Evaluation of Phytoestrogens in Traditional Plant Based Herbal Drug Formulations and their possible consequences to the Gut Flora

A

Dissertation submitted

In the partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

By

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Evaluation of Phytoestrogens inTraditional plant based Herbal Drug formulations and their possible consequences to the gut flora**"in partial fulfillment of the requirement for the award of the degree of **Masters of Science in Microbiology**, Department of Biotechnology(DBT),Thapar university, Patiala as an authentic record of my work during the period of six months from January, 2014 to June 2014, under the guidance of **Dr. Abhijit Ganguli**, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled **Evaluation of Phytoestrogens in Traditional Plant based Herbal Drug Formulations and their possible consequences to the Gut Flora** submitted by Pallak Jain in partial fulfilment of the requirement for the award of Degree of **Masters of Science in Microbiology** to Thapar University, Patiala is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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CONTENTS

Abbreviations

RP-HPLC	Reverse phase- high performance liquid chromatography.
TLC	Thin layer chromatography.
UV	Ultraviolet
MRS	Man, Rogosa and Sharpe
PBS	Phosphate Buffer Solution
P.E	Petroleum ether
GAD	Glutamate decarboxylase
MATS	Microbial Adhesion of solvents
CFU	Colony Forming Units
ng	nanograms

ABSTRACT

The objective of this study was to investigate the metabolic response of *Bifidobacterium adolescentis* and *Lactococcus lactis*, two representative gut flora to phytoestrogens consumed through traditional herbal formulations. Healthy strains are present in the mother`s gut are transmitted to the newborn during vaginal delivery resulting in normal development of the newborn. About 200 herbal formulations collected from different areas of Punjab were analyzed; the results indicated presence of phytoestrogens (daidzein, genistein, formononetin, biochanin A , testosterone, coumesterol) in all samples in notable quantities. In vitro studies were carried out by challenging the formulations to the pure cultures of *B.adolescentis* and *L.lactis*. The results indicated significant inhibition of both the strains , furthermore examination of selected probiotic characteristics indicated prominent detrimental changes suggesting that these strains to be grossly affected by phytoestrogens. Further studies are warranted for understanding the detrimental effect to these gut flora. It may be inferred from this study that a loss of viability, beneficial properties upon consumption of phytoestrogens can be an important cause in impairing overall health of vaginally delivered newborns.

On consuming such spurious formulations, gut flora get affected which results in the reduction in their original log values of the probiotics. Moreover it results in various malformations in the developing fetus. The tolerance of both the strains towards phytoestrogens was examined and it was found that both the strains loose their probiotic attributes when we increase the concentration of the dose. These formulations are generally prescribed for twenty days and there total consumption becomes 69.6 ng which is a very large amount to cause destruction of the flora as well as hormonal imbalance.

Keywords: Phytoestrogens, probiotics, herbal formulations

Introduction

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens and therefore they are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis. They influence the growth and functioning of female and male reproductive tissues, maintain the skeletal and central nervous system, provide cardio protective effects in the cardiovascular system and protect against colon cancer and skin aging. There are numerous effects estrogens on the human body. Both in humans and in animals, endocrine signaling is involved in reproduction and embryo development, growth and maturation, energy production, use and energy storage, electrolyte balance, maintenance and behaviour. Hormones trigger such complex functions by interacting with their receptors that are present at a nuclear and cellular level in various organs and tissues as part of a complex biological feedback system. Any disruption of this balance can cause impairment in the physiological status of the whole organism, especially during the more susceptible developed stages. If the regulatory role of the endocrine system is impaired, abnormal function and development of the reproductive, the nervous and the immune systems may occur. It has been recently postulated that predisposition for certain types of tumours is caused by an altered development during prenatal growth (intrauterine) and in the first years of life (Sharpe and Sakkebaek *et al*., 1993). Several substances, including EDC, shares the ability of interfering with the female reproductive system and possibly implicated in the development of some gynecological pathologies.

Traditional practices on sex selection in North India have also documented the use of traditional herbal formulations, containing high doses of phytoestrogens, in the region for having a male child (Bandyopadhyay S *et al.,* 2007).Several such formulations which claim high efficacy abound in the Indian market and are currently prescribed to rural population. The role of phytoestrogens is not clear in this prospect and these formulations containing phytoestrogens can might have adverse effects on male fetus and the microbiota of the consumer. They are consumed to boost the male population. This can also leads to certain types of malformations in a developing fetus.

Objectives of our study

- To characterize these phytoestrogens in local herbal formulations.
- To understand the effect of these phytoestrogens to the representative members of the gut flora.

In view of the controversies related to congenital malformations ,the study attempted to understand the role of local formulations used as either food adjuncts or as herbal medicines to selected representative of the gut, the characterization of local soy formulations were also attempted.

Review of literature

1.1 CLASSIFICATION OF PHYTOESTROGENS

Isoflavones

Isoflavones are the most well-known among the phytoestrogens. The recognition of 'clover disease' in Australian sheep in the 1940s led to the investigation of estrogenic activity of phytoestrogens . The sheep whose diet was predominately subterranean clover (*Trifolium subterraneum L., Fabaceae*) suffered from a reproductive disorder that reduced the lambing rates and involved abnormal lactation, changes in the sex organs, permanent infertility, prolapsed uterus and maternal dystocia. Naturally occurring isoflavones that have shown estrogenic activity are: the aglycones, daidzein (4′,7-dihydroxyisoflavone) and genistein (4′,5,7-trihydroxyisoflavone); the glycosides, daidzin and genistin; and biochanin A and formononetin, 4′-methyl ethers of daidzein and genistein (Price and Fenwick et al., 1985). In plants, they can often be found as glycosides. In processing, isolation and analysis, these compounds are readily degraded chemically or enzymatiically to the aglycones (Price and Fenwick *et al*., 1985).After mammals consume isoflavones, daidzein and genistein are metabolized in the gastrointestinal tract. Biochanin A and formononetin can metabolize to genistein and daidzein respectively. Isoflavones are primarily found in the Fabaceae family, which has food legumes such as soy, peanut (*Arachis hypogaea L*.) and clover (*Trifolium spp*.). Soy seeds show high levels of formononetin and biochanin A (both 729 μg/g dry weight). Isoflavones have also been found in the *Iridaceae* and the *Euphorbiaceae family* (Dewick *et al*., 1994). They are primarily extracted from soy and red clover. Of all the phytoestrogens, genistein has received the most attention.

Coumestans

Coumestans are another group of plant phenols that show estrogenic activity. Coumestrol was first reported in 1957 by Bickoff and coworkers as a new phytoestrogen that was isolated from ladino clover (*Trifolium repens L., Fabaceae*), strawberry clover (*Trifolium fragiferum L., Fabaceae*) and alfalfa or lucerne (*Medicago sativa L., Fabaceae*) (Bickoff *et al*., 1957). Coumestans are less common in the human diet than isoflavones (Ibarreta *et al*., 2001), yet

similar to isoflavones, in that they are also found in legumes, particularly food plants such as sprouts of alfalfa and mung bean.

Lignans

Lignans were first identified in plants and later in biological fluids of mammals. As a class of compounds they contain a dibenzylbutane skeleton and in plants they aid in the formation of lignin used to construct the plant cell wall.A cyclic pattern observed in the excretion of these phenolic compounds by humans and animals during the menstrual cycle initiated interest in their physiological role (Adlercreutz *et al*., 1987). They were thought to be a new class of endogenous hormones. These compounds were elucidated simultaneously by different researchers and identified as unique mammalian lignans (Setchell *et al*.,1980). The most well-known phytoestrogenic lignans are secoisolariciresinol and matairesinol which are converted by bacterial action in the gut into enter odiol and enterolactone, mammalian lignans not found in plants (Adlercreutz *et al*.,1988). Enterodiol can be further metabolized to enterolactone (Borriello *et al*., 1985). The removal of the sugar moiety through metabolism by intestinal bacteria is common in isoflavones and lignans (Adlercreutz *et al*., 1987). Mammalian lignans, like isoflavones, have a low molecular weight and are considered chemically, biochemically and biologically unique and stable molecules because they have phenolic groups in the meta position of the aromatic rings. Lignans are widespread in foodstuff such as cereals, fruits and vegetables and have not been studied as thoroughly as isoflavones and coumestans (Ibarreta *et al*., 2001). Lignans are commonly found in rye bread (*Secale cereale L*., *Poaceae*) and oilseeds such as flaxseed (*Linum usitatissimum L., Linaceae*) (Thompson *et al*., 1991). Flaxseed contains the most abundant amount of lignans. Lignans are also found in brewed green and black tea and coffee.

