IN VITRO CULTURES OF Morus alba FOR ENHANCING PRODUCTION OF PHYTOESTROGENS

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Plant estrogens have long been associated with health benefits. The potential of tissue culture techniques for the production of several secondary metabolites has been known for many years. Tissue cultures stimulate the production or induce the biosynthesis of novel compounds not found in the mature plant. Tissue culture of *Morus alba*, family Moraceae, is known to contain phytoestrogens, was established on plant-hormone supplemented Murashige and Skoog (MS) medium. Petiole and the stem tissue from mature trees were the best explants for initiation and proliferation of calli. The best callus proliferation was obtained on MS medium containing 1-naphthalene acetic acid (1mg/ml) and benzylaminopurine (0.5mg/ml) for *M. alba*. Comparison of phytoestrogens of Moraceae species from *in vivo* and *in vitro* tissue isolation were carried out. The estrogenic activities of callus extracts were assayed in an estrogen-responsive yeast system expressing the human estrogen receptor alpha. Male callus extracts had higher estrogenic activity than male and female extracts from *in vivo* and *in vitro* tissues. Isolation and characterization of phytoestrogens from above tissues were carried out using solid phase extraction, high performance liquid chromatography and mass spectrometry techniques. Biochanin A, an isoflavonoid, was isolated as one of the compounds in male callus extracts. Biochanin A has been known to have an antiestrogenic activity in mammals. Isoflavonoid compounds have been characterized as strong protein tyrosine kinase inhibitors in variety of animal cells. Isoflavones are
structurally similar to estradiol, and display agonistic and antagonistic interactions with the estrogen receptor. Isoflavones possess therapeutic and preventive properties such as being used for postmenopausal osteoporosis, breast cancer, and inhibition of tumors.
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CHAPTER 1
INTRODUCTION

Metabolism comprises different pathways for the survival of all cells. Secondary products occur in special, differentiated cells that are not required for the primary growth of the organism. Metabolism can be defined as the chemical processes occurring within a living cell or organism that are necessary for the maintenance of life. It could also be described as a large group of enzyme-controlled and regulated chemical reactions that produce energy in the form of ATP, substances needed for the growth and development of tissues, and help the organism survive in different conditions. In metabolism some substances, carbohydrates and lipids, are catabolised to yield energy for vital processes while other substances, necessary for life, are synthesized. Depending on the biosynthetic origin, general occurrence and biochemical role, compounds produced during metabolism are called either primary or secondary metabolites. Primary metabolites are essential for all life forms and include carbohydrates, lipids, proteins and nucleic acids.

1.1 Plant Secondary Metabolites

Secondary metabolites often accumulate in specialized plant cells in smaller quantities than primary metabolites (Pichersky and Gang, 2002). Plant secondary
compounds are usually classified according to their structures (Harborne, 1999). Phenolics, terpenes and alkaloids are three large families generally considered. The production of plant secondary metabolites in plant tissues varies with species, environmental conditions and internal hormonal levels (Bourgaud et al., 2001). Although secondary plant products are very common, this does not mean that every plant can produce every product. Some compounds are restricted to a single species, others to related groups. But they are nearly always found only in certain specific plant organs, often in just one type of cell (and there again only in a certain compartment). Also, secondary metabolites are often generated only during a specific developmental period of the plant. The chemical structure of secondary plant products is without exception more complex than that of primary products. The complexity of products becomes comprehensible when recognizing that many, though by far not all, of them are derived from amino acids or nucleotides. Most of the compounds found in plants belong to rather few families of substances. Only small chemical modifications such as methylations, hydroxylations, intercalations with metal ions, etc. lead to a wide spectrum of functionally different substances.

1.2 Importance of Secondary Metabolites for Plant Life

Due to their multitude of biological effects, plant secondary metabolites have been used for centuries in traditional medicine. Currently, valuable secondary metabolites are extracted from plants and used as pharmaceuticals, cosmetics, fragrances, food additives pigments, flavoring and aromatic compounds and pesticides (Bourgaud et al.,
2001). Good reasons exist for the use of some secondary compounds or, even better, groups of chemically similar compounds as features of classification. But what is true for other morphological features, is also true for these: the presence of a chemical substance in a plant is adaptive although this may not always be as clear as in the case of flower pigments, lignin or cutin. Among the numerous secondary metabolites, isoflavonoids, together with alkaloids and terpenoids are most commonly researched under in vitro conditions. The reason for this may be seen in their multidirectional biological activity (Dutta et al., 2007). Although plants possess a huge diversity of secondary metabolites, structures, functions, and medical benefits for most of these phytochemicals are not yet known.

1.3 What are Phytoestrogens?

Phytoestrogens are a diverse group of naturally occurring compounds that exert estrogenic activities in plants. Phytoestrogens have structural similarity with estradiol. There have been reports of more than 300 plant species that contain estrogenic compounds. The phenolic ring is essential for binding to estrogen receptors (ERs). Phytoestrogens commonly occur in glycosylated forms and importantly the bioavailability of glyco-conjugates differs from that of the unsubstituted aglycones. Phytoestrogens are generally classified in three main chemical groups of compounds: isoflavones (genistein, daidzein), lignans, and coumestans. Isoflavones are found in a variety of plants including fruits and vegetables, but they are predominantly found in legumes such as beans, peas, lentils and soybeans. Lignans are widely found in cereals,
fruits and vegetables. The third class of Phytoestrogens are coumestans, produced in legume sprouts after germination (Luczkiewicz and Glod, 2003).

