dals were made and assessed the bioavailability of isoflavones in masoor and mung dal soups in women. And the bioavailability of isoflavones in dal soups were compared with the bioavailability of soy-milk isoflavones in menopause women under the same condition.

4.5. Clinical parameters of the postmenopausal women

In the present study, the bioavailability of daidzein and genistein were evaluated in the serum of 16 postmenopausal women in who belonged to middle socio-economic class and their mean age was 52.5 ±5.8 years. Other clinical parameters chosen were pulse (68.2 ±6.4 beats/min), systolic blood pressure (SBP) [116.5 ±6.7 (mmHg)],

diastolic blood pressure (DBP) [76.5 ±5.1 (mmHg)] and body mass index (BMI) [25.7 ±5.3 kg/m^2]. Volunteers' women were screened by assessing all the parameters at the Bangladesh Institute of Health Sciences (BIHS) hospital and were enrolled for the study of bioavailability.

Ethical permission was taken from the Ethical Review Committee of Bangladesh through Diabetic Association of Bangladesh (BADAS) for bioavailability studies on women.

Written consent was taken from every postmenopausal woman after full explanation of the nature of test, purpose, and potential risks of all procedures to be used for the study. Personal information of the women was kept confidential. The women were told to have free accommodation &| pathology for biological parameter determination, supply food in the hospital during admission period and small amount honorarium for their contribution in the study.

4.6. *In Vivo* quantification of isoflavones based blood samples

For bioavailability studies, a reported method was followed (211, 212) for identification and quantification of the isoflavones in the blood samples of postmenopausal women after the feeding soymilk, mung and masoor as described in the experimental part.

4.6.1. Soy-milk

Sixteen postmenopausal women were admitted at the BIHS hospital, all the parameters were checked once and found they fulfill the requirement of the biological studies. There was no daidzein and genistein found in the blood samples before serving food. Freshly prepared soy-milk (~350 mL) was served to every woman for assessing bioavailability of isoflavones. Blood samples were drawn at 0, 2, 4, 6, 8, 24, 36 and 48 hours (8 times) to find out *in-vivo* time duration of the presence of isoflavones in each of the woman. A total of 128 number of blood samples were collected in 128 different test tubes from 16 women. The freshly collected blood samples were centrifuged, and the serum of the each blood samples were stored at -20^0C to avoid biodegradation of the targeted compounds.

Samples were injected to the system with one injection of standard after each two or three injections of samples; in order to control whether there was a deviation in the retention times of standards or not. By comparing the retention times of standard peaks with that of the sample peaks; possible daidzein and genistein peaks in chromatograms of the samples were determined. Excellent symmetrical elution pattern was obtained in the chromatograms (Fig No. 16-18) during 4-8 hours duration. The chromatograms showed efficient separation and correct integration of genistein in the blood samples. After 4 hour degradation might have occurred because several small peaks were found earlier than the retention time of genistein. And at 2, 8, 24, 36 and 48 hours no elution pattern of genistein was found. Only 08 postmenopausal women showed the bioavailability of genistein following a single dose of orally administered soy-milk during 4-6 hour.

Genistein was found in the blood samples in women after serving soy-milk. But no peak was found at the retention time of daidzein indicated its non-bioavailability in the blood samples. From the area in the chromatogram each sample maximum concentration amount was calculated was using the same method as used for soymilk samples. The results are expressed as C_{max} (maximum concentration) AUC (area under the curve) and T_{max} (at time of the maximum concentration) (91, 96, 100, 101, 104) (Table 4).

4.6.2. Pharmacokinetics of daidzein and genistein in healthy postmenopausal women after serving soy-milk

The mean C_{max}, AUC and T_{max} of plasma genistein concentration of soy-milk were found to be 1.62 ±0.87 µg/mL, 7.41 ±4.16 µg/mL and 6.0 ±1.85 hour, respectively (Table 4). AUC unit depends on measurement unit. Daidzein and genistein was measured in serum as µg/mL and is given as AUC unit in table.