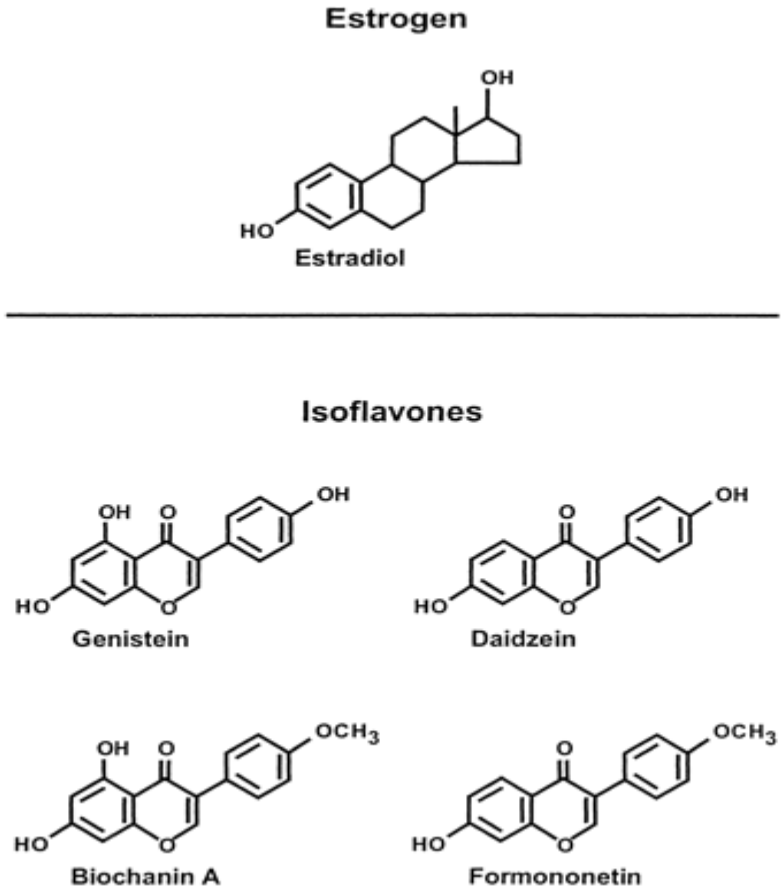


Fig.1.1 Chemical structure of isoflavones. (Franke *et al*., 1996)

1.2 BOTANICAL SOURCES OF PHYTOESTROGENS

Soy

Soy or soybean (Glycine max) belongs to the Fabaceae family and has long been used as a food plant. Of the bean plants it has one of the highest levels of protein and oil (Duke *et al*., 1981). Medicinally it has been reported in ancient Chinese herbals for the healthy functioning of the heart, kidneys, liver and stomach (Duke and Ayensu *et al*., 1985). It was domesticated in China around the eleventh century BC and there are many varieties. The Chinese distinguish the different varieties by color. Black seeds are associated with medicine and have been used for strength and vigor as well as in mixtures for post-partum and sexual disorders (Li *et al*., 1973). The black bean sprouts have been used as a laxative, for rheumatism and hair growth. A ‘bean

relish' made of salted fermented beans has been highly valued in Chinese medicine and has multiple uses including colds, head- aches, hemorrhaging in abortion, threatened abortion, difficult labor, irritability and fever (Li *et al*., 1973). In the 1940s, genistin, the genistein glycoside, was first reported in soybean oil meal and was later shown to have estrogenic activity in mice.

Black Cohosh

Black cohosh (*Actaea racemosa, Cimicifuga racemosa*) is a North American plant in the buttercup family (*Ranunculaceae)* that grows in Eastern North America, from southern Maine to Georgia (Ramsey *et al*., 1997). Black cohosh, also known as baneberry, black snakeroot, bugbane and rattle weed is used for various women's health conditions. Native Americans used the roots and rhizomes for a variety of indications such as stimulation of menstrual flow, dysmenorrhea, and suppression of cough, treatment of diarrhea, childbirth and rheuma- tism (Foster *et al*., 1999). The 19th century American Eclectic physicians recognized black cohosh to be 'very efficacious in maladies of the female reproductive organs.

Red Clover

Red clover (*Trifolium pratense*) in the *Fabaceae* family is a herb that is indigenous to Europe and parts of the Middle East and has naturalized to North America. It is well known as animal fodder. Humans have rarely consumed red clover, although it has been used medicinally. In the beginning of the twentieth century a 'Trifolium compound' that included red clover blossoms along with other botanicals was marketed by pharmaceutical companies for venereal disease, although there was little evidence to support this use (Foster and Tyler *et al*., 1999). Traditionally it has been reported by Native American Iroquis as a gynecological aid for 'the change of life'. The Celtics and Romans employed red clover as a sedative. It has also been used to purify blood, treat skin conditions and for bronchial asthma because it reduces muscle spasm and is a decongestant. The in vivo estrogenic and antiestrogenic effects of red clover extract have been studied in the uterus, vaginal cells and mammary glands of ovariectomized Sprague-Dawley rats (Burdette *et al*., 2002). Uterine weight and thickness were increased with the extract of red clover.

Flax

Flax (*Linum usitatissimum*) in the *Linaceae* family is an herb that is considered one of the oldest continously cultivated plant. Several European Pharmacopoeias have included it as a medicinal plant (Grieve, 1985). The seeds, also known as linseed, are medicinal and have been used to make flour. Flax has been attractive as an oil seed because it contains polyunsaturated fatty acids suchas α -linolenic acid, which may lower cholesterol and have antioxidant effects for health. Flaxseed is considered one of the richest sources of lignan phytoestrogens.

Licorice

The licorice (Glycyrrhiza glabra) plant is a perennial, belonging to the *Fabaceae* family and indigenous to Eurasia. The sweet yellow wood of the licorice root has been consumed for thousands of years in China for its health benefits and detoxification effects as well as its use as a flavoring and sweetening agent. Medicinally it has been used as a demulcent and expectorant and has been shown to have antioxidant and antimicrobial activity. In the United States, licorice is added to tobacco as well as candies, toothpaste and beverages (Wang and Nixon *et al*., 2001)

Hops

(*Humulus lupulus*), a perennial climbing vine in the *Cannabaceae* family, has been extensively cultivated for its bitter properties found in the female flowers used in beer and medicine. Medicinally hops have been valued as a sedative, for inflammation and as a tonic (Foster and Tyler *et al*., 1999). The Native American Cherokee used the plant for inflamed kidneys, as a sedative, for pain relief and for breast and female complaints where the womb was debilitated .The female flowers of hops are considered estrogenic.

Dong Quai

Dong quai (*Angelica sinensis*) is an herb that belongs to the *Apiaceae* family and has been used extensively in traditional Chinese medicine for many years (Mei *et al*., 1991). Dong quai has been referred to as the ‘female ginseng’ and is used for a variety of conditions such as a blood

tonic and decongestant for body organs. The root is used for women as a tonic often in combination with other herbs. Other women's conditions treated with dong quai are dysmenorrhea, irregular menstruation, anemia, constipation and abdominal pain.

Evening Primrose

Evening primrose is a common herb in North America that has been used medicinally by the Native Americans (Grieve *et al*., 1985). A randomized controlled trial of oral gamolenic acid from evening primrose oil on hot flushes showed no significant benefits over the placebo group . For centuries the dried ripe fruit of chasteberry tree has been used medicinally for its beneficial effects on female reproduction and to decrease sexual desire, specifically of monks.

1.3 Mother-to-Infant Transmission of Intestinal Bifidobacterial Strains and its Impact on the Early Development of Vaginally Delivered Infant's Microbiota

The human gut is defined either as the lower part of the alimentary canal the intestinal tract, or the entire gastrointestinal tract: mouth, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine and rectum. The gastrointestinal tract (GI) is designed to comminute and digest food to absorb and secrete nutrients and other compounds, including toxic compounds, and to excrete wastes. There is a persistent chemical and immunological challenge and transient mechanical challenge. To ensure normal function the gut with its microbiota forms the largest sensory, endocrine and immunological organ of the body.The gut microbiota is typically dominated by bacteria and specifically by members of the divisions Bacteroidetes and Firmicutes (Turnbaugh *et al*., 2006).

Bifidobacterium species are one of the major components of the infant's intestine microbiota. Colonization with bifidobacteria in early infancy is suggested to be important for health in later life. There are specific strains of bifidobacteria in the maternal intestinal flora which is transmitted to their infant's intestine. They are *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium catenulatum, Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum*. They were identified to be monophyletic between mother's and infant's

intestine. Factors like nutrition and diet may influence the persistence of family-specific bifidobacteria strains in infants throughout the sampling period, where they become one of the predominant bifidobacteria groups during early infancy. The high abundance of *Bifidobacterium* species in infants is considered to promote development and maturation of the immune system to sustain health. Furthermore, recent studies suggest that the succession of bifidobacteria is important for the proper immunological development. A cesarian delievered infant has been suggested to be initially colonized by bacteria from the environment, hospital staff and other neonates. The influence on the occurrence of mother-to-infant transmission among the neonates differs in the mode of delivery. In newborns, bifidobacteria constituted nearly one-fourth of the bacterial load, reaching the level of 60% by the age of 4 months.*Lactococcus lactis* also constitutes maternal gut flora.

