Adsorptive Removal and Regeneration Study of Biochanin A: An Endocrine Disruptor

A Thesis

submitted in the partial fulfilment of the requirement for

award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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JULY 2015

CERTIFICATE

This is to certify that the thesis entitled "**Adsorptive Removal and Regeneration Study of Biochanin A: An Endocrine Disruptor**" submitted by **Ms. Kamalpreet Kaur (301301006)** in the in partial fulfillment of the requirement for the award of Master of Science in Biotechnology, Department of Biotechnology (DBT), 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 degree or certificate in this or any other University or Institute.

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CANDIDATE DECLARATION

I, hereby declare that the work presented in this thesis " **Adsorptive Removal and Regeneration Study of Biochanin A: An Endocrine Disruptor**" in partial fulfillment of the requirement for the award of Master of Science in Biotechnology, Department of Biotechnology (DBT), Thapar University, Patiala, is an authentic record of my work during the period of six months from January 2015 to July 2015, under the guidance of **Dr. Moushumi Ghosh**, Professor (DBT) and **Dr. Dipaloy Datta**, Assistant Professor, Department of Chemical Engineering, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any degree or diploma.

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ACKNOWLEDGEMENT

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. It gives me immense pleasure to acknowledge with attitude. The help and guidance rendered to me by a lot of people.

First of all, I sincerely acknowledge my gratitude to Almighty GOD for His compassion made me to fell to have and to see the moment whose euphoria is not vanishing from the mind will remain till eterny.

I beat the rays of my illuminant gratitude to my honourable guide, **Dr. Moushumi Ghosh,** Professor, for her excellent guidance. Her discipline, principles, simplicity, caring attitude, constructive criticism and provision of fearless work environment will be cherished in all walks of my life. I am very much grateful to her for valuable guidance and everlasting encouragement throughout my course.

I am deeply indebted to my co-supervisor, **Dr. Dipaloy Datta** Assistant Professor from Chemical Engineering Department; he helped and encouraged me in all the time of research and in thesis writing.

It gives me great pleasure to express my deep sense of gratitude to **Dr. Dinesh Goyal**, Professor and Head, Department of Biotechnology (DBT), Thapar University, Patiala**,** for allowing me to continue my dissertation work by providing necessary laboratory facilities to fulfill of this work.

My vocabulary utterly fails in expressing my accolade to Mrs. Taranpreet Kaur, research scholar, DBT for her support, valuable suggestions, immense affection, and invariant encouragement throughout this work.

I am grateful to laboratory staff Mr. Babban, Mr. Mohinder, Mr. Surinder and Mr. BP Singh

for their timely help and assistance while completing this project.I thank all my teachers throughout my journey to build me as strong to face all conditions of life. I will be remaining thankful till my last breath.

Words of reverence, gratification and affection for my beloved Parents who brought me to this stage and indebted for their big moral support for me in the completion of my higher studies. It was their confidence, affection and prayer for my success and progress which made it possible for me to complete the task relentlessly in time.

Date: 16/7/15 **KAMALPREET KAUR**

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List of Abbreviations

µl	Microliter
nm	Nanometer
rpm	Revolution per minute
IPCS	International Programme on Chemical Safety
EDCs	Endocrine Disruptors
g/l	Gram per litre
mg/L	Milligram per litre
ESCs	Estrogen receptors
ERβ	Estrogen Receptor beta
O-Dma	*O*-demethylangolensin
END	Enterodiol
ENL	Enterolactone
MMP9	Matrix metalloprotein
VEGF	Vascular endothelial growth factor
SERMs	Selective Estrogen Receptor Modulators
FTIR	Fourier Transformation Infrared Spectroscopy Attenuated Total Reflection
FDA	Food and Drug Administration
AC	Activated carbon
BA	Biochanin A
ppm	Parts per million
PAC	Powered Activated Carbon
GAC	Granular Activated Carbon
Conc.	Concentration
O.D	Optical Density
UV °C	Ultra Violet Degree(s) Celsius
hr	Hour
mL	Millilitre
mg/ml	Milligram per millilitre

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ABSTRACT

Phytoestrogens are produced by plants and mimic the hormone estrogen, and act as Endocrine Disruptors (EDCs). Though prevalence of phytoesterogens in water has been documented, few methods are available for the removal of phytoestrogens from water. In this study the removal of phytoestrogen Biochanin A was attempted from water with the help of adsorption method. The adsorbent materials used were bentonite, chitosan and granular activated carbon. The maximum removal of 98.9% at 5 mg/L with 1.5 mg/g was observed in granular activated carbon. The maximum removal with chitosan and bentonite was 46.1% and 44.2% respectively. The adsorption spectra of biochanin A onto granular activated carbon were studied using different parameters like temperature, pH, concentration of adsorbate and adsorbent. The adsorption study, adsorption isotherm and regeneration study were also carried out.

The optimum pH and temperature for adsorption were found to 6 pH and 25°C. The adsorption isotherm models were applied to study nature of adsorption. The equilibrium data were perfectly represented by the Langmur isotherm with R^2 of 0.9515, showing homogeneous adsorption of biochanin A to granular activated carbon and followed second order kinetics. The active carbon was characterized by Fourier transform infrared spectrophotometry. The granular activated carbon can be regenerated by reversing the adsorption of biochanin A on granular activated carbon, making it as good and inexpensive adsorbent for removal of biochanin A.

1. Introduction

Phytoesterogens alter function of endocrine system and subsequently cause adverse health effects on intact organism or its progeny, or populations. They may be synthetic (polychlorinated biphenyls) or natural (phytoestrogen) and are classed as endocrine disrupting molecules. Phytoestrogens are produced by plants in order to protect them from stress and grazing animals and exposed mainly through diet like soy, grains, fruits and vegetables. They first came to light about 50 years ago when it was observed that some plants could have an adverse effect on fertility of livestock. Phytochemicals are ubiquitous group comprised by phenolic compounds. They are important part of human diet which consists of largest class of flavonoids. Flavonoids can be classified into flavones, isoflavones, flavanones, flavanols and anthocyanins and have structure similarity to their parent compound, flavones (2-phenyl benzopyrone).Studies confirm that due to their structural similarity to the estrogens 17β-estradiol, soya isoflavones can exert hormonal effects in humans as estrogen is female sex hormone and play important role in menstrual cycle and in development of sexual characters. They mimic estrogen hormone and binds to estrogen receptor, mainly β estrogen receptors. Their effects may be of benefit in the prevention of many of the common diseases observed in Western populations (such as breast cancer, prostate cancer, menopausal symptoms, and osteoporosis).Various food or food supplements containing phytoestrogen are often been used as an alternative to hormonal replacement therapy (HRT) in women. They are classified into isoflavones, liganin and coumestrol, however, soybeans predominantly contain a mixture of the isoflavones genistin and daidzine which are metabolised by intestinal bacteria, absorbed, conjugated in the liver, circulated in plasma and excreted in urine. They are hydrolyzed to genistein and daidzein in the gastrointestinal tract by action of β-glucosidases resulting in the formation of compounds with a steroidal structure similar to estrogens. Isoflavones have a nonsteroidal structure but possess a phenolic ring that enables them to bind to estrogen receptors (ESRs) and thus act as ESR agonists or antagonists. However there are many factors on which biological effects are dependent like dose, duration of use, protein binding affinity, individual metabolism and intrinsic oestrogenic state. They act in three ways: agonist, antagonist and can interfere or block the way natural hormones and receptors are made but it occurs only if relatively large amount of doses of the substance are present. So depend upon their action they also act as EDCs. A number of cases have been reported which show the adverse effects of phytoestrogen like increased breast cancer, reduced in fertility in males, subfertility (fewer

pregnancies; fewer pups per litter), longer menstrual bleeding and more discomfort during the menstrual cycle change in sex character in fish and rats. Biochanin A is an O-methylated isoflavone, its IUPAC name is 5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one.Basically an 4-*O*-methyl derivative of genistein, it is present in high concentration in food like soy, alfalfa and red clover and act as EDCs by disturbing hormone balances during pregnancy or breast-feeding and also cause adverse effects like excessive bleeding. Recent studies have highlighted the prevalence of phytoesterogens in river water and industrial waste water. High levels of biochanin A have been detected in water and sediments at the creek and pond sites, horse pastures and subterranean flow (within the tile drain system) in the home's septic system and pasture in several US farms (ISTC, 2010).Biochanin A in the sediment samples were also higher than or comparable to concentrations observed prompting adequate strategies for intervention. Adsorption based methods can potentially aid in removal of water borne chemical contaminants, but few studies thus far have attempted to apply selective adsorptive removal of phytoesterogens especially biochanin A. This study attempted to develop a simple and effective system for removal of biochanin A from water using adsorption; three adsorbent materials - chitosan polymer, bentonite clay and granular activated carbon were investigated.

Scope of study

Phytoestrogens are produced by plants and act as endocrine disruptors. The removal of phytoestrogen is an important concern and is separated by solid phase extraction (Anna and Anna, 2014), by bacteria (Mark and Paige, 2011) and by adsorption method (Huajing, 2006). However, very less work is done to remove phytoestrogen from water. In this study, Phytoestrogen was removed by adsorption method with different adsorbents. The work was done in lab scale and further study need to carry to check feasibility at pilot scale and large scale by using adsorbents. The modification of adsorbent can also be done further.

Objectives

- Adsorptive removal of Standard Biochanin A from water.
- Isothermal modelling of Biochanin A adsorption to the adsorbent.
- Regeneration of adsorbent by extracting the Biochanin from adsorbent.

2. Review of literature

2.1 Endocrine Disruptor

It is noted that some chemicals function as hormones in the living body and may pose risks to human health. These chemicals are designated as endocrine-disrupting chemicals, have recently become a social issue. They interact with endocrine systems by either mimic or blocking the hormone (Environment Canada, 1999). The complete mechanisms by which they disrupt endocrine systems are very complex, and not yet completely understood. The three major classes of endocrine disruptors (Snyder, 2003) are:

Estrogenic – compounds that mimic or block natural estrogen

Androgenic – compounds that mimic or natural testosterone

Thyroid – compounds with direct or indirect impacts to the thyroid

The endocrine disruptors may be synthetic or natural hormones, industrial chemicals and pesticides. Out of 553 substances identified for endocrine disruptor, evidence was found for 163 substances including 2 natural estrogens estrone and 17β-estradiol and 1 synthetic estrogen 17α-ethinyloestradiol, while 106 found to be potential endocrine disruption (Commission of the European Communities, 1999). In this work, estrogenic endocrine disruptors were studied. The phytoestrogen are xenoestrogens i.e., false estrogen which mimic estrogen hormone and binds to estrogen receptor. They cause adverse effects to human and animal health, which includes disruption of lactation, the timing of puberty, the ability to produce viable, fertile offspring, sex specific behaviour, premature reproductive senescence and compromised fertility.

2.2 Phytoestrogen

Phytoestrogens are plant-derived xenoestrogens not synthesised within the body but consumed by eating phytoestrogenic plants and so also called "dietary estrogens". They are similar in chemical structure to that of mammalian estrogen, estradiol, and bind to estrogen receptors (ESRs) most preferably with Estrogen Receptor beta (ERβ) (Turner, 2007). This suggests that these compounds may exert tissue specific effects. These non-nutrient bioactive compounds are present everywhere in the plant kingdom and soy foods and flaxseeds are reported as the most abundant dietary sources of phytoestrogens as they possess a wide range

of biological properties that contribute to the many different health-related benefits. (Kenneth *et al.* 2001). Since biological effects are dependent on many factors including dose, duration of use, protein binding affinity, individual metabolism and intrinsic oestrogenic state (Huber, 2013) therefore various other biological effects independent of the ESRs have been ascribed to these compounds. The effects may be antioxidant capacity, antiproliferative and antiangiogenic effects (Huber, 2013). The phytoestrogrns were ingested, metabolised and excreted from body by urine. There are three types of phytoestrogens are present out of which isoflavone is highly taken through diet while lignans and coumestrols are less consumed by people.