Isoflavones, being recognized as phytoalexins, play a key role in defense mechanisms in plants of the family Fabaceae. Isoflavones are, in addition, compounds with broad health-promoting activity. Isoflavones are known to have anti-inflammatory, antifungal and anti-free-radical activities that are typical for the whole group of isoflavonoids. They also feature compounds that inhibit estrogen beta receptors in mammals (Dixon et al., 2002).

1.4 Phenylpropanoid Pathway

Phenylpropanoid-derived isoflavonoids are known to act as primary defense compounds and also signal as plant-microbe interactions. A large number of isoflavonoids are also being used as pharmacological and nutraceutical properties, including chemoprevention and osteoporosis (Alekel et al., 2000; Uesugi et al., 2001) and other postmenopausal disorders (MerzDemlow et al., 2000), antioxidants that improve cardiovascular health (Lichtenstein, 1998; Setchell and Cassidy, 1999; Heim et al., 2002), and it also reduces the risk of breast and prostate cancers in humans (Adlercreutz, 1998; Lamartiniere, 2000). Due to the human beneficial effects of isoflavonoids, these compounds are being researched comprehensively for a better understanding of its synthesis. Efficient engineering methods are being devised for the exploitation of isoflavonoids. Flavanones are immediate precursors for flavonoid biosynthesis and can occur as monomers, dimmers, and higher oligomers. Flavanones
such as naringenin and liquiritigenin undergo migration at the B-ring from 2- to the 3-position by hydroxylation at the 2-position to yield isoflavonones. Isoflavone synthase (IFS) an enzyme, cytochrome P450, NADPH-dependent, dehydrates liquiritigenin or naringenin to yield 2-hydroxyisoflavone, spontaneously or enzymatically. Dehydration of 2-hydroxyisoflavone is catalyzed by 2-hydroxyisoflavanone dehydratase (IFD), which forms genistein and daidzein, isoflavonoids (Dixon, 1999; Akashi et al., 2005). The isoflavonoids can further be metabolized to yield phytoalexins, examples medicarpin, or rotenoids, example 9-demethylmunduserone (Akashi et al., 2005). Subsequent species and tissue-specific enzymatic conversions (e.g. glycosylation, o-methylation, and prenylation) create a wide array of structurally diverse group of flavonoids, and isoflavonoids.

Phenylpropanoids are natural products derived from the amino acid L-phenylalanine. Phenylpropanoid pathway is conserved in all plant species. The pathway consists of plant phenolics that are responsible for cell wall structural roles, have different wood, and establishing flower color. Phenylalanine deaminates by L-phenylalaninelyase (PAL) as seen in Figure 1 to form cinnamic acid, cinnamate 4-hydroxylase (C4H), and then converted to p-coumaric acid. Hydroxycinnamic acids, example sinapic acid, and the monolignols, such as coniferyl alcohol contain $C_6C_3$ phenylpropane skeleton. Complex phenylpropanoids are formed by condensation of p-coumaryl coenzyme A with a unit obtained from acetate from malonyl coenzyme A, 4-coumarate:coenzyme A ligase (4CL). Triamine derivatives can then branch out into different pathways such as flavonoids,
isoflavonoids, lignins/monolignols, coumarins, benzoic acids and stilbenes (Dixon and Sumner, 2002).

In most species, in flavonoid pathway, chalcone synthase condenses \textit{p}-coumaroyl-CoA with 3 molecules of malonyl-CoA to generate 4, 2', 4', 6'-tetrahydroxychalcone (naringenin chalcone). Naringenin chalcone can be further metabolized to (2S)-5, 7, 4'-trihydroxyflavanone (naringenin) by chalcone isomerase to form the primary C15 flavonoid skeleton. In species that synthesize isoflavones, the enzyme isoflavone synthase (IFS), another cytochrome P450 monooxygenase, acts as the key metabolic entry point for the formation of all isoflavonoid compounds (Akashi et al., 1999; Steele et al., 1999; Jung et al., 2000). IFS mediates, the intramolecular aryl migration of both liquiritigenin and naringenin to form 2-hydroxyisoflavanones. Isoflavones are unstable in ambient temperatures and therefore 2-hydroxyisoflavanones in presence of a dehydratase (isoflavone dehydratase, IFD) converts into genistein and daidzein. However, this may not be the only route for their synthesis in plants. Even though phenyl propanoid pathway is the oldest pathway in plants, there are a lot of steps which still have to be elucidated at the molecular level. Very little is known about the flux control, cross talk between the enzymes such as CHS, CHI, IFS and IFD, the association of biosynthetic pathways, enzymes in metabolic channels or regulatory mechanisms in response to different stress conditions (Dakora and Phillips, 1996; Dixon and Sumner, 2003).