Table 4: Individual and mean pharmacokinetic parameters (C_{max}, AUC and T_{max}) of genistein following a single dose of orally administered soy-milk

Soy milk			
Subj No	C_{max} (µg/mL)	AUC (µg/mL)	T_{max} (h)
1	1.15	5.39	6
2	-	-	-
3	-	-	-
4	2.42	10.89	4
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	1.23	5.38	4
10	1.07	4.38	4
11	-	-	-
12	1.23	5.84	8
14	1.53	8.31	8
15	-	-	-
17	0.92	3.23	6
18	3.46	15.85	8
Mean	1.62	7.41	6.0
±SD	±0.87	±4.16	±1.85

4.6.3. Bioavailability of soy-milk isoflavones

In the present study, each postmenopausal women were assigned to receive 350 mL of soy-milk which was made from 100 g soybean which contained daidzein (36.25 µg) and genistein (43.81 µg). In humans, the systemic bioavailability was measured in terms of area under the curve (AUC) and from literature (96, 97), it was found that the AUC of soy genistein was greater than that of daidzein. The mean AUC of genistein and the mean serum genistein concentrations (C_{max}) of soy-milk was found to be 7.41 µg/mL and 1.62 µg/mL respectively in the present study. The C_{max} (5.99 ±3.22

μmol/L) and AUC (27.41 ±15.30 μmol/L) of the soy-milk genistein in the present study was found higher compared to the study done in healthy adults by *Kano et al* (213).

Cassidy et al (91), described that the AUC of genistein was 54.06 ±32.68 μmol/ L after ingestion of soy-milk in postmenopausal women. The AUC of soy-milk genistein (27.41 ±15.30 μmol/ L) of the present study in postmenopausal women was found less compared to the above study (91). The C_{max} (5.99 ±3.22 μmol/ L) of soy-milk genistein in the present study was found higher than the *Cassidy et al* (91) study.

The bioavailability depends on the excretion, degradation; variation of the results might be due to the differences between the races, physiological differences (intestinal condition) and for the ingestion form of isoflavones. Besides this, other factors such as differences in the food matrix (liquid vs solid), composition of habitual diets (fiber, fat, protein) and the less content of isoflavones in the present study also might play the role in the bioavailability among the postmenopausal women.

Generally, times to obtain maximum plasma concentrations were reported at 1–6 h for free genistein and 4-6 h for total genistein (aglycone + conjugates) (214). In the present study, the average time to maximum concentration (T_{max}) of soy-milk was 4-6 hour. Literature said (91) that liquid matrix yielded a faster absorption rate, higher peak serum concentrations and obtained maximum time concentration than a solid matrix.

In the present study, the frequency and timing of the blood sampling was fixed following the literatures which were done on Thai (101), USA (104) and UK (96) menopausal women. No literature was found on the bioavailability of isoflavones in South-East Asian postmenopausal women. The absorption pattern of isoflavones of USA, UK and Asian menopausal women was not to be similar to ours.

4.6.4. Masoor dal

Sixteen post-menopausal women were readmitted at the BIHS hospital, all the parameters were checked again and found they fulfill the requirement of the biological studies. There was no daidzein and genistein found in the blood samples before serving food. Freshly prepared masoor dal (~350 mL) was served to every

woman for assessing bioavailability of isoflavones. Blood samples were drawn at 0, 2, 4, 6, 8, 24, 36 and 48 hours (8 times) to find out *in-vivo* time duration of the presence of isoflavones in each of the woman. A total of 128 number of blood samples were collected in 128 different test tubes from 16 women. The freshly collected blood samples were centrifuged, and the serum of the each blood samples were stored at -20^0C to avoid biodegradation of the targeted compounds.

Samples were injected to the system with one injection of standard after each two or three injections of samples; in order to control whether there was a deviation in the retention times of standards or not. By comparing the retention times of standard peaks with that of the sample peaks; possible daidzein and genistein peaks in chromatograms of the samples were determined. Excellent symmetrical elution pattern was obtained in the chromatograms (Fig No. 19-21) during 4-8 hours duration. The chromatograms showed efficient separation and correct integration of genistein in the blood samples. After 4 hour degradation might have occurred because several small peaks were found earlier than the retention time of genistein. And at 2, 8, 24, 36 and 48 hours no elution pattern of genistein was found. Only 15 postmenopausal women showed the bioavailability of genistein following a single dose of orally administered masoor dal soup during 4-6 hour.