2.3 Structure

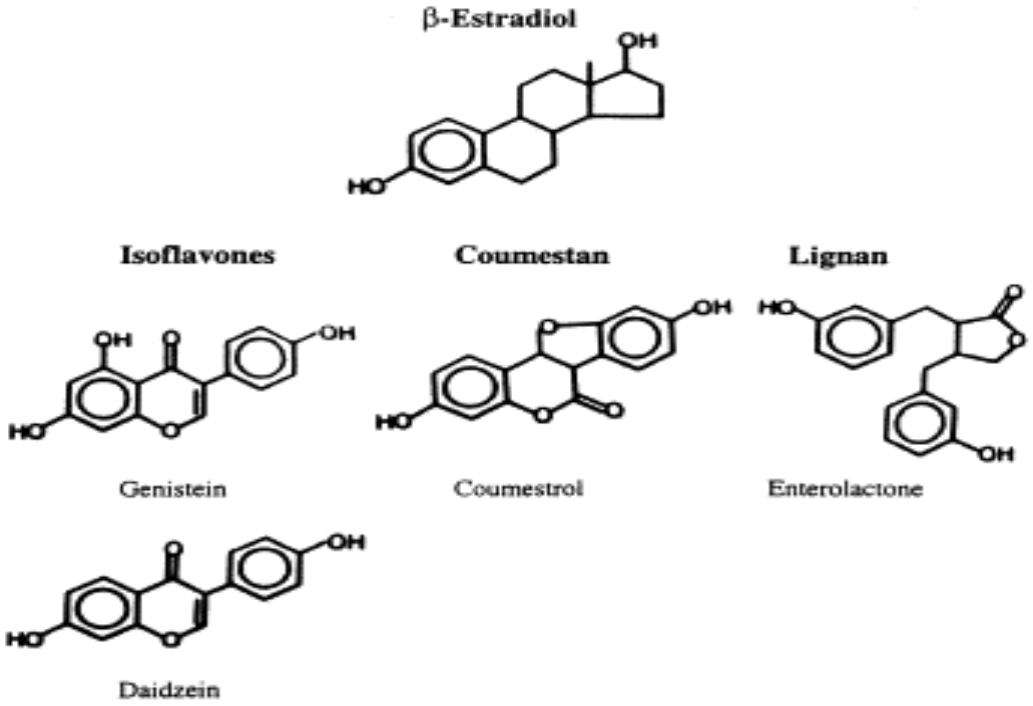

Figure 1: Structure of different types of Phytoestrogen.

(http://content.onlinejacc.org/article.aspx?articleid=1126445)

2.4 Role

Plants produce phytoestrogens as a defence mechanism in order to stop or limit predation by plant-eating animals (Ehrlich and Raven 1964; Guillette *et al*. 1995; Hughes 1988). Instead of protecting themselves with thistles or thorns or tasting bad, these plants produce chemicals that affect the predatory animal's fertility and control their population.

2.5 Classification

The phytoestrogens mostly found in typical human diets can be categorized into two primary classes: isoflavones and lignans. Isoflavones are the most common form of phytoestrogens. They have a common diphenolic structure that resembles the structure of the potent synthetic estrogens diethylstilbesterol and hexestrol. Two of the major isoflavones found in humans are genistein and daidzein. Biochanin A and formononetin are plant precursors which are metabolized to their parent compounds i.e., genistein and daidzein, respectively. In plants, when isoflavones are present in the bound form as glycosides are inactive, but when the sugar residue is removed, these compounds become activated. These plant compounds undergo fermentation by intestinal microflora, with both metabolites and unfermented parent (aglycone) compounds being liable to absorption. Genistein is more selective for endocrine receptor-β than endocrine receptor-α i.e. 7 to 48 fold more as biochanin A is *O*–demethylated which increase its binding with estrogen recerptor-β mainly by 4'-Hydroxyl group. (Barkhem *et al.*1998). The relative estrogenic effect of genistein is approximately 30-fold higher for endocrine receptor-β than for endocrine receptor-α. Once bind to receptors, they act as selective estrogen receptor modulators (SERMS) but not as agonist, like a drug tamoxifen for breast cancer act as endocrine receptor agonist in the uterus and bone but act as antagonist in the breast (Oseni *et al.* 2008).

Most of the phytoestrogens bind to endocrine receptor-β more readily than endocrine receptor-α due the fact of its functionally significance as both endocrine receptor-α and endocrine receptor-β are differentially distributed throughout the body and the brain and appear to upregulate different gene families (Koehler *et al.* 2005). Like in breast tumor cells, by activation of endocrine receptor-β, the suite of genes are upregulated which in turn enhance cell cycle progression and suppress proliferation while activation of endocrine

receptor-α does largely the opposite (Chang *et al.* 2006). Endocrine receptor-β is strongly expressed in breast, bone, the cardiovascular system, uterus, bladder, prostate, lung, ovarian granulosa cells and testicular Sertoli and germ cells (Carlsson *et al.* 1997)

Lignans are compounds possessing a structure of 2,3-dibenzylbutane and present as minor constituents of many plants, where they form the building blocks for the formation of lignin found in the plant cell wall. They are constituents of higher plants (gymnosperms and angiosperms), such as whole grains, legumes, vegetables, and seeds, whereas extremely high concentrations of lignans are found in flaxseed. Although in the biological fluids of man and animals mammalian lignans have been detected (Tham *et al.* 2013).

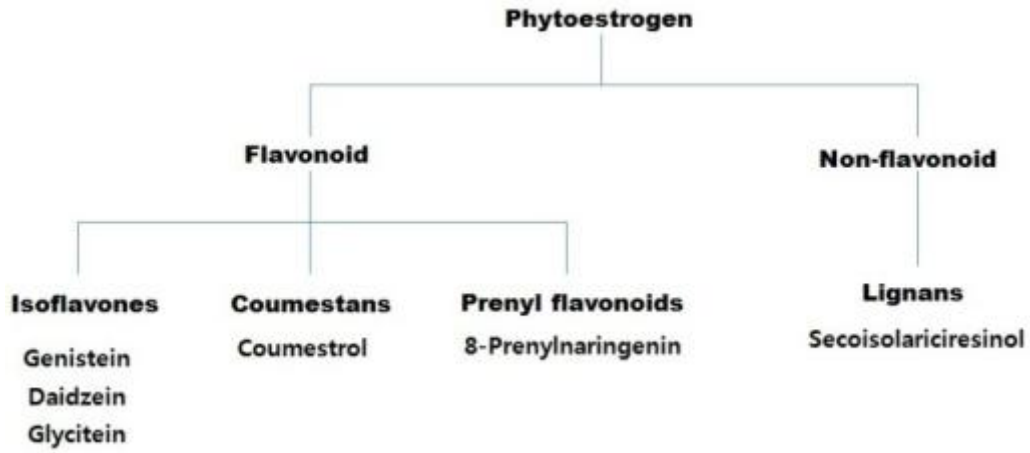

Figure 2: Classification of Phytoestrogens. (Kim and Park, 2012)

2.6 Source of phytoestrogens

There are more than 300 plants found to be contain more than 20 compounds, such as herbs, grains, and fruits. The dietary phytoestrogens are classified into three main classes are isoflavones, lignans, and coumestans (Reinli *et al.* 2009):

Isoflavones (genistein, daidzein, glycitein, and equol) are primarily found in soy beans and soy products, chickpeas and other legumes (Kurzer, 1997).

Lignans (enterolactone and enterodiol) are found in oilseeds (primarily flaxseed), cereal bran, legumes, and alcohol (beer and bourbon).

Coumestans (coumestrol) can be found in alfalfa and clover

(http://crossfitai.com/2012/07/phytoestrogens/)

Figure 3: Sources of Phytoestrogen

2.7 Benefits

Phytoestrogens exert their beneficial effects through several mechanisms that slow cell growth and prevent inflammation in the body. In 1999, the US Food and Drug Administration (FDA) approved that consumption of soy daily is effective in reducing the risk of coronary artery disease which increase the market value of soy products. Numerous studies show that Asian women have lower bone density and lower calcium intake than Caucasian women but still have stronger bones and fewer osteoporotic fractures during menopause due to intake of phytoestrogen rich diet. They also have a lower risk of developing cancer and heart disease (Setchell *et al.* 2003)

Ingestion of isoflavone-rich soy milk for two years increases lumbar bone density by 2.4 percent was shown by one study (Kenny M, 2009). Similarly, the effect of phytoestrogen on risk of breast cancer remains unknown (Patisaul and Jefferson, 2010). It is because the effect varies with exposure during different stages of human development. While eating foods rich in phytoestrogen has helped menopausal women in reducing hot flashes and vaginal dryness but a particular dose or duration of phytoestrogen intake is unknown as their levels and effects in the body are dependent upon individual intake, absorption, metabolism and time of initiation, which can explain the variation in response and benefit between individuals. The bone-sparing benefits of phytoestrogen are mediated by conversion of phytoestrogen to

equol, which approximately a third to half of the population are capable of doing and such metabolic differences might explain the evident inconsistency in the health effects of phytoestrogens (Setchell and Brown, 2003).

2.8 Why we concern?

Phytoestrogens have become one of the more topical areas of interest in clinical nutrition but also show adverse effects in human and animals when taken in higher amount in diet and act as endocrine disruptors. They act as both agonist and antagonist by either increase the release of hormone or by reducing the secretion of hormone respectively. In fact, there effects not depend upon the amount of diet intake but depend upon the conversion of them by intestinal microflora. Some of adverse effects reported were:

Effects on women:

The cases have been reported where women have developed abnormal uterine bleeding due to intake of diet rich in phytoestrogen (Chandrareddy *et al*., 2008)

In vitro, genistein at high doses (>10 M) can inhibit proliferation of ER-positive and ER-negative breast cancer cells but paradoxically promote tumor growth at lower, more physiological doses (Messina and Nagata, 2006).

Feeding of soy-based diets at doses above 11 mg/kg of genistein during development potentially disrupts gonadal development and developed mammary gland hypertrophy (Napier *et al.*, 2014)

In vitro study on mice that both daidzein and equol stimulated the growth of estrogen-dependent breast cancer cells at concentrations between 0.001 and 50 μM (Fultz and Allred, 2006).

Many of the plants have ability to prevent pregnancies or cause miscarriages contain phytoestrogens and other hormonally-active substances are historically noted, like during fourth century BC, Hippocrates noted that the wild carrot (now known as Queen Anne's lace) prevented pregnancies (Riddle 1991) as their seeds contain a chemical that blocks progesterone, a hormone that is essential for establishing and maintaining pregnancy.

The studies report altered ovarian development, altered estrous cycles, problems with ovulation, and subfertility (fewer pregnancies; fewer pups per litter), and infertility (Delclos *et al.* 2001; Jefferson *et al*. 2002, 2005, 2006; Kouki *et al*. 2003; Nagao *et al.* 2001; Nikaido *et al*. 2004; Whitten *et al*. 1993). Other studies on rat found that exposure to genistein during developmental alter pituitary responses that contribute to the ovulation problems (Faber and Hughes 1993; Levy *et al*. 1995). Developmental exposure to genistein altered mammary gland differentiation leading to increased cancer risk is also reported (Hilakivi *et al*. 1999). In addition, mice treated right after birth with genistein had an increased incidence of uterine cancer later in life (Newbold *et al*. 2001).

Effect on Infants

The fetus are also exposed to isoflavones as they can pass from mother to fetus through the placenta, and have been found in human umbilical cord blood and amniotic fluid at levels comparable to concentrations seen in maternal plasma, indicating that fetal exposure is possible (Adlercreutz and Yamada, 1999)

Since development is highly controlled by hormones of the endocrine system, some researchers are most concerned about exposure of unborn fetuses and infants to high levels of phytoestrogens. Human epidemiology studies document adverse effects of genistein. One study found that women eating a vegetarian diet during pregnancy have male offspring with an increased incidence of hypospadias (a birth defect in boys where the penis opening is not located in the normal position at the tip of the penis), possibly due to high maternal levels of soy isoflavones (North and Golding, 2000).

Effect on Nervous System

In prenatal animals the transfer of genistein to the brain from systemic circulation appears to be more efficient than adult animals (Chang and Churchwell, 1970) which indicates that it and other isoflavone phytoestrogens could interfere with the organization of estrogen sensitive neuroendocrine signaling pathways.

Effect on Animals

The fertility of experimental and domestic animals is also adversely affected by diets rich in certain phytoestrogens. For instance, reduction in the number of offspring in wild populations

of California quail (Leopold *et al*. 1976), deer and mice (Berger *et al*. 1977) by phytoestrogens in dry, summertime grasses.

Figure 4: Australian sheep suffering from infertility (Leopold et al. 1976)

Australian sheep suffered from reproductive problems and infertility after grazing in pastures with the phytoestrogen-containing clover Trifolium subterraneum (Bennetts *et al.* 1951)

Phytoestrogen caused a skewing of the sex ratio toward more phenotypic in female zebrafish, but did not cause initiation of vitellogenin in male and undifferentiated fish (Holbech, 2009)

2.9 Factors affecting action of Phytoestrogen

The answer of question whether phytoestrogen act as endocrine disruptor in human and animal or not is very complicated as its action is depend upon many factors like age, health status, level of consumption and composition of an individual's intestinal microflora (Patisaul and Jefferson, 2010). Other factors include the timing of exposure of phytoestrogen to an individual, mostly during puberty and reproductive ages. Due to these all factors the beneficial and adverse effects were vary from individual to individual.