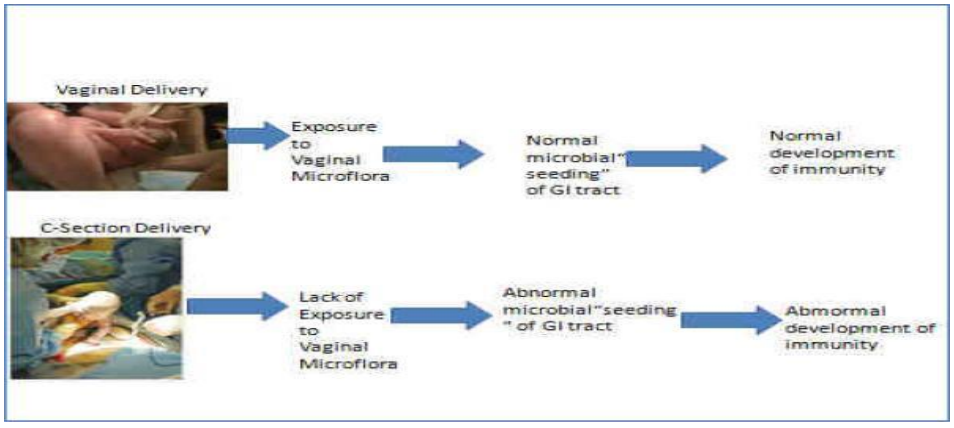


Figure 1.2 Mother to infant transmission of microflora

1.4 Asian and Indian diet with phytoestrogens

The asian diet contain highly processed foods made from legumes, such as tofu, retain most of their isoflavone content, with the exception of fermented miso, which has increased levels. Asians consume soybeans in many forms, including miso soup, tofu, natto (fermented soyabean) ,soy sauce, soya cheese, soya nuts, soya flour, barley miso, ice beans and tempeh. (Neil *et al*., 1993). The Indian diet contain isoflavones include chick pea (biochanin A), peanut (genistein), soyabean (Daidzein and Genistein), spinach (coumestrol), green beans (formononetin). Investigators have proposed the hypothesis that lowered cardiovascular disease, osteoporotic

fractures, rates of breast cancer and hot flushes in Asian populations are related to a diet rich in soy. However, when evaluating this relationship, confounding factors such as lifestyle, diet, socio-cultural and morphological differences that distinguish Asian and Western populations must be considered in the analysis. The isoflavones in modest amounts of ingested soy protein are biotransformed by intestinal microflora, are absorbed, undergo enterohepatic recycling, and reach circulating concentrations that exceed by several orders of magnitude the amounts of endogenous estrogens.

1.5 Diverse effects of Phytoestrogens

The effects of phytoestrogens depend on many various conditions such as dose and route of exposure because these parameters impact the final serum level of the bioactive compound. The timing of exposure is critical in determining the phytoestrogen-induced effects and different tissues have species-specific windows of sensitivity to morphological and functional disruption. The mechanisms and potencies of phytoestrogens are not completely clarified and they may be considered potential endocrine disrupters, and therefore caution should be exercised when taking them. Some concerns have been discussed about the risks associated with phytoestrogens such as increased plasma concentration of isoflavones in babies that ingest soymilk, the ability of non-hormonal secondary plant metabolites to modify sex steroid metabolism, and the effects of phytoestrogens on thyroid (Ibarreta *et al*., 2001). Toxicities associated with herbal medicines that include phytoestrogens have presented in different journals. Some negative effects of phytoestrogens may result because of receiving high levels of isoflavones during fetal development. Studies by Setchell *et al*. (1997) showed that infants fed soy-based formula have high concentrations of daidzein and genistein in their plasma, 13 000 to 22 000 times higher than E2 in early life and proportionately higher than normal adult intake of isoflavones (Setchell *et al*., 1997). This has raised concern about the health benefits and long-term effects that phytoestrogens may have on developing and mature neuronal function and the interaction of phytoestrogens with E2 during perinatal development of the brain.

As potential endocrine disrupters, phytoestrogens may act as antiestrogens and harm the reproductive health of males (Santti *et al*., 1998). Reduced sperm quality, undescended testes and urogenital tract abnormalities were increased in the sons of mothers taking DES compared

with those who did not take them. (Sheehan *et al*., 1998). Animal studies conducted with DES resulted in male genital abnormalities during development including cysts, testicular lesions and lack of growth of the seminal vesicles and therefore concern has been raised about the effects of phytoestrogens on male development (Santti *et al*., 1998). High doses of genistein are shown to alter pituitary responsiveness and basal luteinizing hormone (LH) secretion in castrated post pubertal rats.The potential hazardous effects that estrogen and androgen-like chemicals may have both on wildlife and human health have attracted much attention from the scientific community. Endocrine disruptors (EDCs) are chemicals that have the capacity to interfere with normal signalling systems. EDCs may mimic, block or modulate the synthesis, release, transport, metabolism and binding or elimination of natural hormones. Even though potential EDCs may be present in the environment at only very low levels, they may still cause harmful effects, especially when several different compounds act on one target. EDCs include persistent pollutants, agrochemicals and widespread industrial compounds. Not all EDCs are man-made compounds; many plants produce substances (phytoestrogens) that can have different endocrine effects either adverse or beneficial in certain circumstances. Natural substances such as sex hormones from urban or farm wastes can become concentrated in industrial, agricultural and urban areas; thus, such wastes may be considered potential 'EDCs' for humans and/or wildlife. Much attention has focussed on changing trends in male reproductive parameters in relation to EDC exposure; however, studies on the female reproductive system have been less comprehensive. It also causes fertility problems, fecundability, endometriosis, precocious puberty, breast and endometrial cancer. New sources of estrogenic compounds are attractive for human health because of the numerous effects that endogenous estrogens have on the human body. However some estrogenic compounds have raised concern for human health because of their negative effects.

1.6 Effect of phytoestrogens on male reproductive health

Consumption of phytoestrogen rich diets can cause impaired fertility and reproductive tract disorders in some animals and the apparent decline in human sperm quality. It also affects semen quality, serum sex steroid as well as gonadotrophin levels in human males.

SEXUAL DIFFERENCIATION

Early in life, a bipotential gonad develops, and within the medullary (central) region of that gonad lies the key to gender determination. At the inception of the embryo, as sperm meets egg, sex chromosomes from the mother (X) and from the father (either X or Y) are combined to provide the potential of gender but not the key to sexual differentiation. Sexual differences are determined by a gene on the Y chromosome called the SRY gene. The SRY gene, acting like a transcription factor, mediates chromatin crossing over, which is vital in sexual differentiation. As a result of the presence of that specific gene, the female program of development, which is the modus operandi of biology, is suppressed and the growth of a testis is allowed to occur. In the absence of the SRY gene, a gonad develops into an ovary even if the genetic makeup of the embryo is XY. As a function of the SRY gene, cells of the normal gonad differentiate to produce the hormones and factors that allow for masculinization or feminization. In males, the presence of SRY together with testis-determining factor causes two main cell types to develop in the gonad, namely the sertoli and leydig cells. Leydig cells work in conjunction with the hypothalamal-hypophyseal axis and begin to produce the appropriate hormones for reproductive and urogenital formation. In the testis, testosterone will begin to be the predominantly produced hormone.

The presence of testosterone has a twofold effect on the developing fetus: first, testosterone helps to further the development of the wolffian ducts (the embryonic antecedents of the vas deferens and ureter); and secondly, testosterone is converted into androgens (dihydro-testosterone) via 5-alpha-reductase. The presence of mullerian inhibiting substance (MIS) along with androgens causes the degeneration of the mullerian ducts (the embryonic antecedents of teh upper vagina, cervix, uterus and oviducts) and spurs the development of the wolffian ducts, the penile shaft, the glans, and the descent of the testicles from the abdominal cavity .In females, however, the absence of the SRY gene causes the bipotential gonad to continue developing into an ovary. The ovary does produce testosterone, but most of that testosterone is quickly aromatized (converted by enzymatic action) into estrogens, none of which can be converted back into androgens. The lack of MIS, the lack of significant androgen levels, and the presence of estrogens allow the formation of the ovaries, fallopian tubes, uterus, cervix, upper and lower vagina and clitoris. Together these organs form the main components for reproduction later in

life and for the elimination of wastes in the form of urine upon birth.Significant deviations in the levels of the appropriate sex hormones can cause severe consequences in reproductive and urogenital development, especially at the time of fetal growth and the pre-pubertal period. In humans, the process of masculinization or feminization is not a black and white proposition but a process that takes place on a continuum over the years. The Prader scale measures a person's development on that continuum by looking at the development and at the location of the urethral opening, clitoral hypertrophy, and the location of the testicles or labia compared to the glans or clitoris, respectively. The development of the male body plan, and hence the process of de-feminization, depends on the presence of androgens along with the absence of estrogens, while the development of the female body plan, and hence the process of de-masculinization, depends on the presence of estrogens along with the lack of androgens. Humans differ from other animals in this regard, as animals do not develop so gradually along this continuum and the importance of sexual self-identification for animals is much less significant than it is for humans. Research performed on human males with androgen insensitivity syndrome compared to the classical sexual development models which were created from research on rats, indicates that the rat model does not account for the sensitivity of the hypothalamal-hypophyseal-gonadal axis with fluctuations in hormonal levels, namely androgens and estrogens.Over the years, several researchers have attempted to investigate the extent to which estrogen-mimicking chemicals affect the development of male children *in utero*. The first of such papers, published in 1976, detailed the negative effects of the EMC diethylstilbestrol (DES). Researchers emphasized that treatment of pregnant women with DES affected their male offspring. They found that male offspring of women who had received DES as treatment prior to fertilization or were receiving DES post-implantation for prevention of miscarriage had much higher rates of severe reproductive and urogenital abnormalities. This important double blind study looked at 119 control males and 134 DES-exposed males ages 21-23 via physical examination, urine cytology (pre and post ejaculation), prostate fluid cytology and biopsies (for cyst diagnosis). Researchers compared urogenital pathologies, blood hormone levels and complete semen analysis and found results consistent with their original hypothesis of increased abnormalities in the DES-exposed males. Findings included increased unilateral and bilateral epididymal cysts, increased unilateral and bilateral testicular hypertrophy, decreases in flaccid penis length (hypoplastic penis length less than 4 cm), slight decreases in blood follicle stimulating hormone (FSH) and testosterone

levels, and severely decreased sperm count and sperm motility. The authors concluded, "Administration of DES during pregnancy appears to be followed by latent effects on the male genital tract imparing fertility in a certain number of patients."A paper published in 1983 detailed the extent to which DES caused problems for children and adults whose mothers were treated with DES during preganacy. Researchers found that male children who were exposed to DES during gestation were 80 percent more likely to be born with a genital deformation. Even males who were born with normal-appearing genitalia had decreased testicular volume when fully matured. The findings also showed that the time of exposure during gestation, and the amount of the phytoestrogen ingested, is important factors in determining the extent of the pathology exhibited at birth and during the pubertal years. Although genistein did not adversely affect pregnancy, survival or delivery, exposure in early gestation caused a shortening in AGD and overall feminization of external male genitalia, even at low doses.