Genistein was found in the blood samples in women after serving masoor dal soup. But no peak was found at the retention time of daidzein indicated its non-bioavailability in the blood samples. From the area in the chromatogram each sample maximum concentration amount was calculated was using the same method as used for soy-milk samples. The results are expressed as C_{max} (maximum concentration) AUC (area under the curve) and T_{max} (at time of the maximum concentration) (91, 96, 100, 101, 104) (Table 5).

4.6.5. Pharmacokinetics of daidzein and genistein in healthy postmenopausal women after serving masoor dal

The mean C_{max} and T_{max} of plasma genistein concentration of masoor dal was 4.03 ±3.91µg/mL, and 6.0 ±1.51 h. The median AUC of plasma genistein concentration was [8.76 (2.18-58.34) µg/mL (Table 5).

Table 5: Individual and mean pharmacokinetic parameters (C_{max}, AUC and T_{max}) of genistein following a single dose of orally administered masoor dal

Masoor dal			
Subj No	C_{max} (µg/mL)	AUC (µg/mL)	T_{max} (h)
1	7.51	29.04	6
2	13.72	58.34	4
3	3.23	8.67	8
4	5.76	23.89	4
5	2.59	10.0	4
6	-	-	-
7	3.46	18.58	6
8	3.98	8.41	8
9	1.84	6.62	6
10	10.61	34.47	4
11	0.48	8.76	8
12	0.46	2.17	8
14	1.53	4.37	6
15	1.92	5.81	8
17	2.07	10.69	6
18	1.34	6.73	6
Mean	4.03	15.77	6.0
±SD	±3.91	±15.12	±1.51
Median	2.59	8.76	6.0
Range	0.46-13.72	2.18-58.34	4.0-8.0

4.6.6. Mung dal

Sixteen postmenopausal women were further admitted at the BIHS hospital, all the parameters were checked once again and found they fulfill the requirement of the biological studies. There was no daidzein and genistein found in the blood samples before serving food. Freshly prepared mung dal (~350 mL) was served to every woman for assessing bioavailability of isoflavones. Blood samples were drawn at 0, 2,

4, 6, 8, 24, 36 and 48 hours (8 times) to find out *in-vivo* time duration of the presence of isoflavones in each of the woman. A total of 128 number of blood samples were collected in 128 different test tubes from 16 women. The freshly collected blood samples were centrifuged, and the serum of the each blood samples were stored at -20^0C to avoid biodegradation of the targeted compounds.

Samples were injected to the system with one injection of standard after each two or three injections of samples; in order to control whether there was a deviation in the retention times of standards or not. By comparing the retention times of standard peaks with that of the sample peaks; possible daidzein and genistein peaks in chromatograms of the samples were determined. Excellent symmetrical elution pattern was obtained in the chromatograms (Fig No. 22-24) during 4-8 hours duration. The chromatograms showed efficient separation and correct integration of genistein in the blood samples. After 4 hour degradation might have occurred because several small peaks were found earlier than the retention time of genistein. And at 2, 8, 24, 36 and 48 hours no elution pattern of genistein was found. Only 05 postmenopausal women showed the bioavailability of genistein following a single dose of orally administered mung dal soup during 4-6 hour.

Genistein was found in the blood samples in women after serving mung dal soup. But no peak was found at the retention time of daidzein indicated its non-bioavailability in the blood samples. From the area in the chromatogram each sample maximum concentration amount was calculated was using the same method as used for soymilk samples. The results are expressed as C_{max} (maximum concentration) AUC (area under the curve) and T_{max} (at time of the maximum concentration) (91, 96, 100, 101, 104) (Table 6).

4.6.7. Pharmacokinetics of daidzein and genistein in healthy postmenopausal women after serving mung dal

The mean C_{max}, AUC and T_{max} of plasma genistein concentration of mung dal was 5.15 ±3.02 μg/mL, 17.81 ±9.94 μg/mL and 6.8 ±1.78 h respectively (Table 6).