Plant phenolics contain hydroxyl group attached to the aromatic phenyl ring. All plant species do not show the presence of all classes, for example CHIs are ubiquitous in the plant kingdom and convert naringenin chalcone to naringenin.
Figure 1. Schematic representation of biosynthetic pathways leading to flavonoid and isoflavonoid natural products including genistein, daidzein, and biochanin A. CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FS, flavone synthase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; HI4′OMT, 2,7,4′-trihydroxyisoflavanone 4-O-methyltransferase; I3H, isoflavone 3′-hydroxylase; IFS, 2-hydroxyisoflavanone synthase (from Deavours and Dixon 2005).

In contrast, the other type II of CHIs, appear to be legume specific converting isoliquiritigenin to liquiritigenin (Jez et al., 2000). Members of these classes with specific substitution patterns may be peculiar to certain genera or species. Mostly the isoflavonoids are restricted to the subfamily Papilionoideae of the Fabaceae (Dixon et al., 2002).
Isoflavonoids are derived from the 3-phenylchrome-4-one (3-phenyl-1,4-benzopyrone) structure. Chromone is a derivative of benzopyran with a substituted keto group on the pyran ring. Isoflavonoids are synthesized by the phenylpropanoid pathway in which phenylalanine is used to produce 4-coumaroyl-CoA. This can combine with malonyl-CoA to yield a group of compounds called chalcones, which contain two phenyl rings. The most common isoflavonoids are genistein and daidzein. Genistein undergoes methylation to form compounds like biochanin A and formononetin. Genistein is notable for its estrogenic qualities. Biochanin A is also known for weak estrogenic activities.

In particular, biochanin A (5,7-dihydroxy-4´-methoxyisoflavone) is a member of a class of isoflavonoid compounds characterized by estrogenic activity in humans and animals. It commonly occurs in many species of vascular plants, mostly belonging to the legumes (Fabaceae), and many species of grasses (Poaceae). Biochanin A has also been isolated from fruit trees and shrubs, mainly from the genus Prunus. Studies by Liggins et al., in 2000 showed biochanin A to be abundant in red clover (Trifolium pratense), broom dyers (Genista tinctoria), soybean (Glycine max) and plum (Prunus spinosa).

1.5 Commonly Found Phytoestrogens

Many phytoestrogens have been identified in vascular plants: formononetine, genistein, daidzein, angolensin, phaseolin, pisatin, glyceolin, rygenin, kiewiton, coumestrol, medicarpin, prunetin, biochanin A and pterocarpin. Some of the isoflavone compounds, particularly phaseolin, glyceolin, kiewiton, coumestrol, medicarpin and pisatin, act as phytoalexins which, in many plant species, produce compounds that defend
from the invasion of viral, bacterial and fungal pathogens. Earlier studies have shown that
genistein, daidzein, ekwol, biochanin A and formononetine are the most known and
biologically active compounds with the greatest estrogenic activity (Bruxelles and
Roberts, 2001).

Isoflavones are estrogenic because they have the hydroxyl and methyl groups
resembling estradiol. The number and distribution of hydroxyl and methyl groups on
rings A and B of isoflavonoids bears analogous cyclical structural resemblance to
estradiol and also determines the strength of estrogenic activity. Estradiol is a female
endogenous steroidal hormone. Diethylstilbestrol is a common synthetic analogue.
Estrogenic compounds can compete with steroidal hormones as an agonist or antagonist
(Mikisieck 1995; Pearce et al., 2003).

Some plants are also known to contain steroidal estrogens. The most common
phytoestrogens found in plants are cholesterol, and stigmasterol. The common
phytosterols such as β-sitosterol, campesterol, and stigmasterol do not bind to human
estrogen receptors (ER) and do not exert estrogenicity in female rats (Baker et al., 1999).

Earlier studies have shown that isoflavones, commonly found in fodder, undergo
biotransformation in animal organisms. Formononetin, which has weak estrogenic
activity (found in red clover) possesses a hydroxyl group that undergoes demethylation
and reduction into a compound with higher estrogenic activity.
1.6 Discovery of Phytoestrogens

Plants were originally shown to have estrogenic activity in the 1920s. One of the most dramatic examples of their effects on animals was seen in the 1940s when sheep grazing on clover became infertile in Western Australia (Bennets et al., 1946). It was discovered that the plant chemicals responsible for sheep infertility were coumestans. By 1975, several hundred plants had been discovered to produce estrogenic compounds (Chang et al., 1975; Drane et al., 1975). Between 1954 and 1974, reproductive problems were observed in cattle, guinea pigs, rabbits, cheetahs, and mice (Bradbury and White, 1954; Wright 1960; Leavitt 1963; Adams 1995). Since then, some compounds that had caused concerns about reproductive or related risk of phytoestrogens in humans have been discovered with beneficial aspects.