1.7 Phytoestrogens: Present State of Research

Research in phytoestrogens has increased dramatically in the past several years as seen by the numerous publications. Research is still needed to evaluate the safety of phytoestrogens on human systems, beneficial and harmful doses, gender differences in response to phytoestrogens, differences in the chemical classes of phytoestrogens and the effects phytoestrogens may have with other drugs or dietary products. Phytoestrogens are common in the human diet and able to exert many biological effects that have been observed in cell, animal and human systems. These phytochemicals are postulated to be endocrine disrupters and have the ability to mimic the physiological effects of steroid hormones, specifically estrogen.These group of endocrine disrupters which has been studied extensively are the phytoestrogens (Casanova *et al*., 1999). Phytoestrogens are non steroidal, diphenolic structures found in many plants that have the capacity to bind estrogen receptors and are abundant in fruits, vegetables, legumes, whole grains and especially flaxseed, clover and soy products. They have many similar physiochemical and physiological properties to that of estrogen. On the other hand, soy isoflavones mixtures have been employed for cancer prevention (Faqi *et al*., 2004) and genistein (one of the primary soy isoflavones) has been identified as a potent tyrosine kinase inhibitor that modulated tissue differentiation, cell proliferation, growth and development of neoplasia in several target organs. Genistein and several other isoflavones and their metabolites have induced apoptosis in cancer

cells (Faqi *et al*., 2004). Isoflavones may also be involved in the prevention and alleviation of menopausal symptoms as well as inhibition or delay of cardiovascular disease (Faqi *et al*., 2004). In addition to its positive effects, consumption of phytoestrogens may also be associated with brain aging. Experiments performed by White *et al*., (2000) indicated that regular dietary exposure to soy isoflavones over nine years during middle life (age range 55-74) may be associated with the appearance of accelerated brain aging in later life. Alternatively, further investigations reported that estrogen replacement therapy (ERT), when given to postmenopausal women who were no longer able to produce estrogen, resulted in a decreased risk of Alzheimer's disease and dementia.

Previous research also indicated the role of phytoestrogens in memory and cognition, more specifically, visual spatial memory. As a result of their ability to successfully mimic the actions of endogenous estrogens. Exposure to these compounds may alter fertility by disrupting several aspects of reproduction such as sexual development, timing of puberty, sex-dependent behaviors, testicular and ovarian endocrine functions, gamete production, pregnancy and lactation. The initial recognition of the endocrine disrupting properties of phytoestrogens on reproduction was made in the 1940s, when it was found that ewes grazing in clover pasture developed infertility syndrome due to the exposure to high levels of formononetin, an isoflavone present in red clover (*Trifolium pretense*) This syndrome, referred as "clover disease" resulted in reduced ovulation rates, low lambing rates and structural defects in the reproductive tract. In the wild, one study found high levels of phytoestrogens in the leaves of stunted desert annuals in a dry year, leading ultimately to impaired reproduction when ingested by the California quail (*Lophortyx californicus*). In wet years, these quails bred normally and phytoestrogens were largely absent in these herbs (Leopold *et al*., 1976). Finally in captured cheetahs, cases of infertility and liver disease were attributed to the consumption of a soy-based diet and the exposure to high levels of isoflavones (~50 mg/day) (Setchell *et al*., 1987). Exposure of phytoestrogens and various EDCs that may cause additive or synergistic detrimental effects on both male and female reproductive systems.

1.8 Probiotics and phytoestrogens

Probiotics are organisms such as bacteria or yeast that are believed to improve health. They are available in supplements and foods. The digestive system is home to more than 500 different types of bacteria. They help keep the intestines healthy and assist in digesting food. They are also believed to help in improving and building the immune system. Intestinal microflora especially probiotic bacteria play a key role in the metabolism and bioavailabilty of isoflavones as they hydrolyse the glycoside components using their indigenous β-glucosidase and β-galactosidases in the jujenun, releasing the bioactive aglycone isoflavone form (Setchell *et al*., 2000). Aglycone forms have also been found to absorb faster and in higher amounts in human than their respective glycoside forms (Izumi *et al*., 2000). Intestinal bacteria are known to convert daidzein to its metabolite, equol, which is more potent estrogenic compound than its precursor (Setchell *et al*., 1999). Thus the use of probiotic bacteria to improve the biological activity of phytoestrogen based products during processing formed an integral part of this study. In recent years, the probiotic effects of Lactic Acid Bacteria have gained interest in terms of their functional aspect. Synbiotics refer to nutritional supplements combining probiotics and prebiotics in a form of synergism, hence synbiotics. Probiotics can balance intestinal bacteria by producing organic acid, bacteriocins, and antimicrobial peptides. This may lead to a competitive displacement of intestinal pathogens, the engagement of cell membrane receptors, which activate signaling events leading to cytokine synthesis, including interferons, and cell resistance to viral attack.

Material and Methods

1.1 Collection and Storage

Traditional herbal formulations were collected from the local markets of Punjab (India) and these areas are not being disclosed for the sake of confidentiality**.** Samples were collected and stored in polypropylene bottles at moisture free conditions and transferred to laboratory within 17-24 hours. Moreover they were stored under dark and cool conditions at 28°C.

1.2 Reference standards and Materials

Reference standards for isoflavones namely (Daidzein, Genistein, Biochanin A, Coumestrol, and Formononetin) were purchased from Sigma-Aldrich (USA). All standards used were of analytical grade and prepared in HPLC grade methanol for use.

1.3 Extraction of is flavones from collected samples.

The method developed for isoflavones extraction from samples was adapted from the extraction method reported by Wong (1962), Beck (1964), Francis *et al*., (1965) for analysis of clover forage. Several modifications were made to these methods in part to account for small sample size in present study and to prepare the sample extract for subsequent analysis by High performance liquid chromatography. Approximately 5 gm. of collected sample were ground with washed, dried sand using a mortar and pestle. The ground sample was allowed to stand for 10 min as per Francis *et al*., (1965) to permit hydrolysis of the phytoestrogens glycosides by the herbal drug sample glycosidase.

The sample was then refluxed with 5 ml 2N HCL and 20 ml methanol for 30 min to further hydrolysis. After the 10 min cooling period, the Soya slurry was filtered through what man No.1 filter paper. The filter cake was washed with methanol and discarded. The filtrate was tr ansferred to a 1000 ml round bottom flask with rinsing water. Evaporation to remove a portion of methanol in the filtrate was carried out under vacuum for 25 min using a rotary flash evaporator in conjunction with water bath at 30° C. This temperature allowed rapid removal of methanol yet limited the amount of heat applied to the sample. The remaining aqueous methanol extract was transferred to 250 ml separatory funnel, again rinsing the flask with water. Volume was adjusted

to with water too as previously calibrated 18.5 ml mark. Lipids and chlorophyll pigments are removed by extraction with 3 × 20 ml portions of petroleum ether (b.p 37-58 C).After the addition of petroleum ether the mixture was shaken vigorously for 30 sec and allowed to stand for 10 min to permit the phases to settle and the pigments to transfer to the upper petroleum ether layer.

The final extraction of isoflavones from Soya sample was accomplished with 4 × 20 ml portion of diethyl ether .Each aqueous layer mixture was shaken for 30 sec and allowed to stand for 10 min before the removal of diethyl ether. The ether extract was combined and evaporated under vacuum at 32 C for 15 min to approximately 1 ml. The remaining residue was transferred into a 10 ml volumetric flask with HPLC graded methanol rinses and diluted to volume. Sample was stored in borosilicate glass vials at 5 C until analyzed as depicted below