2.10 Mechanism

The key structural elements that enable phytoestrogens to bind with high affinity to estrogen receptors especially ESR-β and display estradiol-like effects are (Yildiz *et al.*, 2005)

- The phenolic ring is necessary for binding to estrogen receptor

- The ring of isoflavones mimicking a ring of estrogens at the receptors binding site
- Low molecular weight similar to estrogens (MW=272)

Phytoestrogens have been identified in bile, urine, semen, blood, and feces in man and animals. They have 2-phenylnaphthalene type chemical structures similar to those of estrogens and have been found to bind to estrogen receptors (Zava *et al.* 1997). Depending on several factors they may exert both estrogenic and antiestrogenic effects on metabolism. The various factors include their concentration, the concentrations of endogenous estrogens, and individual characteristics, such as gender and menopausal status (Adlercreutz *et al.* 1997). The antiestrogenic activity of phytoestrogens may be partially explained by their competition with endogenous 17β-estradiol for estrogen receptors. This partial estrogenic and antiestrogenic behavior is a common feature of many weak estrogens (Martinex *et al.* 1986).

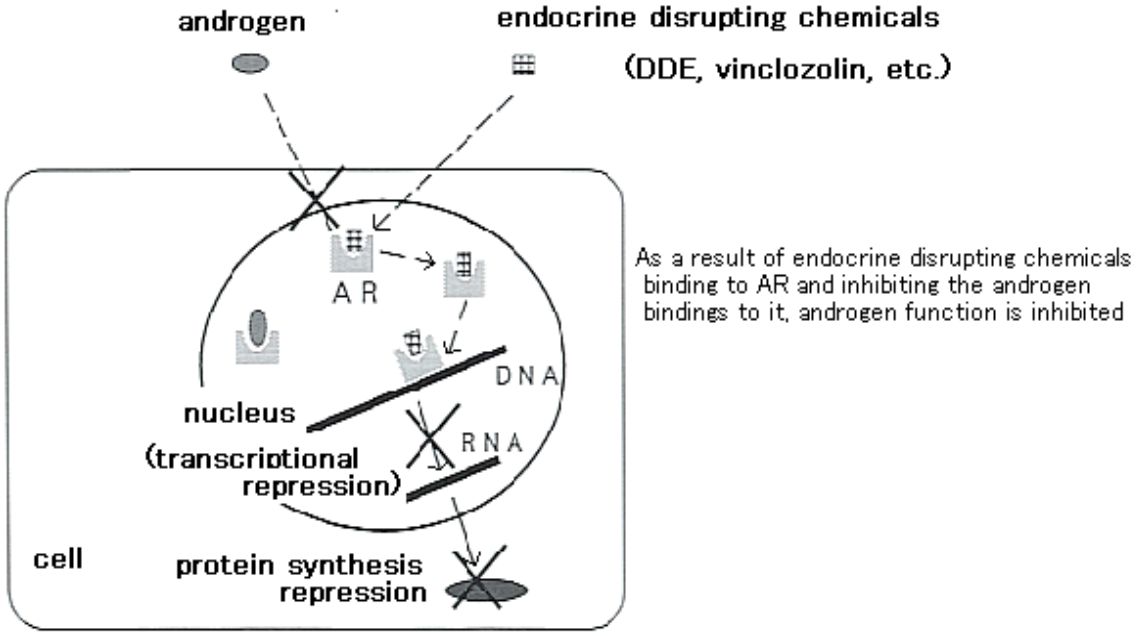

Figure 5: Endocrine disruptor act as antagonist by blocking the endocrine receptor.

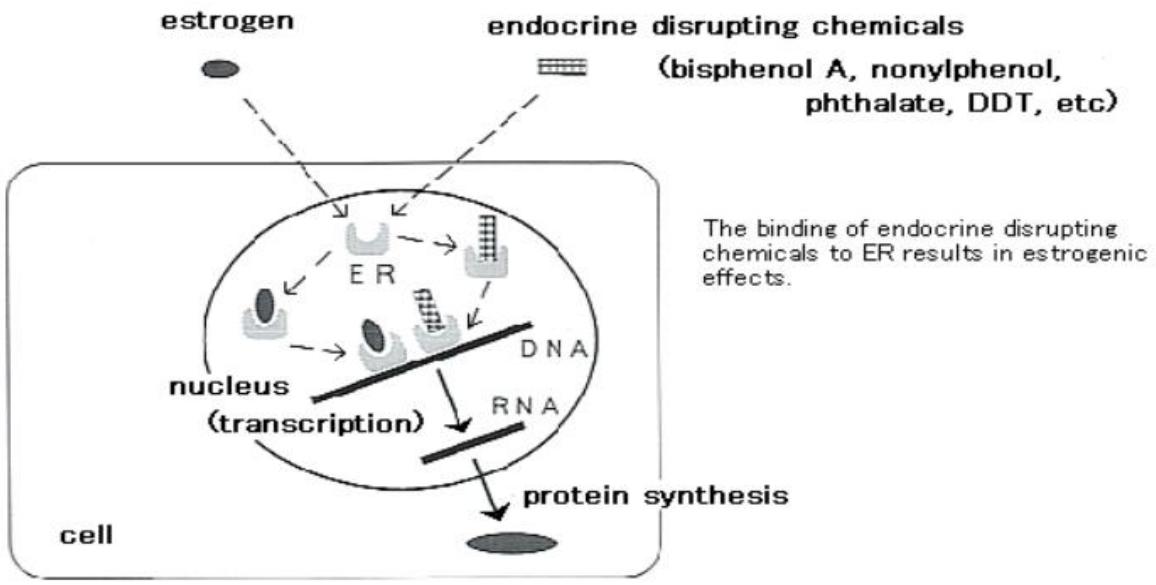

Figure 6: Endocrine disruptor act as agonist by binding to estrogen receptor and synthesising protein.

Metabolism in body

The Phytoestrogen are taken from plant in form of diet and are metabolised in the liver and intestine by enzymes and micro flora respectively.

Isoflavones occur in conjugated form in nonfermented soy products as glycosides, malonylglycosides and acetlyglycosides, where as in fermented form, the unconjugated aglycones are predominating. They are further metabolised in the gut by micro flora. The hydrolysis of β-glycosides, genistin and daidzin was done by an enzyme lactose phlorizin hydrolase present in the apical membrane of the villi of small intestine and by intestinal microflora which convert them into aglycone forms (Axelson and Sjovall, 1984). They were then reconjugated with glucuronic acid and sulfate in the liver and other organs by phase II enzyme UDP-glucuronosyltransferase and sulfotransferase respectively. The metabolities of phytoestrogen phase ll are taken by liver, excreated in bile and passs to intestine, where intestinal β-glucuronidases and sulfatases release aglucones. These aglucones enter bacterial-rich large bowl and processing of heterocyclic ring of isoflavonoids occur. Various processing steps include reduction (i.e. daidzein to equol), ring opening (daidzein to O-desmethylangolensin) and ring cleavage (to p-ethylethanol) takes place.

Lignan phytoestrogen has been found in various foods particularly in flax seeds. Among them secoisolariciresinol and matairesinol are two which converted into mammalian lignans, enterodiol (END) and enterolactone (ENL) and are excreated in urine of humans and rats. Both END and ENL are conjugated with glucuronic and sulphuric acid before excreation because of presence of two phenolic groups. These phytoestrogen have weak estrogen activity and acts as antagonist at high concentration. Due to this importance of phytoestrogen in living organism, the identification of compounds in biological system is very important.

2.12 Prevalence of Biochanin A

Biochanin A is a natural organic compound of class flavonoids and is O-methylated isoflavone chacterised by estrogenic activity in humans and animals (fig 7). Its IUPAC name is 5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one. It is 4-*O*-methyl derivative of genistein. Its molecular weight is 284.27 and molecular formula is $C_{16}H_{12}O_5$. It is in powder form stored at 4°C and soluble in chloroform, methanol and slightly soluble water (less than 1mg in 1 ml) (Kole *et al*. 2010).

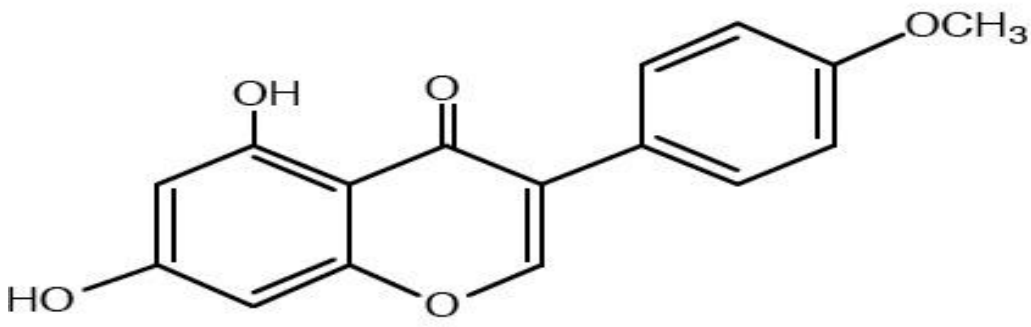

Figure 7: Structure of Biochanin A.

It commonly occurs in many species of vascular plants, mostly belonging to the families' legumes (*Legumi-noseae*), papilonaceous plants (*Papilionaceae*) and many species of grasses and cereals (*Graminae*). The plant species which are the richest source of biochanin A include red clover (*Trifolium pratense*), which contains 0.8% this compound in dry weight of leaves, juniper (*Genista tinctoria*), soybean (*Soja hispida*) and plum (*Prunus spinosa)* (Czerpak *et al.* 1998). biochanin A is metabolised in body to genistein, which have more

estrogenic effect than any other phytoestrogen (fig 8).

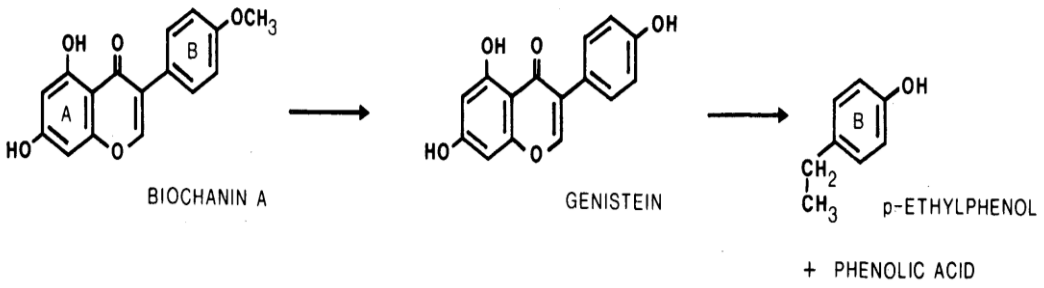

Figure 8: Metabolism of Biochanin A

The studies has been undertaken to study effects of biochanin A and observed that animals fodder (red clover, white clover) contains estrogenic isoflavones, especially formononetin and biochanin A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter and show clear estrogenic effect on uterine weight of immature rats (Saloniemi *et al.* 1995).Recent study show significant decrease ($p<0.05$) in the number of live pups per rat and male/female ratio were observed in the pups exposed transplacentally to biochanin A when compared to control pups (Soujanya and Reddy, 2014). Other study done to know it's effect during perinatal period by using rats as test model and observed decrease in fertility index and number of implantations due to decrease in the 3β-hydroxy steroid dehydrogenase and 17β-hydroxy steroid dehydrogenase enzymes in perinatally biochanin A exposed males which may decreases the testosterone levels. Hence decreases daily sperm count and quality of the sperm which may ultimately alters the various reproductive parameters in female (during pregnancy) after mating (Branham *et al.* 2002). So, biochanin A is causing adverse effect, however there is very less study done on effects of biochanin A on animals and humans.

2.13 Removal methods

The methods used for removal of phytoestrogen were by Solid Phase Extraction Method which allows simultaneous purification, extraction and concentration of various organic compounds from real samples. In this three-phase system is used i.e. aqueous/organic/aqueous, where organic solvent, immobilized in pores of inert support (membrane phase), separates aqueous donor from aqueous acceptor phase (Anna and Anna, 2014). Other method reported is by bacterial degradation in waste water treatment plants. The

phytoestrogen is removed by degrading its ammonia group and its degradation will depend upon time bacteria. In this study, Adsorption method is used for removal of phytoestrogen with help of different adsorbent materials.