1.7 Structural Resemblance of Phytoestrogens with Estradiol and Its Significance

Phytoestrogens act as estrogens in different *in vivo* and *in vitro* assay systems by binding to estrogen receptors (ER) (Pearce et al., 2003; Miksicek, 1995). Genistein, Daidzein and Daidzin, as shown in Figure 2, have ring structures similar to estradiol. The hydroxyl groups present in rings A and C of Estradiol correspond to hydroxyl groups on Genistein, Daidzein and Coumestrol at approximately the same positions (Kudou et al., 1991). These hydroxyl groups are located in such a position as to enable binding of the named chemicals to ER proteins to form ligand-binding complexes, thereby, activating transcription of target genes (Suetsugi et al., 2003).
Figure 2. Comparison of the chemical structures of the phytoestrogens Genistein, Daidzein, and Daidzin with 17β-estradiol, the female sexual hormone and ligand for estrogen receptors (adapted from Zhao et al., 2002).

Some of the phenolic phytoestrogens are believed to act as plant antibacterial and antifungal agents, as well as insect deterrents (Rivera-Vargas et al., 1993). Most animal research on phytoestrogens has focused on their potential protective effects against age related diseases, such as cardiovascular diseases, osteoporosis, and hormone-induced cancer (Barnes, 1998; Le Bail et al., 1998). Isoflavones are known to possess therapeutic and preventive properties. Compounds such as genistein, daidzein and their derivatives have proven to inhibit the formation of tumors (Danzo, 1998). Phytoestrogens have also been associated with antiestrogenic activities by altering the ER action in breast and other tissues, thus reducing the risk of cancers (Ingram et al., 1997; Nikov et al., 2000). Isoflavones have been shown to act both as attractants and as animal deterrents.
The biological activities of isoflavones range from properties that suggest important functions in the plant-environment interactions to pharmacological properties in animal cells that may or may not reflect corresponding functions or activities in plants (Dixon, 1999). Natural roles of isoflavones are in both positive and negative plant microbial interactions. For example, isoflavones function in establishing of symbiotic relationships between plant and rhizobial bacteria which is a positive plant microbial interaction (Pueppke et al., 1996). Conversely, production of isoflavonoids results in phenolics that repel the rhizosphere microbes (Weisskopf et al., 2006).

A correlation was found between high levels of estrogenic activity and the formation of functional gynoecium in female flowers of mulberry, and of vestigial gynoecium in mulberry male flowers (Maier et al., 1997). The occurrence of endogenous phytoestrogens may suggest a possible pattern or strategy in the reproduction of these dioecious species.

1.8 Tissue Culture and Its Importance

Tissue culture opens up an extensive area of biotechnological research into the potential use of in vitro cultures to produce highly valuable secondary metabolites, including compounds of medical applications (Verpoorte et al., 2000, 2003; Kieran et al., 1997). In vitro plant tissue cultures are used for: 1) for micropropagation of a large number of genetically uniform and pathogen-free, economically important clones in a limited time and space (Bhau and Wakhlu, 2003; Zobayed and Saxena, 2003); 2) an alternative to whole plants for production of secondary products for biochemical and
developmental studies (Brisson et al., 1988; Seo et al., 1993); and 3) a source of useful novel phytochemicals that are not produced or have not been detected in differentiated plant tissues (Federici et al., 2003).

Phytochemical research has provided valuable information about the presence of flavonoid compounds in plant tissue cultures. Therefore, in vitro plant tissue cultures are considered an attractive source of biologically active compounds and several approaches have been used to increase their accumulation in cultures. Thus, plant cultures can become new sources for beneficial phytochemicals, independent of season and climate conditions (Luczkiewicz et al., 2003).

Plant cell cultures have been used to produce novel useful compounds, especially secondary metabolites involved in drug development (Staba et al., 1982). For example, Vinblastine and Vincristine, alkaloids obtained from periwinkle, Catharanthus roseus, are used as anticancer agents (Garnier et al., 1996; Datta and Srivastava, 1997). Diosgenin, a steroid saponin of fenugreek, Trigonella foenum graecum, has anti-fungal and anti-bacterial, and medicinal properties to treat diabetes, high cholesterol, wounds, inflammation and gastrointestinal ailments. Recent studies have shown that diosgenin may possess anticarcinogenic properties as well (Jayadev et al., 2004). Generally, plants such as Hypericum perforantum and Arnica montana do not withstand large field cultures due to pathogen sensitivity (anthracnose), which has led scientists to consider plant cell tissue and organ cultures as an alternative way to produce useful secondary metabolites (Bourgaud et al., 2001). Therefore, cultivation of plant tissues on synthetic
media offers an efficient alternative to the traditional cultivation in the fields or greenhouses for the production of metabolites of interest.

Examples of plant tissue cultures are callus, cell suspension and protoplast cultures. Traditionally, plant tissue explants were used to induce callus, but they are also useful for obtaining cultures of differentiated tissues, such as hairy root cultures, known for the production of secondary compounds in some plant species (Yoojeong et al., 2002). Callus produces an undifferentiated mass of parenchymatic cells that can be subcultured indefinitely to regenerate whole plants under proper hormonal conditions. The induction and growth of callus tissue is dependent on the composition of the medium, mostly on the concentration of the plant growth hormones (auxins and cytokinins), and on plant genotypes (Gonzalez et al., 2001). When friable callus is grown in liquid medium on a shaker, it becomes a cell suspension culture that can be used for production of secondary metabolites of interest. For example, Hypericin and Pseudohypericin have been isolated from Hypericum perforatum in cell suspension cultures (Bais et al., 2002).