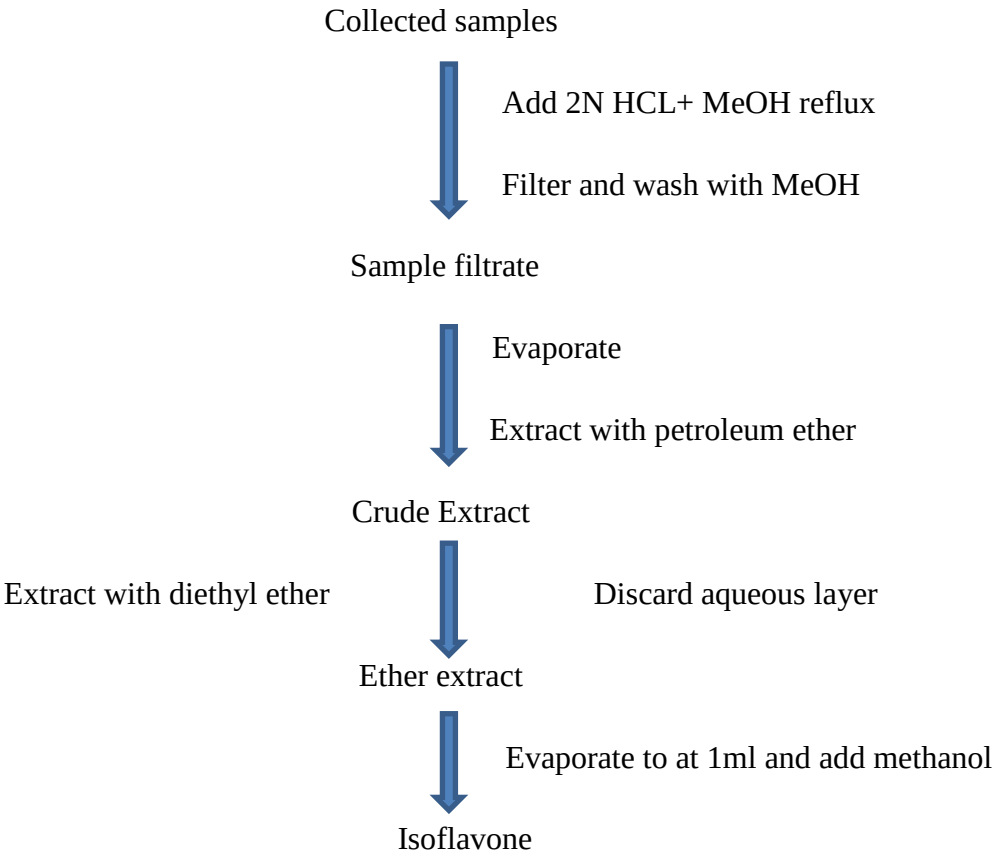


Figure 3.1 Extraction procedures of phytoestrogens

1.4 Detection techniques for phytoestrogens

Thin layer chromatography

The thin layer chromatography method was developed by beck (1964). Beck tested 60 solvents and solvent mixture to resolve the estrogenic isoflavones from clover. Chloroform/methanol (89: 11, v/v) was found to be the only solvent system to give a satisfactory separation on silica gel plates. Formononetin and daidzein could be visualized as blue-white fluorescent spots under UV 11 light (257 nm). Genistein and biochanin A could be visualized as orange brown spot after spraying diazotinized sulphanilic acid.

High performance liquid chromatography

All HPLC analyses were performed using a Reverse phase column (C-18) octadecylsilane (4 mm I.D ×30 cm) by(Murphy *et al*.,1978). Detector used was UV-VIS variable wavelength detector. Colum temperature was maintained at 30 ˚C. Detection of phytoestrogens was made at 254 nm (optical bandwidth 8 nm). The methanol, water used for this purpose was HPLC graded. Separation was achieved by using a linear methanol-water gradient system at a flow rate of 1.0 ml/min. Methanol and water reservoirs each contained 1 % glacial acetic acid (v/v) and 0.01 M ammonium acetate HPLC grade by (Casteele *et al*. 1982). The gradient was programmed to increase from 53% to 58% reservoir B (methanol) over 30 min. total analysis time per sample, including column equilibration was 60 min. Preliminary peak identification was based on a comparison of retention times of phytoestrogens standards and unknown peaks in the sample extracts.

1.5 Behaviour of representative gut flora to phytoestrogens

Overnight grown cultures of *L. lactis, B. adolescentis* and *B. bifidum* were treated with different concentrations (5 to 50 mg/ml)) of extract in the presence of MRS media in a automated high throughput microplate reader and absorbance was measured at 600 nm. Viability was measured as log CFU/ml on MRS agar media by plate count method.

Four day challenge test was performed on *bifidobateria* by adding extract in different concentrations with MRS media .Optical Density was observed at 600 nm after every 12 hours

1.6 Spectrophotometric analysis of testosterone.

1) Interaction of steroids with bovine serum albumin (BSA)

The sample solution consists of progesterone and estrogen mixed each with stearic acid and BSA in three different concentrations and the absorbance is noted at 410 nm.

a. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 5mg BSA

b. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 10mg BSA

c. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 15mg BSA

d. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 5mg BSA

e. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 10mg BSA

f. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 15mg BSA

2) Interaction of steroids with alcohols

The sample solution consists of progesterone and estrogen mixed each with ethanol and propylene glycol in the following concentrations and the absorbance of each sample is noted at 410nm.

a. 0.5ml progesterone (2mg/ml) + 2 ml ethanol

b. 0.5ml progesterone (2mg/ml) + 2 ml propylene glycol

c. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol

d. 0.5ml estrogen (0.5mg/ml) + 2 ml propylene glycol

3) Interaction of steroids with stearic acid

The sample solution consists of progesterone and estrogen mixed each with ethanol and stearic acid in three different concentrations and the absorbance is noted at 410 nm.

a. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.1 ml stearic acid(0.5mg/ml)

b. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.2 ml stearic acid(0.5mg/ml)

c. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.4 ml stearic acid(0.5mg/ml)

d. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.1 ml stearic acid(0.5mg/ml)

e. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.2 ml stearic acid(0.5mg/ml)

f. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.4 ml stearic acid(0.5mg/ml)

Selected assays for probiotic functions of gut flora challenged with phytoestrogens

1.7 Effect of formulations on human beneficial functions of representative gut flora

As the mean intestinal bile concentration is believed to be 0.03% to 0.3% (w/v) (Prasad, etal., 1998), the residence time of food in small intestine is approximately 4 h, the experiment was carried out at this concentration of bile for 4 h. MRS medium containing 0.3% and 3% bile (Oxoid) was inoculated with active overnight grown cultures (incubated for 16-18 h). Survival of the strains were then examined by monitoring growth for 4 hours after inoculation and viable colonies were enumerated for every hour with pour plate technique and by monitoring OD at 620nm and plotted against CFU/mL by calculating with the graph optimized for CFU/mL and OD.

1.8 Glutamate Decarboxylase Activity

A rapid assay (Cotter *et al.*, 2001) was used to determine qualitatively the glutamate decarboxylase (GAD) activity in the isolate with isoflavones (daidzein and formononetin). One-milliliter volumes of overnight cultures in MRS were washed in quarter strength of Ringer's solution and resuspended in 0.5 ml of test reagent adjusted to pH 3.0 with 1 M HCl. The reagent consisted of 90 g l-1 NaCl, 1g L-glutamic acid, 0.3 g l-1 Triton X-100 and 0.05 g l-1 bromocresol green, made up in distilled water. The development of a blue coloration after 4 h of incubation at 37 degree C indicated a positive result.

1.9 Microbial Adhesion to Solvents

Microbial adhesion to solvents (MATS) was measured according to the method of Rosenberg *et al*. (1980) with some modifications (Fontaine *et al*., 1996). *L.casei* treated with isoflavones were harvested in the stationary phase by centrifugation at 5000 g for 15 min, washed twice, and resuspended in 0.1 mol-1 KNO3 (pH 6.2) to approximately 108 CFU ml-1. The absorbance of the cell suspension was measured at 600 nm (A0). One milliliter of solvent was added to 3 ml of cell suspension.

After a 10-min preincubation at room temperature, the two-phase system was mixed by vortexing for 2-min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A1) was measured. The percentage of bacterial adhesion to solvent was calculated as (1 - A1/A0) * 100. Three different isoflavones were tested in this study. *L.casei* adhesion to xylene reflects cell surface hydrophobicity or hydrophilicity.

Results and Discussions

4.1 Selection and Screening

Two hundred (200) samples were collected from different parts of Punjab and stored in polypropylene bottles at moisture free conditions. They were transferred to laboratory within 17-24 hours and were stored under dark and cool conditions at 28°C. The phytoesterogens were extracted from the samples using a established protocol .Out of two hundred samples ninety percent of the samples contain genestein,diadzein and coumestrol and fifty percent of the samples contain formononetin and biochanin A as observed from the initial results using thin layer chromatography. Out of 200 samples, ninety percent of the samples contain testosterone.