Adsorbent

An adsorbent is defined as material that has the ability to extract certain substances from gases, liquids, or solids by causing them to adhere to its surface without changing the physical properties of the adsorbent. The adsorption method is used for removal of biochanin A. The adsorbent materials used for removal of biochanin A from water may be polymer, clay or granules. The various adsorbents used were hydrogel beads of chitosan, bentonite clay and granular activated carbon.

Chitosan

It is a linear polysaccharide composed of both deacetylated and acetylated unit. The monomer of chitosan is β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is made up of shrimp and crustacean shells by treating it with the alkali sodium hydroxide (Wang and Sun, 2007). The application of chitosan has been widely recognized in various fields such as biomedical engineering, pharmacy, dentistry, ophthalmology, biotechnology, chemistry, cosmetics, textile, oenology, agriculture and photography (Rinaudo, 2006). The chitosan used in treatment of waste water because of various advantages like effectively remove metallic ions from the water, cost effective, environment friendy, non-toxic, bio-degradable and renewable properties (Grégorio and Marie, 2008; Kumar, 2000). The solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups present in the polymeric chain of chitosan and therefore also depend on the proportion of acetylated and non-acetylated D-glucosamine units present. The chitosan is soluble in acids because amino groups (pKa from 6.2 to 7.0) are completely protonated in acids with pKa smaller than 6.2 making them soluble. They are insoluble in water, organic solvents and aqueous bases but is soluble after stirring in acids such as acetic, nitric, hydrochloric and phosphoric (Guibal, 2004; Klug *et al*., 1998; Kubota *et al*., 2000).

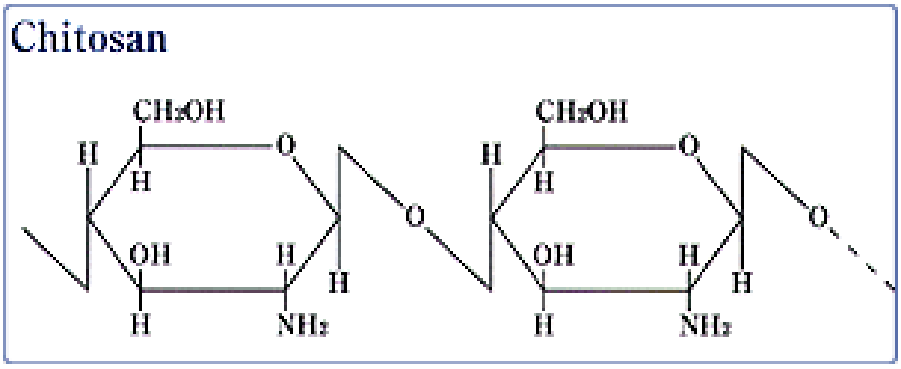

Figure 9: Structure of chitosan showing β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine bonding.

The hydrogel beads of chitosan were prepared for the adsorption of biochanin A from the water. The beads were prepared by dissolving in acetic acid and pour in chilled sodium hydroxide and ethyl acetate solution, to form a spherical surface. The beads were dried and when added in adsorbate, they absorb the biochanin A and swell up (Wang, 2007). The absorbance was noted at 266 nm after the separation of beads.

Bentonite

It is impure aluminium phyllosilicate clay consist mainly of montmorillonite and other smectite groups. These may include lesser amounts of other clay minerals such as kaolin, mica, illite, as well as non-clay minerals like quartz, feldspar, calcite, and gypsum. The bentonite name was given in 1898 by Wilbur C. Knight, an American geologist, after a rocky formation near Fort Benton. The bentonite clay has large surface area per unit weight. The structure consists of 2:1 mineral with one octahedral sheet and two silica sheets, which forms a layer. Due to the broken bonds around the edges of the silica–alumina units it carries a net negative charge on it, which is balanced by exchange of cations (Bhattacharyya and Gupta, 2008). So, bentonite clay is used as good ion exchanger adsorbent for heavy metals (Sajidu *et al.*, 2008). Different types of bentonite clay formed are potassium (K), sodium (Na), calcium (Ca), and aluminium (Al) bentonite clay. The bentonite clay can be modified as organophilic bentonite has been used too as appropriate adsorbent for organic compounds (Cowan and White, 1962; Cowan, 1963; Tiller *et al*., 1984). The removal mechanism of bentonite depends upon its pretreatment (Sajidu *et al*., 2008).

Activated carbon (AC)

Activated carbon is processed carbon and can be prepared from coconut shells, wood, peat and carbon (Pis *et al.* 1996). Activated carbons are carbonaceous materials which can be separated from other elemental carbon by the oxidation of the carbon atoms found on the outer and inner surfaces (Mattson and Mark, 1971). These carbonaceous materials are characterized by their large specific surface areas, well-developed porosity and easily modified surface-containing functional groups (Baker *et al*. 1992, Zongxuan *et al*., 2003). Therefore activated carbons are widely used as adsorbents for the removal of organic chemicals and metal ions of environmental or economic concern from air, gases, potable water and wastewater (Hendawy, 2003) Adsorption is a process where solid particle is used for removal of soluble particles from water. Activated carbon is specifically produced to have a very big internal surface (between 500 – 1500 m^2/g). Activated carbon comes in two forms: Powered Activated Carbon (PAC) and Granular Activated Carbon (GAC) (Metcalf and Eddy, 1991).The granular activated carbon is used for removal of organics (such as unwanted taste and odours, micropollutants), chlorine, fluorine or radon from drinking water or wastewater. During adsorption the biochanin A adheres to the surface of granular activated carbon and get trapped in small pores (Amirault *et al.* 2003).

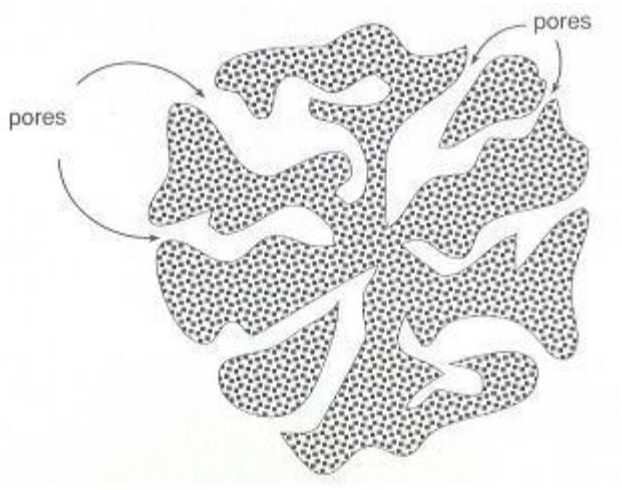

Figure 10: A carbon particle showing numerous pores (Lemley *et al,* 1995).

The study of adsorption of biochanin A on granular activated carbon is affected by various parameters. Such parameters include the concentraton of adsorbate, concentration of adsorbent, effect of pH, effect of change in temperature and contact time of adsorbate and

adsorbent (Radovic and Castilla, 2001). Granular activated carbon is costly as compared to bentonite and chitosan and, therefore regeneration of granular activated carbon is required. The methods mostly used for regeneration are thermal reactivation (Bagreev *et al.* 2001), chemical and solvent regeneration (Martin, 1997), microbial regeneration (Aizpuru, and Malhautier, 2003), electrochemical regeneration (Narbaitz, *et al*., 2009), ultrasonic regeneration (Lim and Okada, 2005) and wet air oxidation (Shende *et al.* 2002). In this study, regeneration of granular activated carbon can be done by thermal method. In this method the adsorbed substances were evaporated at higher temperature. It utilise the exothermic nature of adsorption which results in desorption, polymerisation or cracking of adsorbed organics. It removes the organic adsorbate from the pores of granular activated carbon which re-expose the pores of granular activated carbon by regenerating its original surface characteristics. For this the used adsorbent was regenerated by heating them in muffle furnace for 30 minutes at 350°C. After treatment the adsorbent is regenerated n number of times but due to regeneration, the 5–15 wt% of the carbon bed is burnt off resulting in a loss of adsorptive capacity (Miguel *et al*., 2001). Also due to high energy process, the high temperature is required making it both an energetically and commercially expensive process (Sabio and Gonzalez, 2004).

2.14 Characterises of Adsorbent

Chitosan	Biodegradability, biocompatibility, antimicrobial, non-toxicity, and anti-tumor.
Bentonite	Large surface area, good ion exchanger, hydration, swelling, water absorption and viscosity.
Activated carbon	surface area, product density; mesh size, abrasion resistance and ash content

Table 1: Characterises of Adsorbents

2.15 Adsorption isotherms

The adsorption process is surface phenomenon and defined as the adhesion of atoms, ions, or molecules from a gas, liquid, or dissolved solid to a surface. This creates a film of the

adsorbate on the surface of the adsorbent, while in absorption; the adsorbate will penetrate inside the adsorbent. The process of adsorption usually studied with graph is called as adsorption isotherm. It represents the amount of adsorbate adsorbed on the surface of adsorbent and pressure at constant temperature (Hamdaoui *et al.* 2007). The various isotherms models study including Langmuir, Freundlich, Elovich, Temkin, Flower-Guggenheim and Kiselev.

Langmuir model: This model is used to study uniform adsorbance on the surface of adsorbent. It is represented by

$$\frac{C_e}{q_e} = \frac{1}{Q_o b} + \frac{C_e}{Q_o}$$

Where, Ce (mg/L) is concentration of biochanin A solution at equilibrium, q_e (mg/g) is amount of sorbed biochanin A at equilibrium, Q_o is maximum sorption capacity, K_L is Langmuir constant.

Freundlich model: it is used to study multi-layer adsorbent in which the concentration of adsorbate on the adsorbent surface increase with increase in the concentration of adsorbate.

Freundlich equation

$$\ln q_e = \ln K_F + \left(\frac{1}{n}\right) \ln C_e$$

Where K_F and n are Freundlich constant.

Temkin model: this model assumes that due to adsorbent-adsorbate interactions, heat of adsorption of all molecules in a layer decreases linearly.

$$q_e = B\ln A + B\ln C_e$$

Where q_e is (mg/g) is amount of sorbed biochanin A at equilibrium, A is Temkin equilibrium binding constant (L/g), B is theTemkin constant related to heat of sorption (J mol^{-1}).

3. Material and methods

***3.1a Material*:** Conical flasks (50 ml), measuring cylinder, quartz cuvettes, falcon tubes, microcentrifuge tubes. Biochanin A phytoestrogen was used as adsorbateand purchased from Santa Cruz biotechnology (CAS 491-80-5). The adsorbents used were bentonite (Loba Chemie), chitosan (Sigma Aldrich Chemie, batch 06714 SJ) and granular activated carbon (SD Fine- chem. Limited, Mumbai).

3.1b Method

3.1.1 Preparation of biochanin A solution

A stock solution of 0.2 mg/ml was prepared by dissolving 2 mg in 10 ml of distilled water with help of sonication till it dissolved completely.

3.1.2 Preparation of standard curve of biochanin A

Biochanin A standard curve was prepared by diluting the stock solution of biochanin A at different concentration (mg/L) of 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 and 100. The final volume of solution prepared is 5 ml and volume was made up to with distilled water. The absorbance was measured with spectrophotometer (Champion UV-500, model number AQ1205017) at optical density (O.D.) 266 nm.

3.1.3 Study of equilibrium time of biochanin A and bentonite

A stock solution of biochanin A of 0.4 mg/ml was prepared by dissolving 6 mg of biochanin A in 15 ml of distilled water. A 20 ml of biochanin A solution of concentration 10 ppm was prepared and 2 mg of bentonite was added in solution and dissolved completely. The incubator shaker was set at 25°C, 150 rpm for 20 hours. The samples were withdrawn at different intervals and adsorbent was separated by centrifuge. The supernatant was taken without disturbing pellet. The absorbance was measured at O.D. 266 nm.

3.1.4 Preparation of chitosan solution and beads

Take 1 g of chitosan was added into 50 ml of 2% acetic acid and mixed properly in a beaker. The mixture was incubated overnight. The chitosan solution was dropped by syringe into beaker containing solution of 100 ml 3% sodium hydroxide with 1.5 ml ethyl acetate solution, to obtain spherical gel beads. The beads were mechanically stirred for 3 hr. The

sodium hydroxide solution was removed and beads were washed with deionised water for 2-3 times. (Wang, 2007).