1.9 Importance of Mulberry for the Study

Osage-orange, Maclura pomifera, red mulberry, Morus rubra, and white mulberry, M. alba (Figure 3) belong to the Moraceae family of angiosperms containing 55 genera and 1,000 species. Both Maclura and Morus genera are dioecious with greenish-yellow unisexual flowers grouped in inflorescences (Harrar and Harrar, 1962; Smith and Perino, 1981).
Osage-orange is the only species of its genus and a native to the Red River Valley between Texas, Oklahoma and Arkansas (Harrar and Harrar, 1962). Mulberry species are perennial woody plants of considerable economic importance because of their foliage, which constitutes the food for the mulberry silkworms, *Bombyx mori*, and because the males are widely used in landscape as “fruitless mulberry.”

![Morus alba](image)

**Female**

**Male**

**Figure 3.** Foliage and inflorescences of the *Morus alba*. A. *M. alba* male; B. *M. alba* female.

There are several species of mulberry worldwide. In the USA, red mulberry and white mulberry are distributed from the northeastern areas to Florida in the south to Texas in the west. *Maclura* and *Morus* both contain phytoestrogens that activate the estrogen receptor in a transgenic yeast system (Maier *et al.*, 1995; 1997) and thus could produce phytochemicals of interest for medical research in prevention and or treatment of cancers. The Moraceae family of dioecious flowering plants is native to warm, temperate, and subtropical regions of Asia, Africa, North America, and southern Europe. Mulberry trees grow fast initially and have a slow growth later. Their fruits are multiple compound, 2–3 cm long. In several species, mulberries begin as white to pale yellow with pink edges, becoming red during ripening and dark purple to black when fully ripened.
Since *in vitro* plant tissue cultures have proven to be attractive sources of biologically active compounds that can be independent of the season and climate conditions (Luczkiewicz and Glod, 2003), one aim of this study was to establish *in vitro* tissue cultures.

Mulberry trees are conventionally propagated by grafting and cutting (Honda 1972). Studies have been done on the establishment of callus culture, regeneration of plants, micropropogation, cryopreservation of germplasm, synthetic seed, and the formation of secondary metabolites in cell cultures. *Morus* species, in addition to producing many secondary metabolites, are a rich source of prenylchalcone and prenylated 2-arylbenzofuran. Morin (2,3′,4,4′,6-pentahydroxybenzophenone) is a constituent of the heartwood of *M. alba* (Haley and Bassin 1951; Spada *et al* 1956). Quercitin, rutin, and quercitin-3-triglucoside (Moracetin) were obtained from an aqueous methanolic extract of mulberry leaves (Naito 1968). Later four prenylated flavonoids, Mulberrin, Mulberrochromene, Cyclomulberrin, and Cyclomulberrochromene were isolated along with betulnic acid from the stem and root bark of *M. alba*, (Deshpande *et al.*, 1968). Beta–tocopherol was isolated from root bark (Kang *et al.*, 1999). Mulberry constituents are comprised of two molecules of prenylphenols (Nomura 1988). Leaves of *M. alba*, when infected with *Fusarium solani f. spp. Mori*, produced a natural Diels-Alder-type adduct Chalcomoracin, known as a phytoalexin and as 1-deoxy-nojirimycin found in leaves, root and bark (Nomura 1988).

Mulberry is a crop of economic importance in the sericulture industry. Previous studies indicate that estrogen / estrogen-like compounds occur during the development of
female reproductive structures in Osage-orange and mulberry (Maier et al., 1997) and quaking aspen, *Populus tremuloides* Michx. (Khaleel, 2003). *Bacillus thuringiensis* has been reported on the adaxial as well as the abaxial surfaces of the leaf (Ohba, 2007). Mulberry supplies leaves to raise silkworms. Mulberry leaves, bark, and branches have been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen the joints, facilitate discharge of urine and lower blood pressure. It is only recently that their mechanism action has been related to their antioxidant activity.

The chemical composition of mulberry leaves includes rutin, quercetin, isoquercitin and other flavonoids. In most mulberry-growing countries, especially in China and India, production is focused on enhancing the foliage. The leaves, the sole food source of the silkworm (*Bombyx mori* L.), the cocoon of which is used to make silk, have a large ecological and economical importance. In Europe, mulberry is appreciated more for their fruits than for their foliage. However, almost all the parts of the tree are used for pharmacological studies all over the world. The leaves have been shown to possess diuretic, hypoglycemic, and hypotensive activities, whereas the root bark of mulberry has been used for antiinflammatory, antitussive, and antipyretic purposes. Mulberry fruits can be used as a warming agent, as a remedy for dysentery, and as a tonic, sedative, laxative, odontalgic, anthelmintic, expectorant, and emetic (Ercisli and Orhan et al., 2006). In Italy, the berries of *Morus nigra* and *Morus alba* are consumed fresh or in the form of various confectionary products such as jam, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine (Ercisli and Orhan et al., 2006).
One of the aims of this study was to enhance the production of phytoestrogens in \textit{in vitro} cultures of Moraceae species, isolate and chemically characterize some of the phytoestrogens in callus extracts and fractions in order to obtain a reliable source for beneficial phytoestrogens.