Table 6: Individual and mean pharmacokinetic parameters (C_{max}, AUC and T_{max}) of genistein following a single dose of orally administered mung dal

Mung dal			
Subj No	C_{max} (µg/mL)	AUC (µg/mL)	T_{max} (h)
1	6.23	25.78	8
2	9.34	28.44	6
3	3.08	10.20	8
4	1.48	5.23	4
5	5.63	19.44	8
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
14	-	-	-
15	-	-	-
17	-	-	-
18	-	-	-
Mean	5.15	17.81	6.8
±SD	±3.02	±9.94	±1.78

Pharmacokinetics data were compared according to food items, utilizing analysis of variance (ANOVA) test; *p* value of ≤0.05 was considered as a level of significance and no significant differences in the AUC of genistein was observed within three food items (soy-milk vs masoor dal vs mung dal). Similarly, no significant differences in the T_{max} and C_{max} among these food groups were observed. All statistical analyses were performed with the software SPSS 17.0 for Windows (SPSS, Inc. Chicago. IL. USA).

4.6.8. Bioavailability of masoor and mung dals isoflavones

No studies were found regarding bioavailability of isoflavones in non-soy food like mung and masoor dals. The AUC (65.91 ±36.78 μmol/L) and C_{max} (19.05 ±11.17 μmol/L) of mung genistein and the AUC (58.35 ±55.95 μmol/L) and C_{max} (14.91 ±14.46 μmol/L) of masoor genistein was found similar in Bangladeshi menopausal women compared to the studies (91, 96, 97, 100, 213) of bioavailability of isoflavones in soy-foods.

Moreover, after consumption of soy-milk the mean AUC of genistein in the present study was found to be 7.41 μg/mL which was less from mung (17.81 μg/mL) and masoor dals (15.77 μg/mL) and the comparison was not significant ($p \leq 0.05$) and the mean serum genistein concentrations (C_{max}) of mung (5.15 μg/mL) and masoor (4.03 μg/mL) were also showed higher compared to soy-milk (1.62 μg/mL) but not significantly. Daidzein from mung, masoor dal soups and soy-milk did not show any peak at its a retention time and this was specified the non-bioavailability of daidzein in blood samples though genistein from above three foods gave some peak on allocated time but not full profile from which we might calculate serum concentration curve.

South-East Asia region people mainly consume masoor and mung dals which contain more or less amount isoflavones of soy-milk. Finally, it might say that different ethnicity, appropriate selection of time for collecting blood after ingestion of food, quantity of individual and total isoflavones in food played important role in the bioavailability of isoflavones in Bangladeshi menopausal women.

4.7. Health effects of isoflavones

Menopause women suffer from hot flushes, night sweating and osteoporosis. There are several reports (142, 143, 147, 149, 150, 160, 162, 163, 177) that taking soy food stuff as dietary supplement in for longer duration reduces risk of hot flush, night sweating and osteoporosis in menopausal women and isoflavones present in the bean are the main active ingredients of biological effects.

Albertazzi et al (197) made a double-blind and randomized placebo-controlled study on 104 postmenopausal women for three months to find out health benefit. Postmenopausal women received soy protein containing food and found significant reduction of hot flush frequency. The pattern of hot flush reduction frequency was similar to the estrogen replacement therapy. Similar findings has been reported by *Upmalis et al* (215) that soy isoflavones containing extract significantly decreased hot flushes in postmenopausal women. In another study made by *Kotsopoulos et al* (216) found slight improvement of menopausal symptoms in healthy postmenopausal women after taking 3 months of soy dietary supplements. *Kyung et al* (217) reported that isoflavones are safe and effective alternative therapy for menopausal symptoms along with the benefit of atherosclerosis. *Yamori et al* (218) found isoflavones containing soy protein inhibit postmenopausal osteoporosis. Another study (101) also stated that continuous intake soy food inhibits osteoporosis.

From the all these studies revealed that isoflavones rich food stuff are beneficial for postmenopausal women. Considering earlier studies isoflavones present in soybean/milk were identified and quantified by LC-PDA with respect to certified isoflavones standard. Presence of isoflavones in soybean was further confirmed by LC-MS/MS. Similarly isoflavones present in the masoor and mung dals were studied.

Bioavailability of isoflavones in blood stream by serving soy-milk, mossor and mung dals were done and 4–6 hours duration of isoflavones genstein in the blood stream was found in each case of study. This is very similar to the study done by others (214).