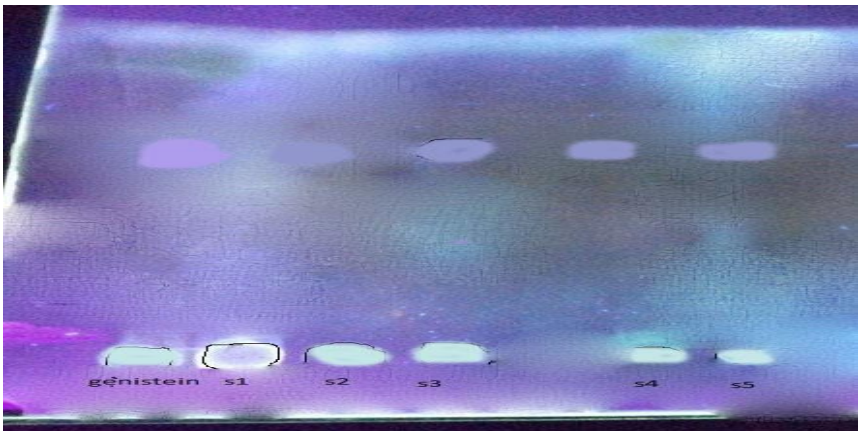


Fig.4.1 Thin layer chromatography Spot of Genistein

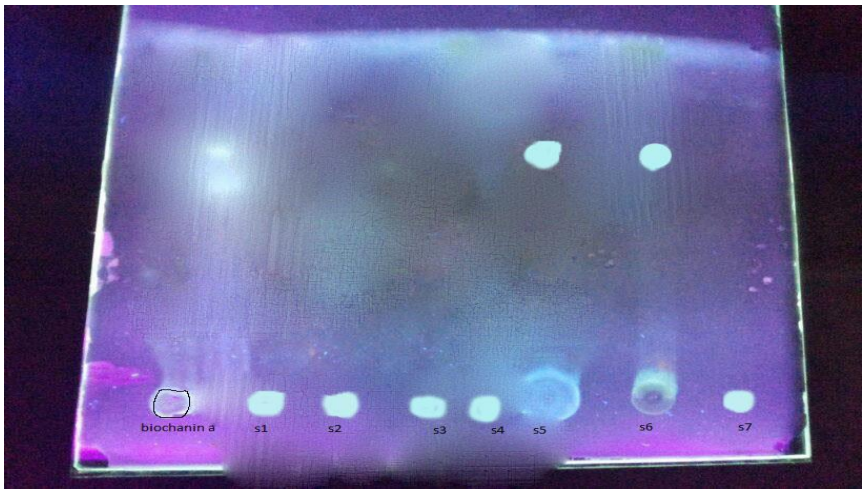


Fig 4.2 Thin layer chromatography spot of Biochanin A

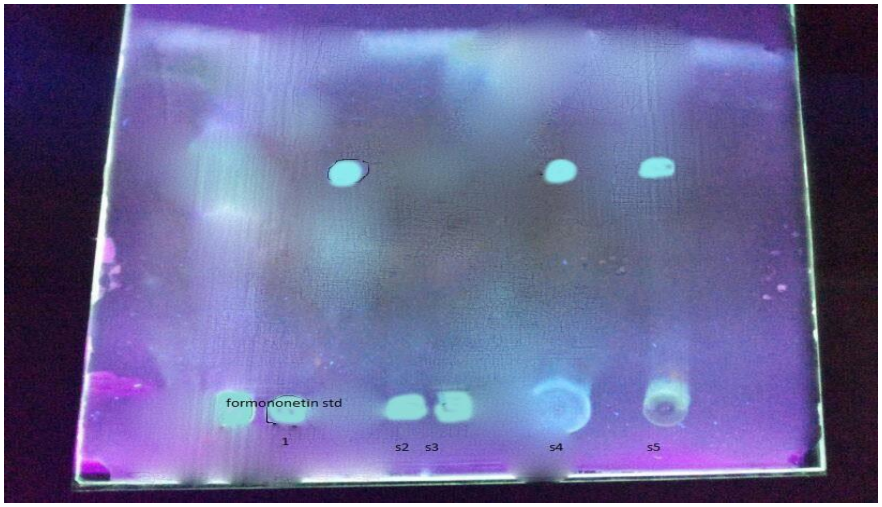


Fig 4.3 Thin layer chromatography spot of Formononetin

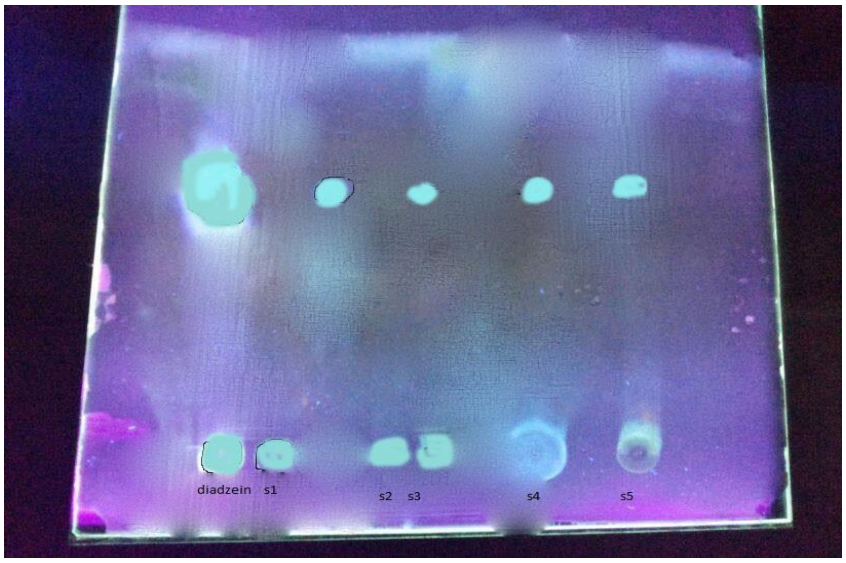


Fig 4.4 Thin layer chromatography spot of diadzein

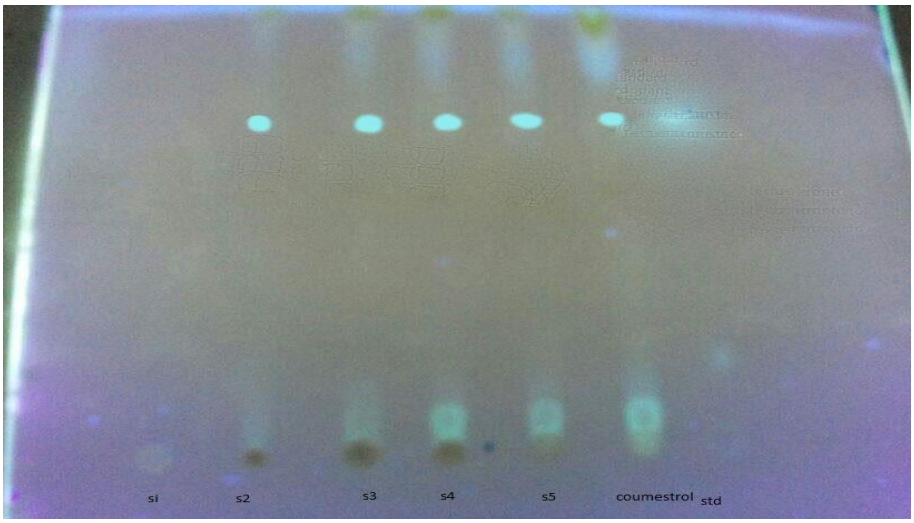


Fig 4.5 Thin layer chromatography spot of coumestrol

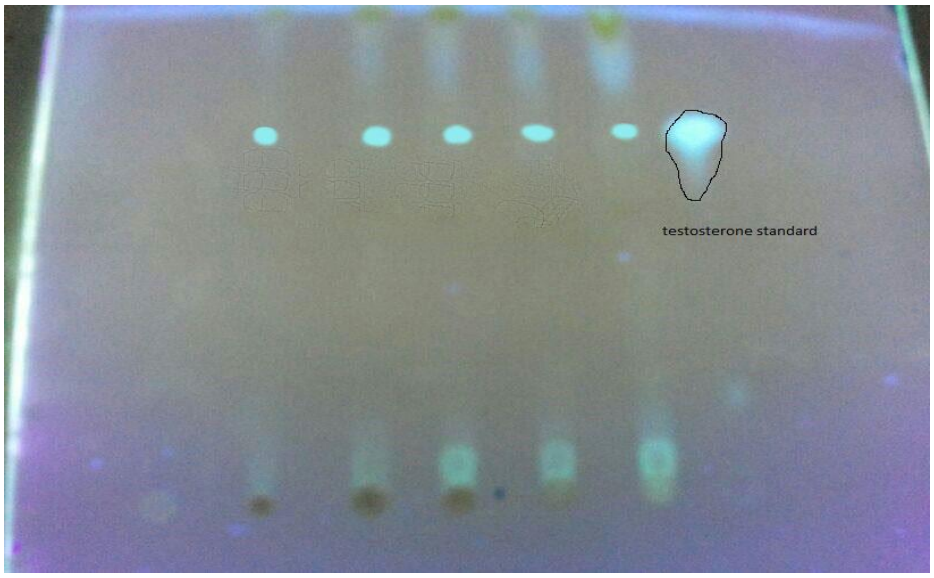


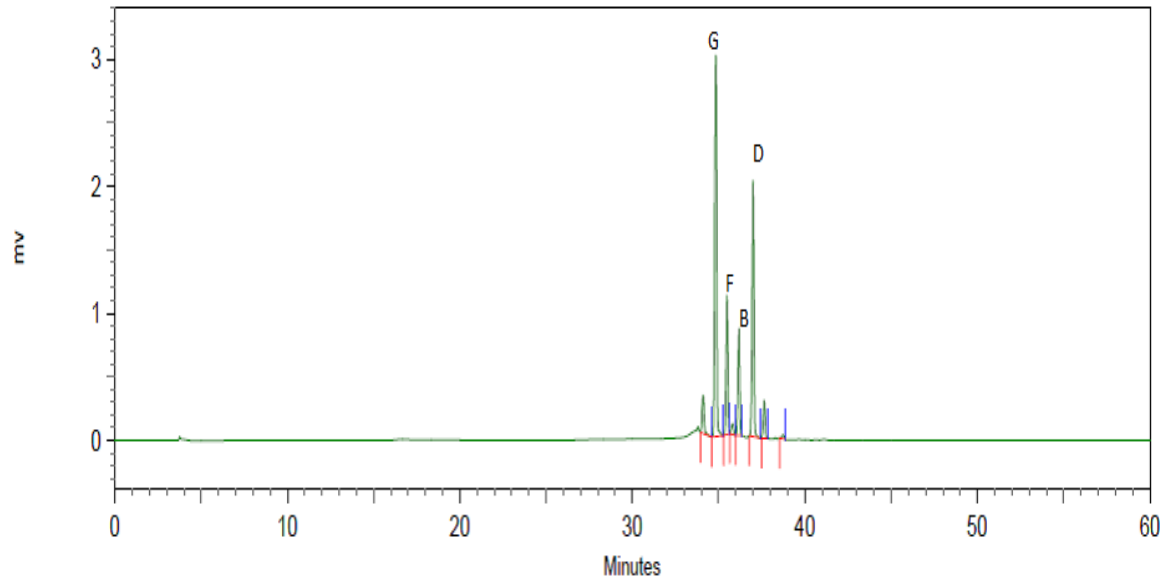
Figure 4.6 Detection of testosterone

4.2 Determination of phytoestrogens

HPLC

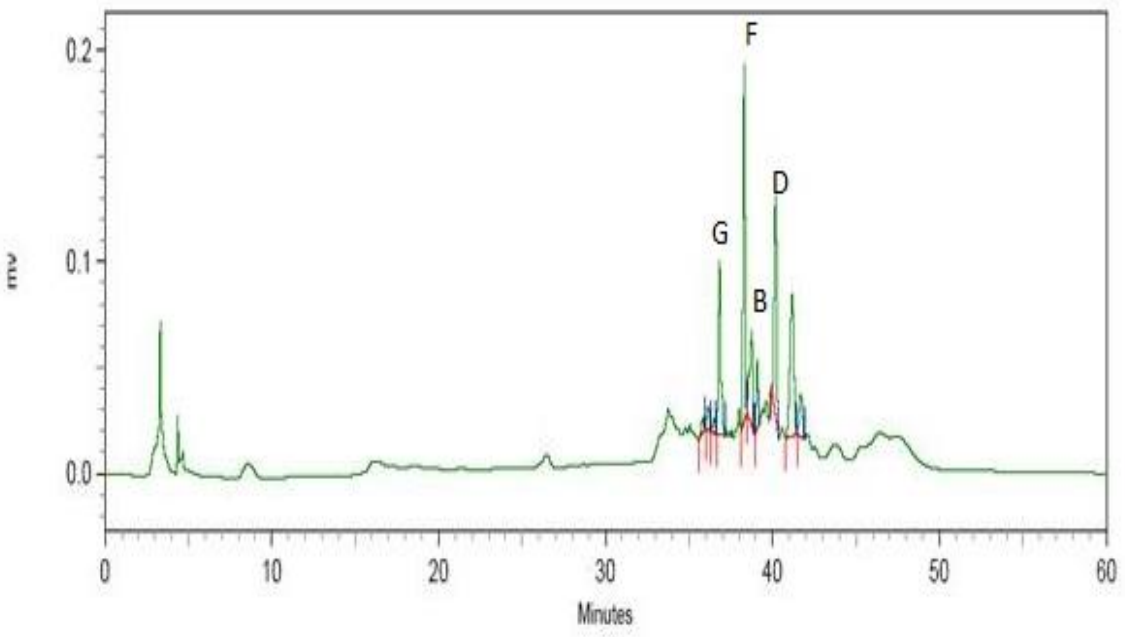
High Pressure Liquid Chromatography was performed for the analysis of the samples quantitatively as per the method of Casteele *et al*. (1982). Fig 4.7 shows the HPLC chromatogram for four standard phytoestrogens namely genestein (G), formononetin (F), biochanin (B) and diadzein with the corresponding peaks at 3.5, 3.7, 3.8 and 3.9 Retention time respectively. The standard phytoestrogens were prepared in the concentration of 0.8ng/ml)

Fig 4.7 HPLC Chromatogram of Standards genestein (G), formononetin (F), biochanin (B) and diadzein (D)



The sample prepared was analyzed earlier for the presence of phytoestrogens by TLC was confirmed by HPLC. The chromatogram for the sample confirmed the presence of genestein (G), formononetin (F), biochanin (B) and diadzein (D) at corresponding RT at 3.5,3.7,3.8 and3.9 respectively. The sample contains 0.08 ng/ml of genestein, 0.01 ng/ml of formononetin,1.61 ng/ml of biochanin and 0.04 ng/ml of diadzein.

Fig. 4.8 HPLC Chromatogram of sample Genestein (G), Formononetin (F), Biochanin (B) and Diadzein(D)