1.10 Goals and Objectives

The main goal of this work was to conduct a practical investigation to identify phytoestrogens in mulberry. In particular this work aims to address the following questions:

1. Establish and enhance the \textit{in vitro} cultures for \textit{M. alba}

2. Determine estrogenic activity of \textit{in vitro} cultures and contrast it with adult plant

3. Determine HPLC profiles of \textit{in vitro} cultures and contrast it with adult plant

4. Determine a specific compound having estrogenic activity in mulberry

For establishing the \textit{in vitro} cultures of male and female of \textit{Morus alba}, different concentrations of auxins and cytokinins were used on Murashige and Skoog (MS) medium. The estrogenic activities of the established cultures were also noted. MS medium with NAA (1 g/l) and BAP (0.5 g/l) was the best medium for the study. The estrogenic activities of \textit{in vitro} tissue extracts were compared with the adult plant tissue extracts. The \textit{in vitro} tissue extracts had three times higher estrogenic activities than adult plant tissue extracts.

The extracts were further fractionated with the help of solid phase extraction. The active fraction or peaks were identified with the help of high performance liquid
chromatography (HPLC). The HPLC profiles of \textit{in vitro} tissues were different from the adult plant tissues. The concentration of the solvents methanol and water was varied to obtain well separated fractions.

An estrogenically active fraction from the male differentiated tissue extract was chosen for further analysis. This fraction was further subjected to Mass spectrometry fragmentation to identify the compound present in mulberry. The estrogenically active compound was biochanin A, which is structurally similar to 17β–estradiol. Biochanin A has a methyl group at the A ring instead of the hydroxyl group and functions as a weak phytoestrogen.
CHAPTER 2
MATERIALS AND METHODS

2.1 Plant Tissue Culture

2.1.1 Plant Material and Preparation of Explants for Tissue Culture

Samples of *M. alba* male were taken from trees located on Ruddell Street, Denton, Texas and from *M. alba* females located in the area surrounding the Texas Woman’s University, Denton campus. Explants such as young green stem, leaf and petiole were used to induce callus. Explants were surface sterilized for 15-20 minutes in 15% bleach solution to which 2-4 drops of Tween-20 (Fisher Scientific, New Jersey) was added and then were rinsed three times with sterile distilled water and placed on callus inducing medium. The explants were excised to 1 mm$^3$ and cuts were made with a scalpel on the explant surfaces to stimulate growth of callus.

2.1.2 Initiation and Maintenance of Callus Cultures

The protocol for establishing *in vitro* tissue cultures is diagrammed in Figure 4. The following type of tissue culture was established: callus culture. Different hormonal concentrations were supplemented to Murashige Skoog (Murashige and Skoog, 1962) basal medium containing 2% sucrose and 0.3% phytagel w/v (Table 1) and assayed for the initiation and growth of callus. Concentrations of auxins and cytokinins were varied in the MS medium composition to obtain high callus yield (Jain *et al.*, 1996).
Among the callus-inducing media, MS 1, [MS basal medium supplemented with 1-napthalene acetic acid (NAA) (1 g/l) and benzylaminopurine (BAP) (0.5 g/l)], proved to be the most effective for establishing callus from *M. alba*. Explants were incubated for two weeks in Petri plates in complete darkness at 25±2°C. In order to maintain and
propagate cultures, calli were excised from the original explants, cut into roughly 1 mm³ pieces, and subcultured on fresh MS 1 medium for *M. alba*. The different chemicals used for tissue culture were obtained from Sigma-Aldrich, Missouri.

2.2 Estrogenic Activity Determination or Analysis

2.2.1 Isolation of Estrogenic Fractions from Callus Extracts and Preparation of Plant Extracts for Estrogenic Activity Analysis

Specimens, such as calli and branches with leaves, were chopped into small pieces and their fresh weights were recorded. Extracts were made by homogenizing the tissues in 100% ethanol (1:4 fresh w/v) for 5 minutes with a PowerGen 700 homogenizer. The homogenates were extracted at room temperature for 48 to 72 hours with occasional shaking and then centrifuged at 3,200 rpm for 30 minutes. The mix was filtered using Whatman 54 filter paper and was stored at -20°C until use.

2.2.2 Estrogenic Activity Assays

Crude extracts, solid phase extraction (SPE) and high performance liquid chromatography (HPLC) fractions were assayed for estrogenic activity using a transgenic yeast system expressing the human receptor alpha (hERα) (Figure 5). The genetically engineered yeast cells contain both an expression plasmid (YEPE10) and a reporter plasmid (YRPE). Through the expression plasmid, the cells produce ER molecules, which are biologically active and manifest the same biological properties as those expressed in mammalian cells (Santiso-Mere *et al.*, 1991).
The reporter plasmid expresses β-galactosidase molecules, whose activity can be detected visually in a colorimetric reaction and measured spectrophotometrically. The transactivation of the β-galactosidase gene by the estrogen receptor is dependent on the availability of a specific ligand and the presence of estrogen response element (ERE) upstream of lacZ promoter in the reporter plasmid (Figure 5).