3.1.5 Equilibrium time of biochanin A and chitosan

A 20 ml of biochanin A solution of concentration 10 ppm was prepared and chitosan beads were added in solution. The incubator shaker was set at 25°C, 150 rpm for 20 hrs. The samples were taken at different intervals and absorbance was measured with spectrophotometer at O.D. 266 nm (Wang *et al*., 2007).

3.1.6 Equilibrium time of biochanin A and Granular Activated Carbon

A 20 ml of biochanin A solutions of concentration 10 ppm was prepared and 0.1 mg of granular activated carbon was added in solution. The incubator shaker was set at 25°C, 150 rpm for 20 hrs. The samples were taken at different intervals and adsorbent was separated by centrifuge. The absorbance was measured with spectrophotometer at O.D. 266 nm (Jing, 2012).

3.1.7 Effect of concentration of Granular Activated Carbon on biochanin A removal

The adsorbent concentration effect was carried out to evaluate the optimum concentration of granular activated carbon for effective removal of biochanin A. The concentration (in g) is lay of range 0.5, 0.7, 1, 1.2 and 1.5. The granular activated carbon was measured and added in flask containing 20 ml solution of 10 ppm, of biochanin A. The solutions were incubated at 25°C for 15hr, 150 rpm and measured with spectrophotometer O.D. at 266 nm (Wang, *et al*, 2012).

3.1.8 Effect of concentration of biochanin A on its own removal

The adsorbate concentration study was done at fix concentration of adsorbent to find the optimum concentration of **b**iochanin A for its effective removal. The concentration (in ppm) was taken in of range 5, 7, 10, 12 and 15. The 1.5 g of granular activated carbon was weighted and added in flask containing 20 ml solution of different concentration of biochanin A. The solutions were incubated at 25°C for 15 hrs, 150 rpm and measured O.D. with spectrophotometer at 266 nm (Jing, *et al*, 2012).

3.1.9 Effect of pH on absorbance of biochanin A on Granular Activated Carbon

About 20 ml of solution of biochanin A of concentration 10 ppm was prepared in a conical flask and pH adjusted at range of 2, 4, 6, 10 and 12 with help of conc. Hydrochloric acid and sodium hydroxide. 1.5 g of granular activated carbon was added in conical flask containing solution and incubated for 15 hr at 25°C, 150 rpm in an incubator shaker. The granular activated carbon was separated after 15 hr by centrifugation and O.D. was measured at 266 nm with spectrophotometer (Wirasnita *et al.* 2014).

3.1.10 Effect of temperature on absorbance of biochanin A on Granular Activated Carbon

Adsorption is also affected by change in temperature, thereby; adsorption study at different temperature was done. 1.5 g of granular activated carbon was added in conical flask containing solution of biochanin A (10 ppm). The range of temperature (°C) is 10, 20, 30 and 40. The incubator shakers were set at different temperature but for same time and rpm i.e. 15 hrs and 150 rpm. After 15 hrs, O.D. was measured at 266 nm with spectrophotometer.

3.1.11 Mechanism of biochanin A binding by Granular Activated Carbon

To determine the structure and functional group present on adsorbent, the characterization of granular activated carbon was done with Fourier Transfer Infra Red (FTIR)(Agilent Resolution Pro carry 660). It is method used to determine the surface functional groups responsible for the adsorption of biochanin A uptake by granular activated carbon (Wirasnita *et al.* 2014). The change in surface functional group is detected by comparing the IR spectra of both granular activated carbon (control) and biochanin A - granular activated carbon (after adsorption). The change in peak explains the functional group responsible for adsorption.

3.1.12 Regeneration study of Granular Activated Carbon

Regeneration of adsorbent granular activated carbon was achieved by thermal method. The adsorbent used was collected after 15 hrs and washed with distilled water. The granular activated carbon was air dried and regenerated by evaporating biochanin A in muffle furnace (Prefit India) for 30 minutes at 350°C. This granular activated carbon used as adsorbent for removal of biochanin A. This regeneration cycle was repeated for five times and O.D. was taken at 266 nm with spectrophotometer (Cazetta *et al.*, 2013).

4. Results and discussion

The adverse role of phytoestrogens to mammals upon continued exposure has been established. The primary effect of phytoesterogen lies on its binding with estrogen receptor, mainly β-estrogen receptor. biochanin A was selected as principal phytoesterogen for removal studies, since a prevalence of this in diverse areas predominated by agricultural practices have been noted. The biochanin A is removed with help of adsorbents like granular activated carbon, bentonite, chitosan.

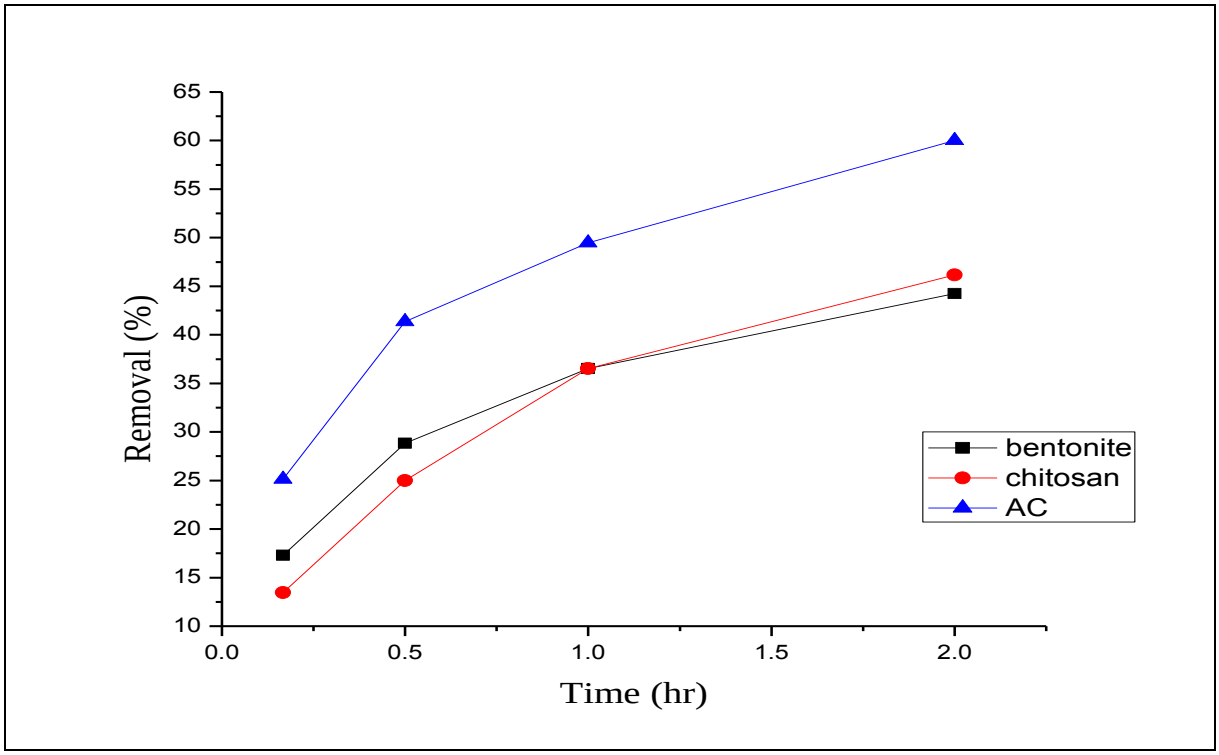

Figure 11: Comparison of Adsorbents (bentonite, chitosan and granular activated carbon).

The comparison of all adsorbents was done with biochanin A for 2 hr at 25°C and 150 rpm. As shown in figure, the maximum removal percentage was done by granular activated carbon at concentration of 10 mg/L of biochanin A. The removal percentage of chitosan and bentonite and granular activated carbon were 46.1%, 44.2% and 60% respectively. Therefore, granular activated carbon is selected for the removal of biochanin A for further studies.

4.1 Preparation of standard curve of biochanin A

The calibration curve prepared with different standard concentrations of biochanin A is depicted below (Fig 12). The regression coefficient (R^2 value of 0.9592) indicated suitability of the assay procedure and was therefore adopted for further analysis.

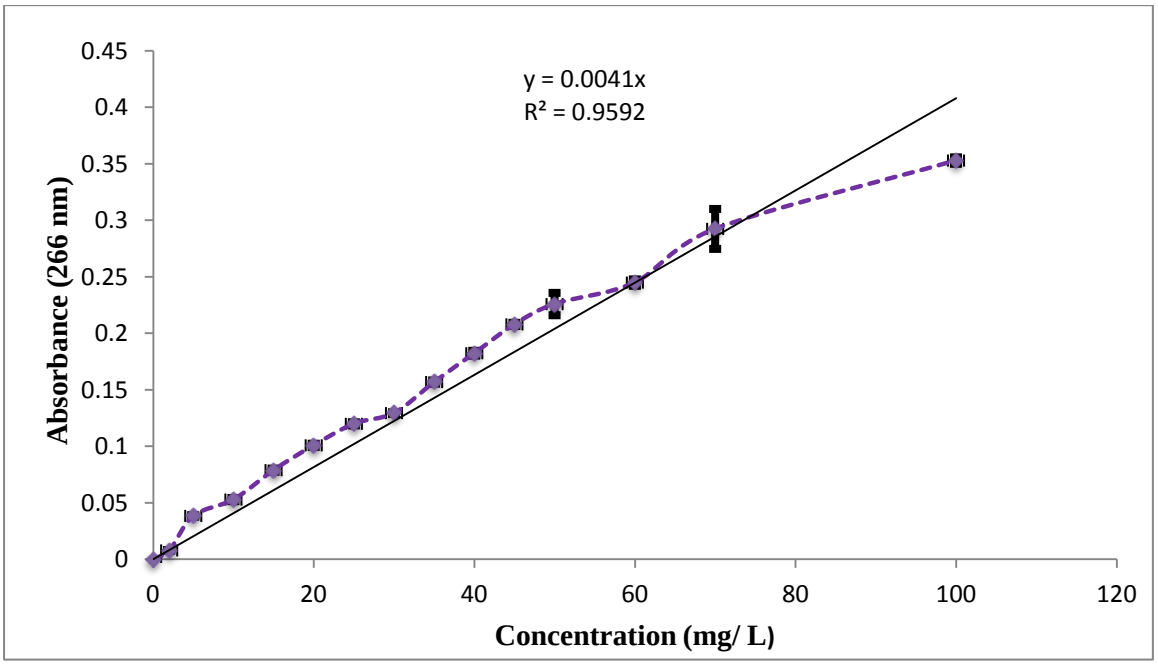

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Figure 12: A standard curve of biochanin A with R^2 value of 0.9592.

Kinetic studies on adsorption of Biochanin A.

4.2 Equilibrium time study

The equilibrium time was determined to estimate the time at which maximum adsorption of biochanin A could be achieved. The adsorption of biochanin A increased from zero minutes to 15 hr, after which equilibrium was attained and no adsorption was detected. The percentage removal and maximum adsorption capacity also increased with increase in time as shown in Figure 13. The maximum absorbance was observed at 15 hr with percentage removal and maximum adsorption capacity (q_e) of 87.3% and 5.83 mg/g respectively. However a strict comparison could not be made since similar studies have not been conducted thus far. The adsorption process was initiated with rapid adsorption first due to

availability of readily accessible sites, followed by slower adsorption after 14 hr as the accessible sites were decreased. After 15 hrs a plateau was achieved where saturation of granular activated carbon taken place.