Hot flush and night sweetening are not the clinical parameters to measure qualitatively and quantitatively. Osteoporosis need to be studied following menopausal women for a long period of time by serving isoflavones containing food stuff and gradual improvement of bone texture of the women.

Presence of daidzein and genistein in soybean and soy milk is the good indication of health benefit for menopausal women. Again bioavailability, another important factor for beneficial health effect will be on menopausal women. In the present study, the average time to maximum concentration of soy-milk, masoor and mung dals soup was

found to be 4-6 hour. This is the good sign of beneficial health effect in postmenopausal women.

4.8. Limitations of the study

i. Due to limited resources, financial and technical support, it has not been possible to do the extensive chemical analysis of isoflavones in our locally produced mung, masoor dals and soybean seed.

ii. In the present study, frequency and timing of the blood sampling was fixed following the literatures and was found not appropriate for our study samples. For this, it has not been possible to get full profile from which it might likely to calculate serum concentration curve.

iii. It was not easy to collect urine samples of the study subjects due to their non-cooperation.

5. Conclusions

Menopause is an important but obligatory physiological phenomenon which creates a number of biological, psychological and social problems imposing additional risk and challenge in the life of a woman. Presently, HRT is used frequently to improve the quality of life of menopausal women, but due to its expensiveness and probable side effects it is now seriously thought that an alternative therapy is necessary.

In 1940s, it was first realized that some plant-derived compounds could cause estrogenic effects in animals (219). Sheep grazing on pastures containing red clover had multiple fertility problems. The clover in these pastures had high amounts of the isoflavones (220). Evidence was beginning to accumulate that isoflavones which belong to phytoestrogens may begin to offer protection against a wide range of human conditions, including breast, bowel, prostate, and other cancers; cardiovascular disease; brain function; alcohol abuse; osteoporosis; and menopausal symptoms **(**133**)**. The basis for these effects has not been established yet, but the weak estrogenic activity of isoflavones may be a factor in conferring these properties (134).

According to literature, no studies have been undertaken to clarify effective types and forms of isoflavones, optimal intake, and individual variation in isoflavones bioavailability among Bangladeshi postmenopausal women. The present study aimed to analyze the quantity and type of total isoflavones in soybean seed, mung and masoor dals and to measure the bioavailability of each type of isoflavones in postmenopausal women of ours country.

The conclusions of the study are given below:

i. Two isoflavones daidzein and genistein were identified in soybean [*Glycine max* (L.) Merr.], mung dal [*Vigna radiata* (L.) R. Wilczek] and masoor dal [*Lens culinaris* (Medik.)].
ii. Daidzein, and genistein were found to be 36.25 µg/100 g, and 43.81 µg/100 g in soybean; 37.66 µg/100 g, and 37.33 µg/100 g in masoor dal; 27.66 µg/100 g, and 44.00 µg/100 g in mung dal respectively.

iii. The estimated mean of total isoflavones in locally produced soybean, masoor and mung dals was found to be 80.05 µg/100 g, 74.99 µg/100 g and 71.66 µg/100 g respectively.

iv. The assessed mean of maximum concentration of isoflavone genistein was found to be 1.62 µg/mL for soy-milk, 4.03 µg/mL for masoor dal soup and 5.15 µg/mL for mung dal soup.

v. The calculated mean of area under the curve (AUC) of isoflavone genistein was found to be 7.41 µg/mL for soy-milk, 15.77 µg/mL for masoor dal soup and 17.81 µg/mL for mung dal soup.

vi. The average time to maximum concentration (T_{max}) of soy-milk, masoor and mung dal soups was 6 hour.

6. Recommendations

Being a very popular subject of interest for the research, isoflavones seem to remain on the agenda of many countries as a priority for the coming years. Therefore, it is highly recommended that different varieties of legumes, should be analyzed for their isoflavones content and estrogenic activities which will contribute to food and medicine industries.

For our own perspective, few more recommendations are given as follows:

- The form of isoflavones (aglycones vs glycosides) should be known before ingestion of isoflavones rich foods.
- The extent of daily intake of isoflavones should be longer in menopausal women for getting beneficial effect.
- Complete pharmacokinetic characteristics of daidzein and genistein should be known through the exploration of mode of action of isoflavones among Bangladeshi postmenopausal women.