**Figure 5.** Estrogen-responsive transcriptional system in *S. cerevisiae* strain BJ3505 used to determine the estrogenic activities of crude extracts, SPE and HPLC fractions (adapted from Santiso-Mere *et al.*, 1991).

Yeast cells were maintained in a minimal medium containing Casamino acids, 2% dextrose, 1% yeast nitrogen base without amino acids and 0.0012% adenine sulfate. A solution of 17β-estradiol prepared in 95% ethanol was used to standardize the plant extracts. Serial dilutions of 17β-estradiol were used to construct a standard curve.
Readings were taken with the spectrophotometer at 282nm. The estradiol standard curve was used to estimate ‘estrogen equivalents’ in the plant extracts. In general, the A282 for male and female extracts were similar, or those for male extracts were slightly lower than the female extracts. Therefore the volumes of male and female extracts used to inoculate the yeast cultures were adjusted such that equivalent amounts of A282 absorbing material were compared.

To test estrogenic compounds in plant tissue extracts, yeast cells containing the receptor expression plasmid and reporter plasmid were grown in minimal medium with 100µM CuSO4, overnight at 30º C. The addition of copper sulfate to the medium induces the production of ERs since the estrogen receptor gene is under the control of yeast metallothionein (CUP1) promoter. Extracts and fractions were standardized by an estradiol standard curve, 200 µg estrogen equivalents were used for each assay. Yeast was grown overnight on a shaker at 230 rpm and 30ºC. Yeast cells were lysed by vortexing them in the presence of glass beads followed by centrifugation at 4ºC for 10 minutes. Protein content in the supernatant was measured by the Bradford method (Bradford, 1976). The supernatants were analyzed for protein content and β-galactosidase activity. Total protein content was estimated according to Bradford.

The level of β-galactosidase expressed in the yeast cells was measured according to the method described by Miller. Typically, each fraction was assayed in triplicate using 5-15µg of yeast cell protein. Cultures inoculated with estradiol and Genistein were used as positive controls. Yeast cultures alone in minimal medium without CuSO4 were used as negative controls.
2.3 Fractionation and Isolation of Phytoestrogens

2.3.1 Solid Phase Extraction (SPE)

For SPE, Alltech C\textsubscript{18} columns were employed for the separation of crude extracts into three different fractions: solid matrix (SM), F20 (eluted with 20\% methanol), and F80 (eluted with 80\% methanol). The SPE columns were activated with 5ml of methanol and 5ml of water. Crude extracts (3ml) were dried down under nitrogen gas flow and were resuspended in 1ml of 80\% methanol in 3ml of distilled water. The mixture was passed through a Millipore 0.22 μm sterilizing filter onto the activated SPE column to obtain the SM fraction. The column was eluted further with 20\% methanol to obtain F20 and then with 4ml of the 80\% methanol to obtain F80. Each fraction was dried under nitrogen gas flow and resuspended in 100\% ethanol for fractionation by HPLC. Each fraction was assayed for estrogenic activity in transgenic yeast, and assayed spectrophotometrically for phytoestrogens.

2.3.2 High Performance Liquid Chromatography (HPLC)

The estrogenically active SPE fractions were further fractionated using a Gilson 322 pump high performance liquid chromatography system combined with a Gilson 155/156 UV/V detector on C\textsubscript{18} columns. The solvent system consisted of methanol (A) and water (B). The gradient profile was 0-20 minutes from 40 to 90\% A, 20-45 minutes at 90\% A, 45-46 minutes from 90 to 100\% A, and 46-60 minutes at 100\% A. The flow rate was 0.5 ml/min with detector wavelengths at 260 and 280 nm. The HPLC chromatograms of the SPE F80 fractions for \textit{M. alba} male and female callus extracts, \textit{M.}
male and female adult plant extracts were compared. High performance liquid chromatography fractions were taken every 1.5 minutes, assayed for estrogenic activity and subjected to spectrophotometric analyses.

2.3.3 Spectrophotometry

Spectra in UV-visible range of 200 and 400 nm were taken for crude extracts, SPE and HPLC fractions in a Shimadzu UV Mini 1240 spectrophotometer. Solutions of estradiol and Genistein in ethanol were used as controls and ethanol 100% was used as a blank. Spectrophotometric scans were compared for possible identification of the chemical classes for phytoestrogens in active fractions.

2.3.4 Electrospray Ionization Mass Spectrometry (ESI–MS)

In electrospray, HPLC extracts were passed through a nozzle. HPLC extract was introduced to the source in solution through a syringe pump with the flow rate set to 1μl min\(^{-1}\). HPLC extract flew through the electrospray needle at a potential difference of 35eV. This forced the droplets from the needle with a surface charge of the same polarity as the charge on the needle. The droplets were repelled from the needle towards the source sampling cone on the counter electrode. The charged liquid in the nozzle became unstable as it was forced to hold more and more charge. Soon the liquid reached a critical point, at which it could hold no more electrical charge and, at the tip of the nozzle, it blew apart into a cloud of tiny, highly charged droplets.
Exciting potential opportunities are offered by ESI-MS for an ionization and by tandem mass spectrometry (MS/MS) for a precise determination of molecular weight and structural characterization of molecules at trace levels in complex matrices. Two different approaches are offered in principle by ESI-MS to characterize unknown species and help in the identification of the phytoestrogen present in the HPLC extract of *M. alba*:

a) the exact mass of the molecular ion was compared with a standard to identify the molecular species,

b) fragmentation patterns obtained from MS/MS experiments were used to obtain information about the structure of the molecule.