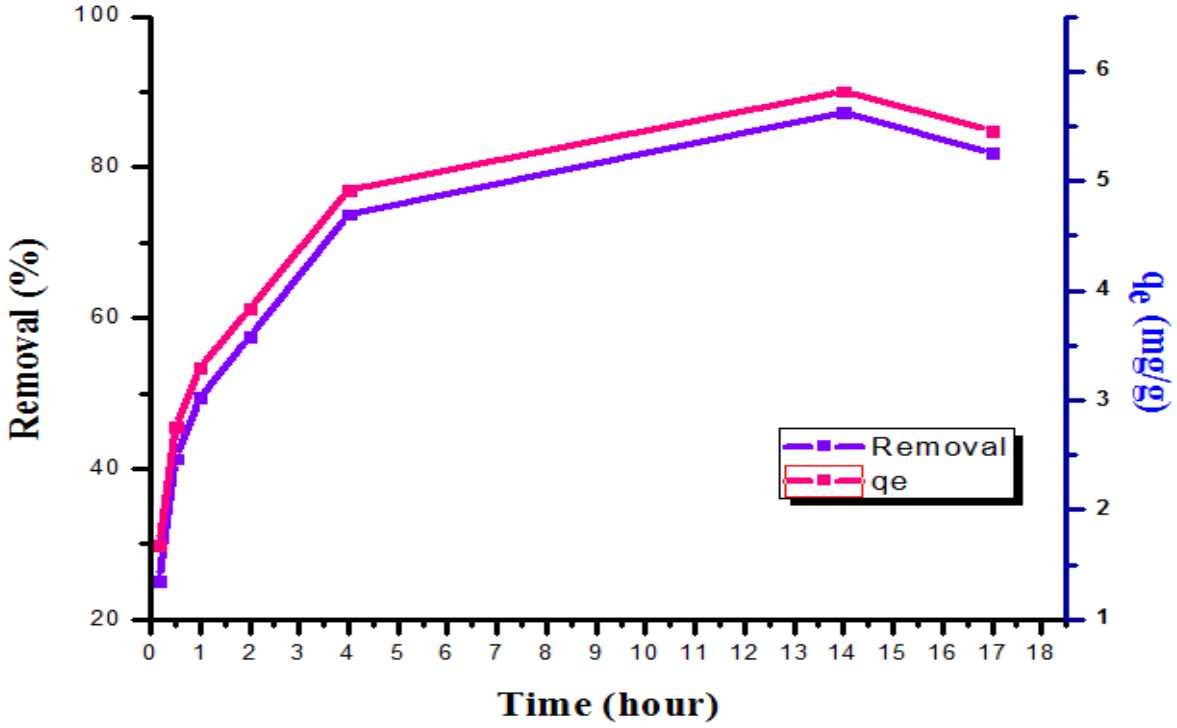

Figure 13: Effect of contact time on biochanin A adsorption (at initial conc. of 10 mg/L, granular activated carbon conc. of 1 g/L)

4.3 Effect of granular activated carbon concentration on adsorption

In order to understand the role of concentration of granular activated carbon on biochanin A adsorption, different concentrations of granular activated carbon was examined. Granular activated carbon of concentration 1.5 g/L shows maximum removal of biochanin A as shown in Figure.14. The removal percentage of biochanin A at 1.5 g/L of adsorbent was found to be 89.7% with q_e 5.98 mg/g. It was observed that with increase in the concentration of adsorbent, the absorbance of biochanin A also increased. The percentage removal also increased (with a decrease in q_e) with increase in concentration of granular activated carbon. The percentage removal is also increasing while q_e is decreasing with increase in concentration of granular activated carbon. The increase in the percentage removal with

increase in adsorbent dose is due to the greater availability of the exchangeable sites or surface area at higher concentration of the adsorbent (Charles and Odoemelam, 2010).

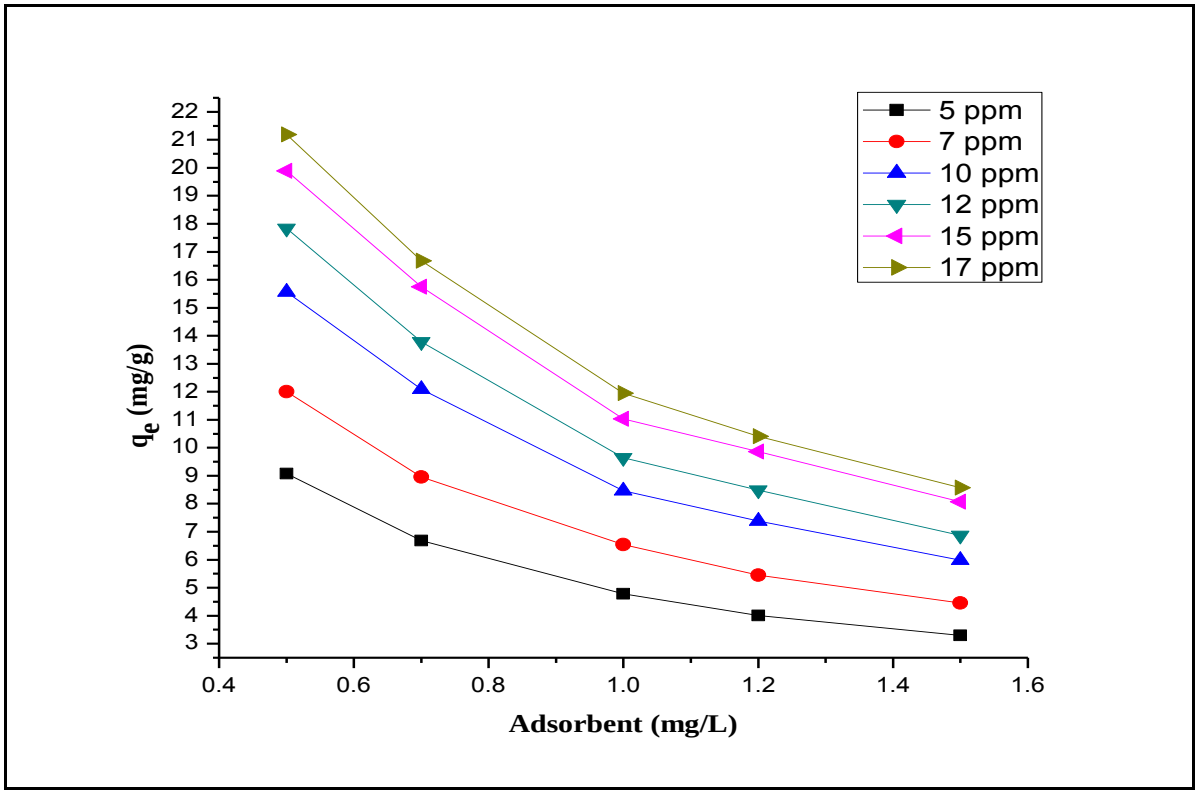

Figure 14: Effect of granular activated carbon on adsorption of biochanin A.

4.5 Effect of biochanin A concentration on adsorption

The biochanin A at different concentrations was used to estimate the optimum concentration of biochanin A at different concentration of granular activated carbon (0.5 - 1.5 g/L). The biochanin A concentration (ppm) used was 5, 7, 10, 12 and 15. Fig 13 depicts that with increase in concentration of biochanin A, the absorbance of biochanin A decreased. The maximum absorbance capacity increased with increase in absorbate while percentage removal declined. The maximum adsorption was observed at concentration 5 ppm with removal percentage and q_e of 98.9% and 3.29 mg/g. These results may be explained by the fact that, at low biochanin A concentration, the ratio of surface active sites to biochanin A is high; hence the biochanin A could interact with the sorbent to occupy the active sites on the carbon surface sufficiently and can be removed from the solution. But with the increase in biochanin A concentration, the number of active adsorption sites is not enough to accommodate biochanin A (Rao, 2011).

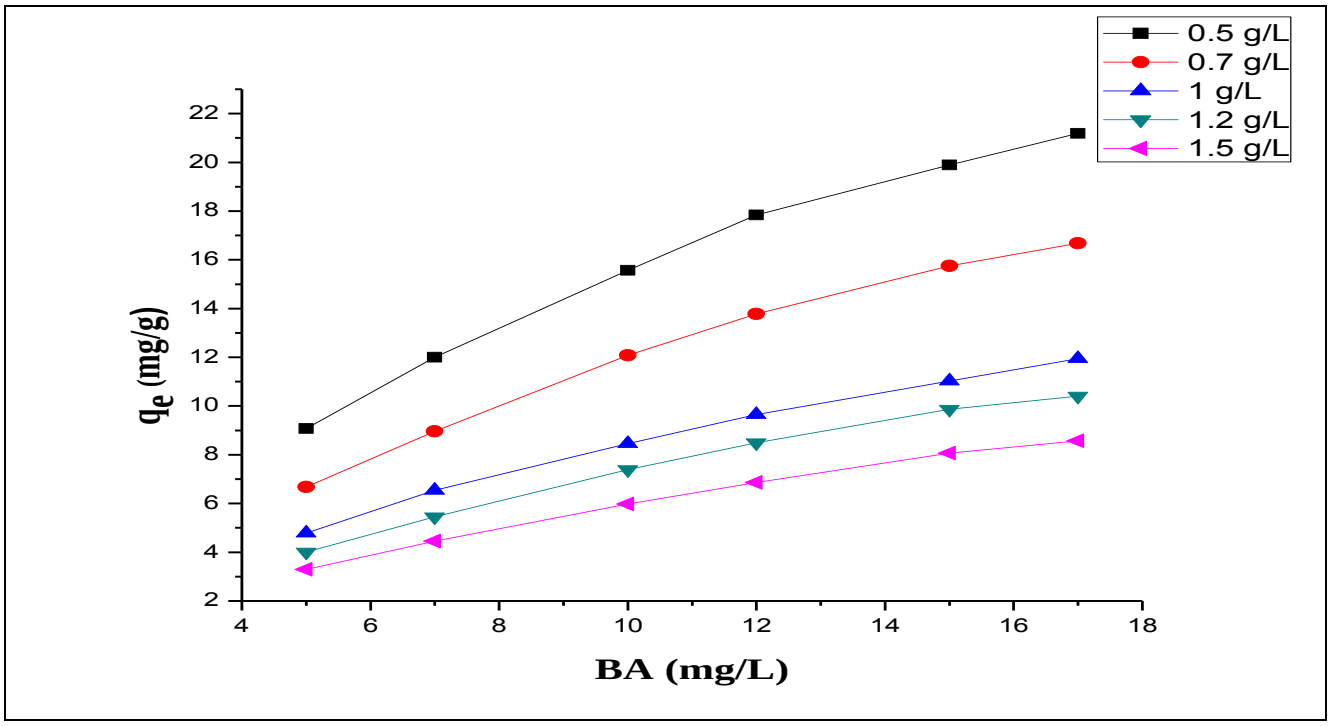

Figure 15: Effect of biochanin A on adsorption at different concentration of granular activated carbon (0.5 - 1.5 g/L).

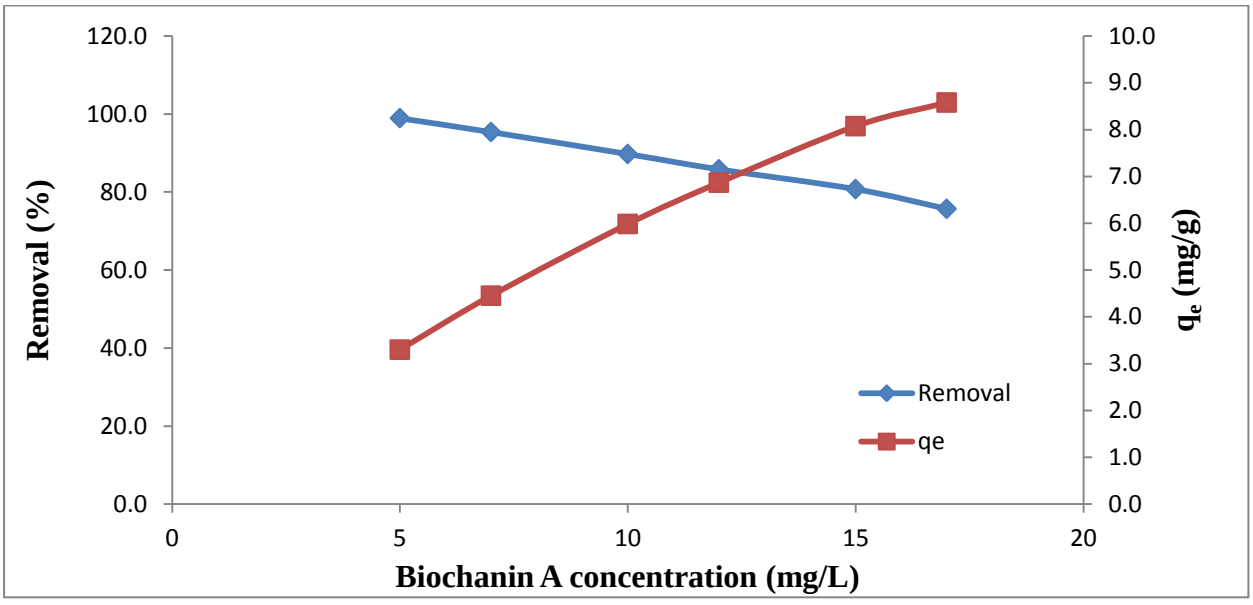

Figure 16: Comparison of q_e and removal percentage with biochanin A showing maximum removal of 98.9% at 5 mg/L.