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সম্মতি পত্র (প্রথম ধাপ)

আমাদের দেশের দরিদ্র আর্থ-সামাজিক অবস্থা, নিরক্ষরতা, অজ্ঞতা এবং অপর্যাপড় স্বাস্থ্যসেবার কারনে মেনোপোজ মহিলাগণ পর্যাপড় পরিচর্যা পাচ্ছেননা। সকলের পক্ষে Hormone Replacement Therapy (HRT) গ্রহণ করা সম্ভব নয় এবং ইহার পার্শ্বপ্রতিক্রিয়া রয়েছে। এইসকল কারনে সহজলভ্য খাবার যেমন সয়াবীন এবং ডাল হতে রাসায়নিক বিশেণ্ডষণের মাধ্যমে Isoflavones নামক উপাদানের গুনগত এবং পরিমাণগত বৈশিষ্ট্য নির্ধারণ করা হবে । এর উপর ভিত্তি করে বিভিন্ন পরিমাণে ও গ্রহণযোগ্য সয়াবীন এবং ডালের তৈরী খাবার মেনোপোজ মহিলাদের দেওয়া হবে যার মধ্যমে রক্তের Isoflavones মাত্রা সম্পর্কিত তথ্য সংগ্রহ করা হবে।

- রক্তের Isoflavones মাত্রা সম্পর্কিত তথ্য সংগ্রহ করার জন্য ৫ মিলি রক্ত সংগ্রহ করা হবে এবং নির্দিষ্ট সময়ের ব্যবধানে ৮ বার নেওয়া হবে ।
- আমার দেওয়া এই তথ্য মেনোপোজ সম্পর্কিত গবেষণার কাজে ব্যবহৃত হবে এবং মেনোপোজ মহিলাদের সেবার ধরণ উন্নত করার ক্ষেত্রে অবদান রাখবে।
- গবেষণার সময় যদি রোগীর গোপন তথ্য প্রকাশ পায় তবে compensation প্রদান করা হবে ।

ক্রমিক নং ঃ-

আমি ----------মেনোপোজ মহিলাদের সয়াবীন এবং ডালের তৈরী খাবার গ্রহন এবং তার উপকারীতা সংক্রান্ড় গবেষণায় প্রথম ধাপে অংশগ্রহনকারী হিসাবে তথ্য প্রদানে আমার সম্মতি প্রদান করিতেছি।

আমার দেওয়া সকল তথ্য গবেষণার কাজে ব্যবহৃত হবে এবং মেনোপোজ মহিলাদের সেবার ধরণ উন্নত করার ক্ষেত্রে অবদান রাখবে।

স্বাক্ষরঃ-

তারিখঃ-

ঠিকানাঃ-

দ্রষ্টব্যঃ এই গবেষণার কাজে অংশগ্রহনের জন্য অংশগ্রহনকারীকে প্রতি সাক্ষাৎকারের জন্য বিশেষ ভাতা প্রদান করা হবে।

BIOAVAILABILITY AND HEALTH EFFECTS OF ISOFLAVONES FROM BANGLADESHI SOYBEAN AND LENTILS IN POSTMENOPAUSAL WOMEN

SCREENING FORM

Sl. No: Date:

1. Name:
2. Age:
3. Address:

   Present:

   Permanent:

4. Patient source:
5. Religion:
6. Current marital status: Married/ Unmarried / Widowed/ Separated/ Others (specify)
7. Family history of hypertension: present/absent
8. Family history of DM: present/absent
9. Present history of Subject:
   a) Hypertension: present/absent
   b) DM: present/absent
   c) Other

10. Past history
    a) Any surgical history (e.g. hysterectomy)
    b) Thyroid
    c) Other
11. Did you take any Antibiotics or HRT last 01 month? Yes/no
12. Menopause_____(Age)____

General examination:

a) Ht (m)_____ b) Wt (Kg)_____ Desire wt (kg)_____

d) Anemia_____

e) Pulse_____ f) BP_____

Investigations:

a) S Glucose (Fasting)

b) After 2 hrs Glucose (75g)

_____

Interviewer Signature