On the basis of above calculations, we calculated the amount of phytoestrogens administered during 10 day course and it comes out to be 6.96 x 10^{-8} grams.

4.3Growth kinetics of *Bifidobacterium adolescentis* in the presence of phytoestrogens

The growth kinetics of *Bifidobacterium adolescentis* in MRS supplemented with varying concentrations of phytoestrogens was evaluated over a period of 48 hours. Each experiment was performed in triplicates and result was the average of three readings. Lag phase was four hours and It grew exponentially for 16 hours . Upon increasing the concentration of the dosage the inhibition of growth was noticed.There was significant decline in highest cell density i.e four fold decrease in this case.Specific growth rate was 6.14. There was 2 log reduction in the growth *of Bifidobacterium adolescentis.*.

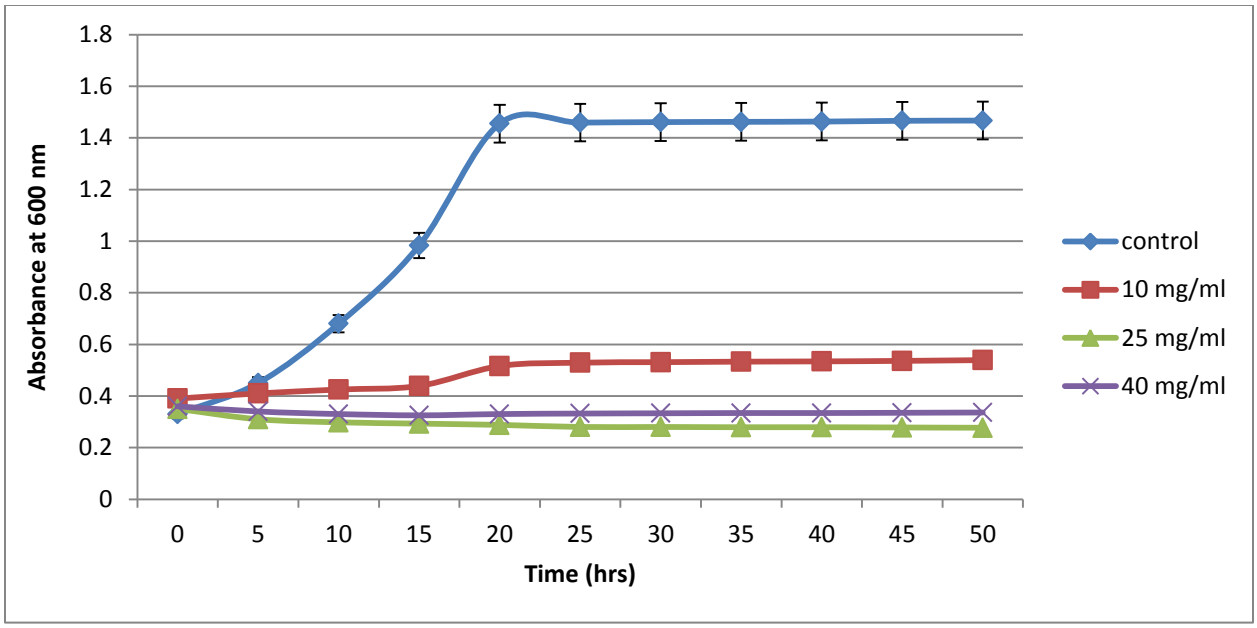


Fig. 4.9 Growth kinetics *of bifidobacterium adolescentis* in MRS supplemented with varying concentrations of herbal formulations

Table 4.1 Viability obtained after 4 day challenge test for *bifidobacterium adolescentis*

SERIAL NO.	CONCENTRATION(mg/ml)	LOG CFU
1	Control	8.6
2	5	7.4
3	10	6.3
4	15	5.3
5	20	4.2
6	25	3.0
7	30	2.9

4.4 Growth kinetics of *Lactococcus lactis* in the presence of phytoestrogens

The growth kinetics of probiotic strain *L. lactis* in MRS supplemented with varying concentrations of phytoestrogens was evaluated over a period of 18 hours. Each experiment was performed in triplicates and result was the average of three readings. Lag phase was two hours and It grew exponentially for 14 hours. With an increase of concentration of the dosage, inhibition of growth was prominient. There was significant decline in highest cell density i.e three fold decrease in this case. Specific growth rate was 2.2. There was 1 log reduction in the growth of *Lactococcus lactis*