4.5 Effect of pH

Earlier studies have indicated that adsorption is affected by change in pH of solution; this may be explained by considering the change in number of hydrogen ion which affects the binding of biochanin A with granular activated carbon (Figure 17). The pH of solutions at different pH values indicated that with increase in pH the adsorption of biochanin A increased; maximum adsorption was observed at pH 6 with removal percentage of biochanin A as 98.92%; the q_e was 6.54 mg/g. Both the removal percentage and q_e first increased and then decreased after attaining pH 6. The net charge on the surface of granular activated carbon is responsible for binding of biochanin A, a favourable binding may be postulated at pH 6. This is explained as increase of the pH to above the pHPZC (point of zero charge) of the adsorbent leads to the presence of net negative charge on its surface. As the biochanin A has the same charge which results in repulsive electrostatic interactions and decreasing the adsorption capacity.

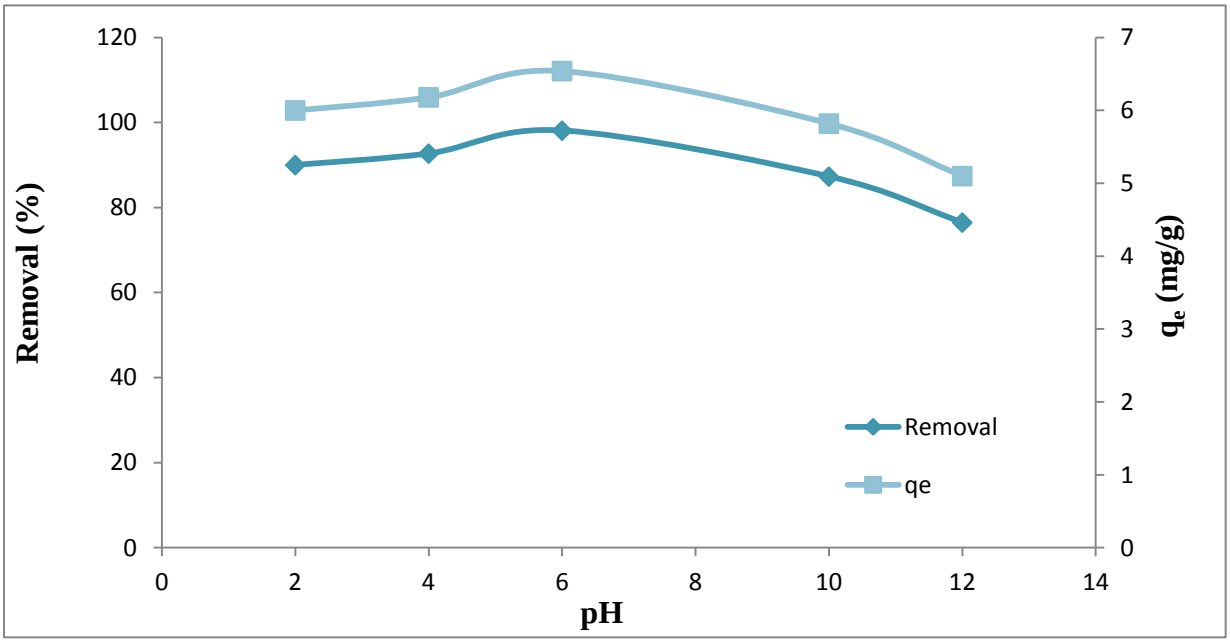

Figure 17: Effect of pH on biochanin A adsorption at fix conc. of biochanin A (10 mg/L) and granular activated carbon (1.5 g/L)

4.6 Effect of Temperature

The adsorption was significantly affected with the change in temperature (Figure 18). A temperature range of 10-40°C was used to evaluate adsorption; as observed with rise in temperature, the adsorption of biochanin A by granular activated carbon decreased. The percentage removal and q_e also reduces with rise in temperature. The optimum temperature for adsorption study was found to be 20°C with removal percentage and maximum adsorption capacity to be 98.1% and 6.5 mg/g respectively. It can be explained as the adsorption spectra characterized by π-π^* transition exhibit a large red shift and an increase in total adsorption intensity with decreasing temperature when hydrogen bonds are formed between the solute and solvent molecules (Mitsuo, 1960).

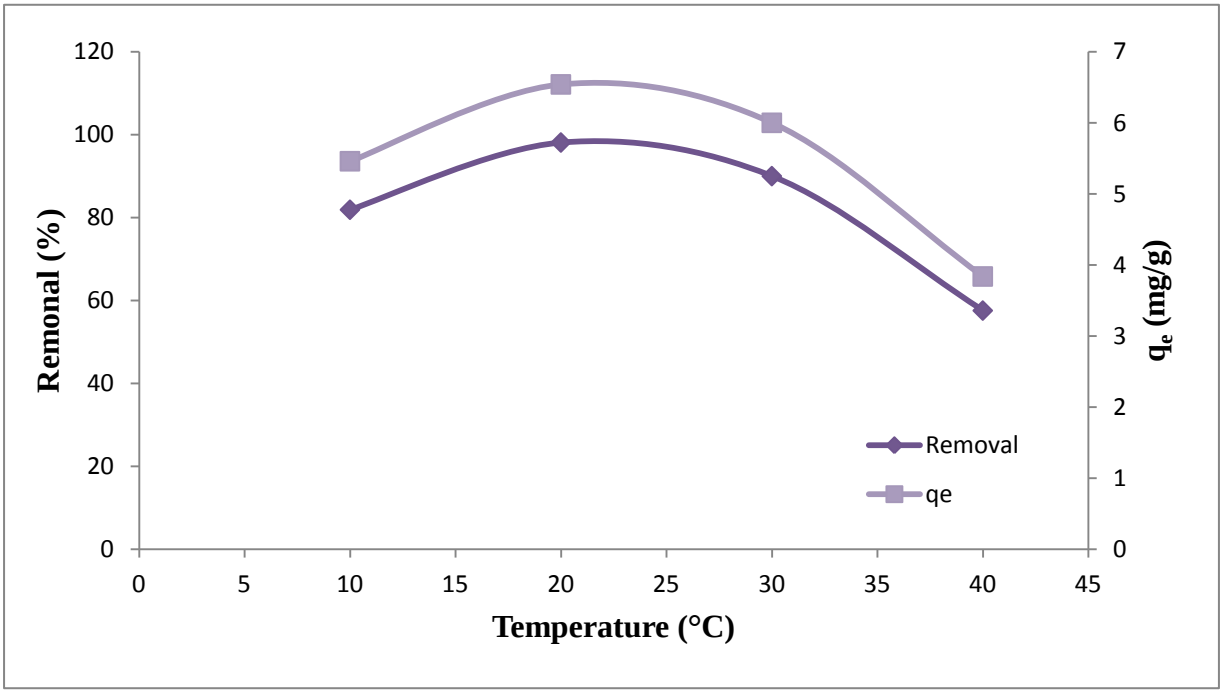

Figure 18: Effect of temperature on adsorption of biochanin A.

4.7 Isotherms

Isothermal study is a basic technique for determining the nature of adsorption between granular activated carbon and biochanin A. It indicates the distribution of biochanin A in equlilbrium phase. Langmuir, Freundlich and Temkin models are most commonly used for evaluating the adsorption isotherm.

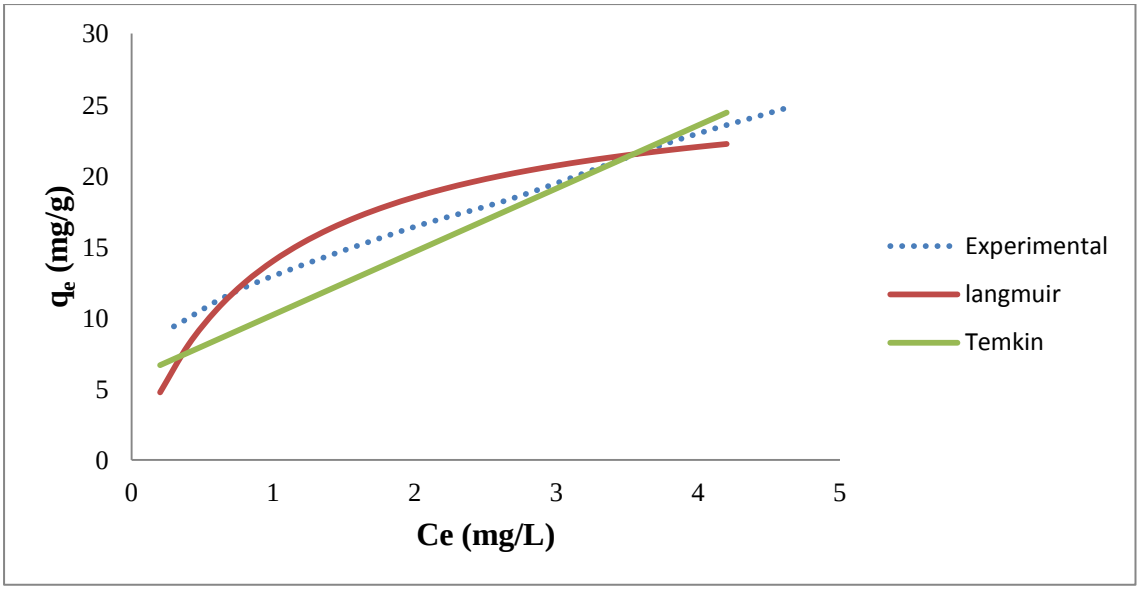

Figure 19: **Adsorption Isotherm for biochanin A onto granular activated carbon**

Name	Equation	Constant	R- square	SSE
Langmuir	$\frac{C_e}{q_e} = \frac{1}{Q_o b} + \frac{C_e}{Q_o}$	b = 1.062	0.9515	20.68
Temkin	$q_e = B\ln A + B\ln C_e$	A = 1.597 B = 1.3	0.8756	53.06

Table 2: Isotherm models applied to data showing equation, constants, R- square and SSE.

The Langmuir and Temkin models were applied to data and study the adsorption isotherms. For the Langmuir isotherm model, the C_e/q_e was plotted against C_e. The slope and intercept is equal to $1/q_m$ and $1/(K_L q_m)$. The graph gives a less linear curve with correlation factor (R^2) of 0.9515 as shown in figure 19 and table 2. The Temkin model generates a correlation of 0.8756 with a linear relationship. The correlation factor for Langmuir and Temkin models were compared and evident that Langmuir model provide a better fit for explaining the adsorption of biochanin A on granular activated carbon. The Langmuir isotherm model suggests that biochanin A - granular activated carbon has a homogeneous surface and each active site of biochanin A - granular activated carbon can accommodate only one molecule of biochanin A (Chandra *et al*., 2007).

4.8 Mechanism of biochanin A binding by granular activated carbon

It is imperative that the high adsorption capacity of activated carbon stems from their inherent chemical composition, therefore to ascribe relevant functional group(s) responsible for binding of biochanin A with granular activated carbon, FTIR was carried out. Figure 20 a and b depict the FTIR spectra of granular activated carbon (control) and granular activated carbon after biochanin A absorption. The FTIR of figure 20a shows various peaks of adsorption. The medium peak at 3400–3250 cm^{-1} is attributed to N–H stretching of 1˚, 2˚ amines and amides. The strong adsorption peak at 1320–1000 cm^{-1} is attributed to C–O stretching and presence of alcohols, carboxylic acids, esters and ethers functional groups. The peak between 550 - 850 cm^{-1} is attributed to stretching of C-Cl of alkyl halides.

Figure 20b presents the adsorption peak of granular activated carbon after adsorption. The broad and sharp peak occurs in range between 3500–3200 cm^{-1} after adsorption, shows the stretching of O–H functional groups and bonding of hydrogen, indicating the binding of functional groups of biochanin A. The peak shows the presence of alcohols and phenols after adsorption. The frequency of sharp peak at 1087 cm^{-1} lies in range of 1320–1000 cm^{-1} attribute to stretching of C–O bond and presence of alcohols, carboxylic acids, esters and ethers functional groups. The intensity of medium peak of 793 cm^{-1} lies in range 550 - 850 cm^{-1} and is attributed to stretching of C-Cl of alkyl halides.