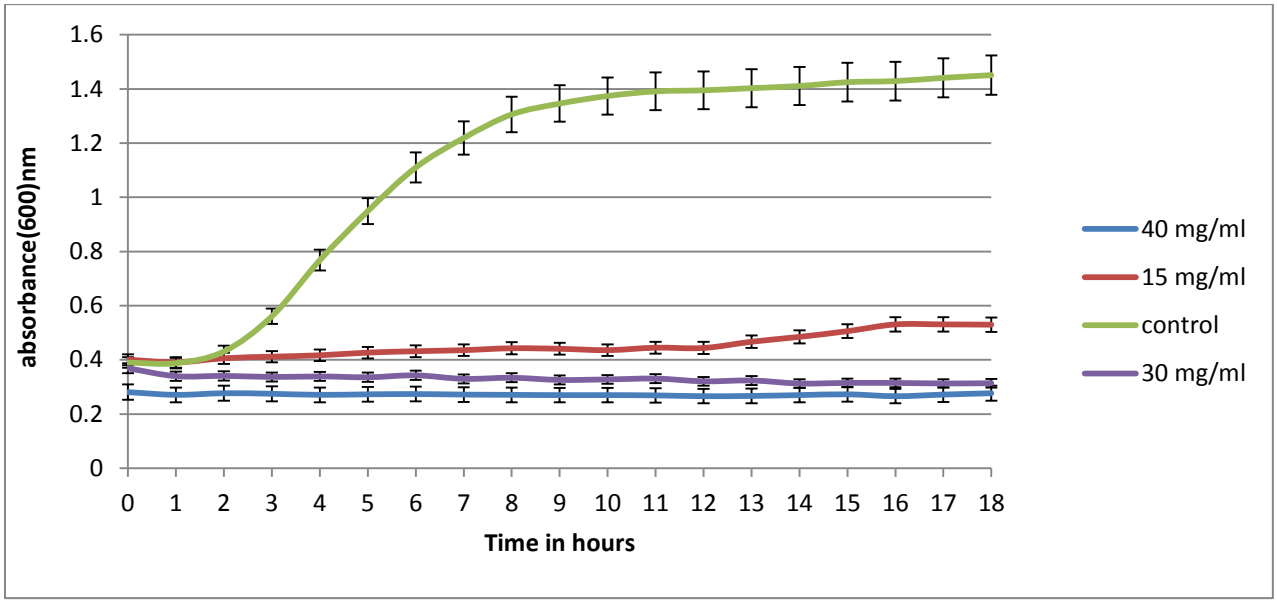


Fig.4.9 Growth kinetics of *Lactococcus lactis* in MRS supplemented with varying concentrations of herbal formulations

Table 4.2 Viability obtained after challenge test for *Lactococcus lactis*

SERIAL NO.	CONCENTRATION(mg/ml)	LOG CFU/ml
1	control	8.6
2	20	7.5
3	25	6.4

4	35	5.1
5	50	4.9

For Bifidobacterium bifidum growth was observed to be completely inhibited by the concentrations of phytoestrogens indicated above.

MATS

MATS method was used to evaluate the hydrophobic/ hydrophilic cell surface properties of *L.lactis and* bifidobacterium so as to compare them with the cell surface properties of *both the strains* grown with phytoestrogen extract .The hydrophobicity observed was in the order Control>Extract The percentage adhesion with Xylene was found to be 77% (*Lactococcus lactis*) and 70% (*Bifidobacterium adolescentis*) of Control and 40% (*Lactococcus lactis*) and 30%(*Bifidobacterium adolescentis*) when treated with phytoestrogen extract The fact that a high percentage of *L.lactis and Bifidobacterium adolescentis* cells adhered to xylene, a polar solvent, demonstrated hydrophobic cell surface of this strain. The results indicated that *Lactococcus lactis and Bifidobacterium adolescentis* were significantly ($p<0.05$) less hydrophobic when treated with phytoestrogens.

GAD ACTIVITY

Among gram-positive bacteria, GAD acid resistance system is the only amino acid decarboxylation system that has been associated with acid response. The GAD system as an acid defense mechanism has been described for *L.lactis* among lactic acid bacteria (Cotter *et al.*, 2001). A slight GAD activity was observed by *L.lactis and bifidobacterium adolescentis* treated with phytoestrogens indicated the malfunctioning of this acid defense mechanism in these strains upon phytoestrogen challenge. It may be presumed that a lack or defective acid tolerance system may impair the cells to survive the acidic conditions in the gut and thus affect their viability.

Table no 4.3 GAD activities of both strains

SERIAL NO	CULTURE	GAD ACTIVITY
1	control	+ + +
2	*Lactococcus lactis*	+ +
3	*Bifidobacterium adolescentis*	+

BILE SALT TOLERANCE

Small intestine and colons of humans and animals contain relatively high concentrations of bile acid in the range of 0.3-3% (Prasad *et al*., 1998) which can inhibit growth of many bacteria. Both the strains were screened for its ability to tolerate bile salts as tolerance to these salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Marteau *et al.,* 1997). One log reduction is observed between treated and untreated indicating an inhibitory effect of the phytoesterogens on the cultures

Overall the results of this study indicated a potential inhibitors effect of the phytoesterogens administered during traditional sex selection practices especially on gut flora. More studies are required to systemically elucidate these initial findings.

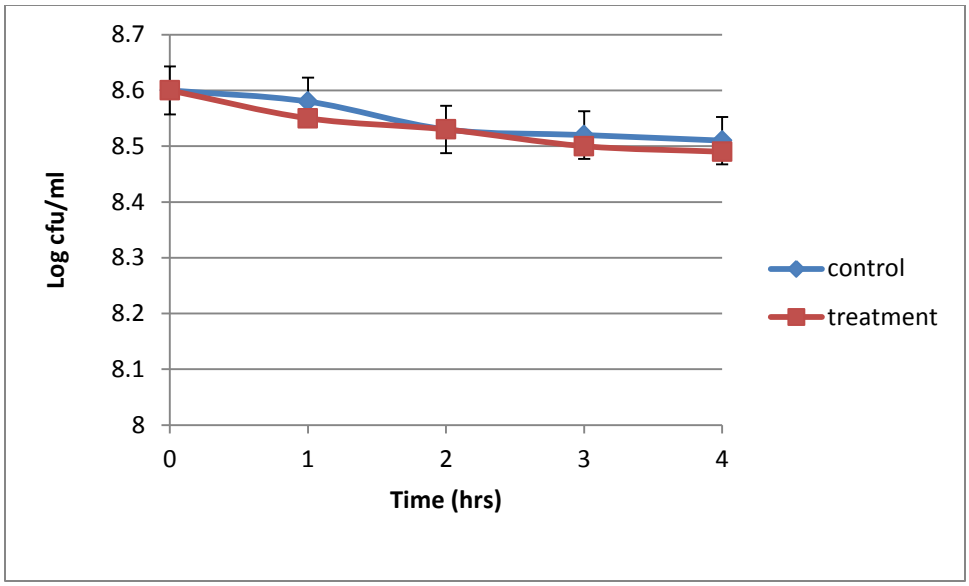


Fig.4.10 Bile Salt Tolerance of *L.lactis*

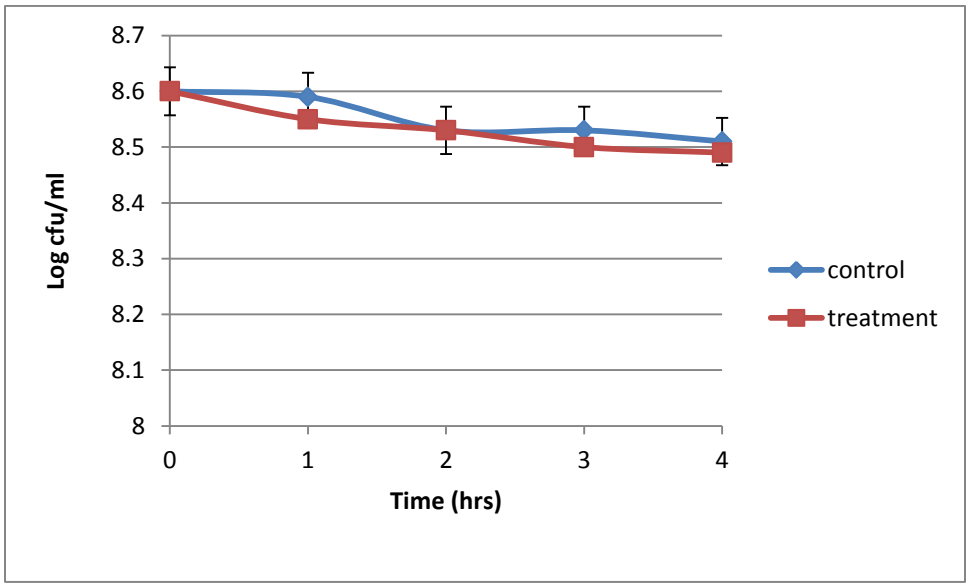


Fig.4.11 Bile Salt Tolerance of *Bifidobacterium adolescentis*

Conclusion

Two hundred samples of herbal formulations used illegally for sex selection practices were analyzed for phytoesterogen content. All samples tested positive for phytoesterogens, these were later quantified by HPLC. The phytoestrogens containing samples were inhibitory to the survival of representative gut flora namely: *L. lactis* and *B. adolescentis* .Potential loss of some beneficial probiotic attributes was also observed suggesting the detrimental effect of these formulations. Further detailed studies are important in concluding the possible threats of these formulations and their role in physiology, mental development of the newborn.

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APPENDIX

7.1 Man Rogosa Sharpe (MRS) Broth

Proteose peptone	10g/ml
Beef Extract	10g/ml
Yeast Extract	5g/ml
Dextrose	20 g/ml
Polysorbate 80 (Tween 80)	1g/ml
Ammonium citrate	2g/ml
Magnesium sulphate	0.1g/ml
Mangaese sulphate	0.05g/ml
Dipottassium phosphate	2g/ml
Sodium acetate	5g/ml
Distilled water	1000ml

Ringer's solution (Full Strength)

Component	**Amount (g/l)**
Sodium chloride	9
Potassium chloride	0.42

Anhydrous Calcium chloride	0.24
Sodium bicarbonate	0.2