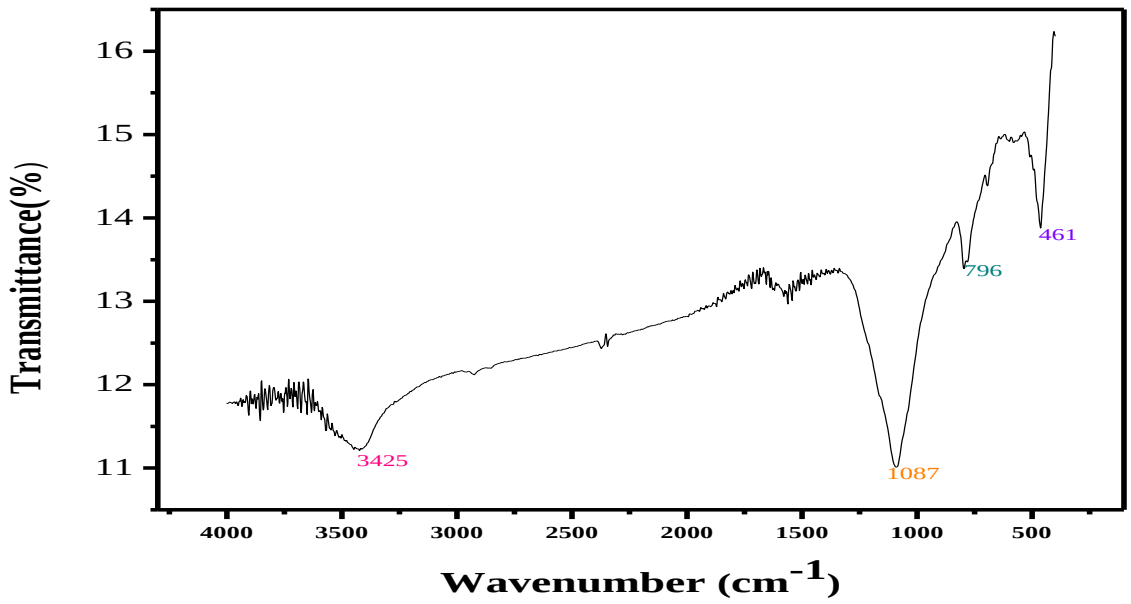

Figure 20a: FTIR spectra of granular activated carbon (control).

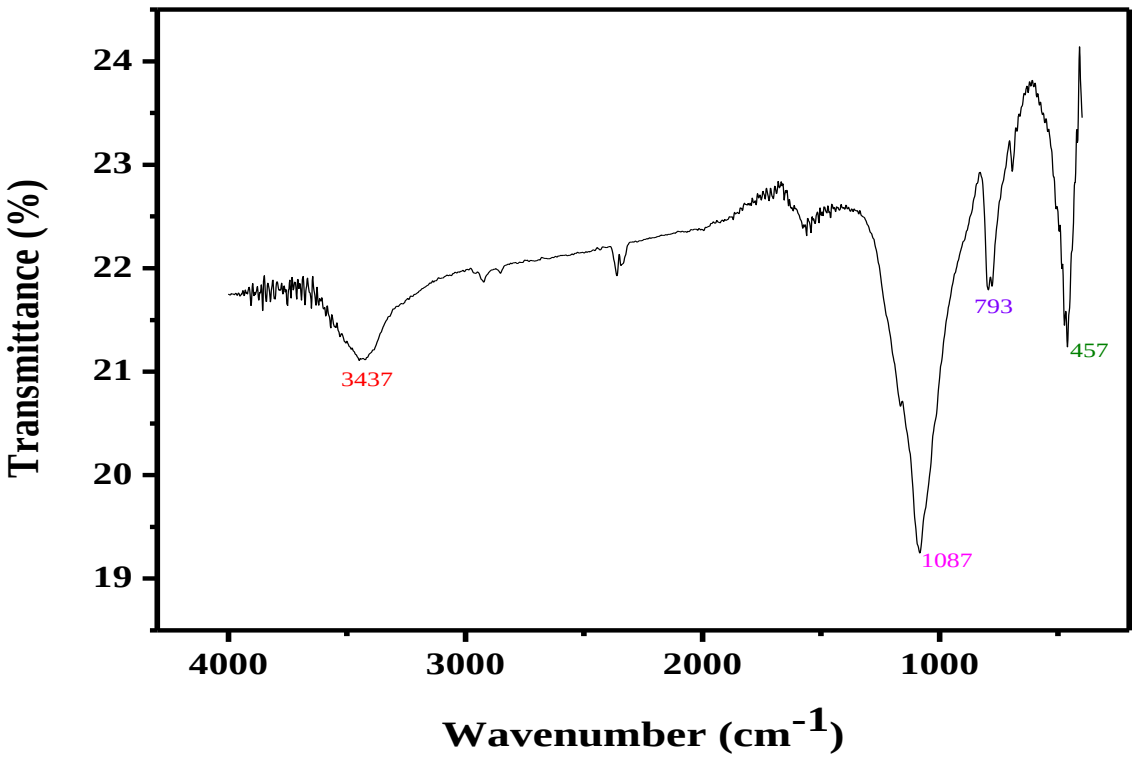

Figure 20b: FTIR spectra of granular activated carbon after biochanin A adsorption.

Overall, the results of the studies indicate that of adsorption of biochanin A on granular activated carbon is affected by various parameters. Such parameters include the concentration of adsorbate, concentration of adsorbent, effect of pH, effect of change in temperature and contact time of adsorbate and adsorbent; these results are in agreement to those reported by Radovic and Castilla, (2001).

4.9 Regeneration of Granular Activated Carbon

In majority of applications, the disposal of adsorbent as waste is not an economic option and therefore regeneration is carried out. It is regenerated with thermal method at 350°C for 30 minutes and this cycle was repeated for five times and observed that with increase in number of regeneration cycles, the adsorption capacity of granular activated carbon was reduced (fig 21). The percentage removal and maximum adsorption capacity were reduced from 87.2% to 33.2 % and 5.82 mg/g to 2.21 mg/g respectively after five cycles. The poor efficiency of regenerated carbon adsorbents used for further biochanin A removal indicates that biochanin A is strongly bound to the adsorbent surface and the availability of number of active sites are not much increased during regeneration.

Huajing *et al.* (2009) investigated two types of zeolites, dealuminated Y (DAY) and silicalite-1for adsorption capacity of estrogen and compared with CentaurÂ® activated carbon (CAC). DAY showed the highest adsorption capacity, while silicalite-1 was the least effective in removing estrogen. Moreover, DAY required four hours to reach adsorption equilibrium; much less than the eight days needed for CAC to reach equilibration. The Freundlich adsorption isotherm was found to best represent the data for adsorption of estrogen on DAY. UV at Î» = 254 nm degraded estrogen in water much more effectively than long-wave UV (Î» = 365 nm). Regeneration of the contaminant-saturated DAY was accomplished with Î» = 254 nm UV light. In this study, regeneration was attempted for cycles, an attempt to desorb biochanin A using reported methods such as ultrasonication/UV light etc were not used. The primary goal is recovery of intact biochanin A. A notable decline in removal was observed after 4^{th} cycle indicates probility of stronger residence of biochanin A and consequently non removal from matrix affecting fresh adsorption.

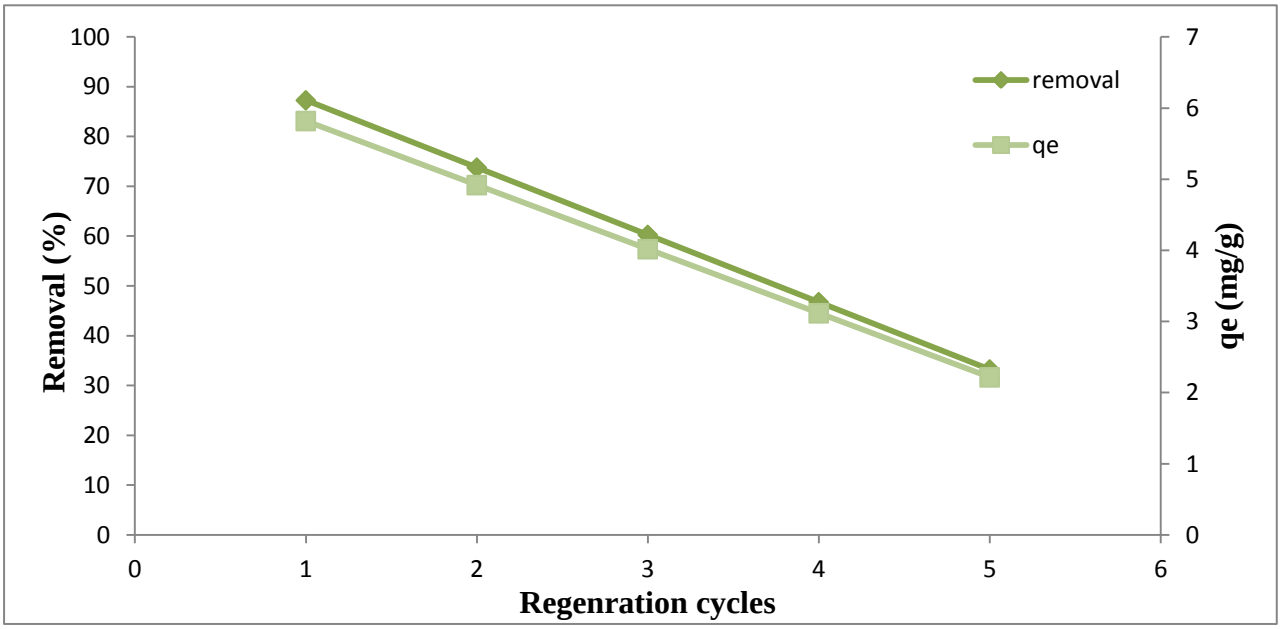

Figure 21: Effect of regeneration of granular activated carbon on biochanin A adsorption.

4.10 Reuse of Granular Activated Carbon

During regeneration, the same biochanin A adsorbed granular activated carbon which reduces both the weight and adsorption capacity of granular activated carbon due to its oxidation (fig 22).

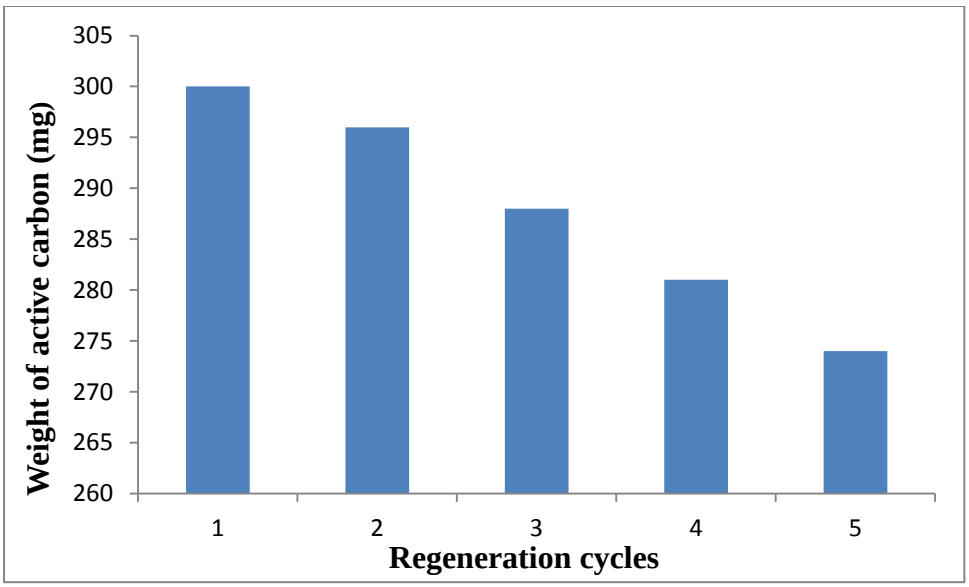

Figure 22: The amount of granular active carbon reduced after regeneration.

5. CONCLUSION

The percent removal of biochanin A increased with increase of adsorbent dose, contact time due to more number of active sites but decreased with increase in concentration of adsorbate and temperature due to less number of active sites and molecules come in motion at higher temperature respectively. The adsorption system is pH dependent, the adsorption efficiency increased when solution changed from acidic to alkaline condition may be because in acidic condition the OH^- of biochanin A bind with H^+ of granular active carbon but in alkaline pH, there is less H^+ charge on granular active carbon which cause decrease in adsorption.

The isotherm models are applied and best fit of isotherm is Langmuir model with correlation of 0.9515, which show homogeneous adsorption of biochanin A onto granular activated carbon and only one molecule of biochanin A will bind on each active site of granular activated carbon.

These carbon adsorbents showed low adsorption capacities for further removal of biochanin A solutions in continuous cycling process. The regeneration of the granular activated carbon is carried out by thermal process. The poor efficiency of regenerated carbon adsorbents used for further removal indicates that biochanin A is strongly bound to the adsorbent surface and the availability of number of active sites are not much increased during regeneration.

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