2.4 Statistical Analysis

Averages and standard deviations were calculated for all experiments with at least two replicates. Callus initiation and proliferation time were used for comparing T-tests and for comparing estrogenic activities among samples.
CHAPTER 3

RESULTS

3.1 Results of Male and Female *M. alba* Callus Cultures

Secondary metabolites are present in minute amounts within the cell. Tissue culture increases the production of secondary metabolites. The first objective of this study was to establish tissue cultures of male and female *Morus alba*. For establishing tissue cultures of *M. alba*, concentrations of auxins and cytokinins in the basal Murashige and Skoog (MS) medium were varied. Appropriate ratios of auxins and cytokinins required for initiating calli were identified as 1 g/l NAA and 0.5 g/l BAP, (MS 1). To obtain calli, young white mulberry stem explants were grown in basal MS 1 medium supplemented with 2% sucrose and 0.3% phytagel.

Once the tissue cultures were established the next question to address was to proliferate the *in vitro* tissue to enhance estrogenic activity. The MS 1 medium was further modified to enhance the production of callus tissue as well as increase the phytoestrogenic activity in callus extracts. Table 1 displays the various concentrations of plant growth hormones for the proliferation of callus. After initiation of callus the tissue cultures were transferred to six different media to measure proliferation. The media used for this study were as shown in Table 1. The white male mulberry callus cultures did not survive in MS 3 and MS 5 media. The female callus cultures of *M. alba* did not survive in MS 3, MS 4, MS 5 and MS 6. MS3 and MS6 did grow for a week and then the growth
stopped. V MS4 and MS5 well in the beginning but leached out a lot of phenolics which hindered the growth.

**Table 1.** Composition of Murashige and Skoog media assayed for establishing callus cultures of white mulberry.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Auxin</th>
<th>Cytokinin</th>
<th>Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA (g/l)</td>
<td>BAP (g/l)</td>
<td>Thiamine–HCl (g/l)</td>
</tr>
<tr>
<td>MS 1</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>MS 2</td>
<td>1.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>MS 3</td>
<td>0.8</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>MS 4</td>
<td>1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>MS 5</td>
<td>1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>MS 6</td>
<td>1.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MS 7</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Rhizogenesis, initiation of roots in callus cultures, was observed in the male and female species of *M. alba* cultures after the second subculture. The cultures were transferred to fresh media frequently to avoid rhizogenesis.

The average growth of callus was approximately 0.5 grams per week. The cultures male and the female grew at near identical rates during the course of the experiments.

To study the initiation and proliferation of callus cultures samples were collected throughout the year 2006 and subcultures were established by December 2006. The calli were established for proliferation in MS 1, MS 2 and MS 7 media by December 2006 and
growth rates on MS 1, MS 2 and MS 7 were similar. The cultures were assayed for estrogenic activity after the second subculture.

Explants of *M. alba* were collected from trees in March 2008 and callus cultures were established. Male explants were collected from trees located on Ruddell Street in Denton, Texas and female explants from trees located on Oakland Street in Denton, Texas. *In vitro* explant tissues, such as stem and petiole swelled within 2-3 days, and callus initiated within 15 days post inoculation.

Initially explants were collected from trees located on Texas Street in Denton, Texas. The male and female plants were in close proximity to avoid differences in nutrient concentration. The explants collected from this location showed high contamination and therefore were not considered for further experiments. Experiments were done to obtain enough callus cultures from 1 mm³ pieces of the explant. The proliferation of callus was not enough to last to the end of the experiment. For future work cell suspension techniques could be tried to obtain enough callus culture from a single explant.

3.2 Results for the Estrogenic Activities of Callus and Adult Plant Tissues Extracts and Its Fraction Obtained through SPE and HPLC

Ethanolic extracts of mulberry callus were prepared and screened for transcriptional activity of the reporter gene in the estrogen-responsive yeast system.

The estrogenic activity of the callus cultures obtained from three different media MS 1, MS 2 and MS 7, were established. Initially about 10 grams of tissue was weighed and 40 milliliters of ethanol was blended with the plant tissues. The estrogenic activity of
callus cultures established in MS 2 and MS 7 were lower than the MS 1 media seen in Figure 6.

**Figure 6.** Estrogenic activity of the three different proliferation media MS 1, MS 2 and MS 7. Yeast Culture = 19.26±8.7 MU, Estradiol = 4559.29±223.4 MU, and Genistein = 1492.18±67.7 MU. Each datum point represents the average of 3 experiments ± S.D.

The callus cultures that were established on the proliferation medium (MS 1, MS 2 and MS 7) by December 2006 were tested for estrogenic activity using the yeast system expressing the human receptor alpha (hERα). The callus cultures were assayed for estrogenic activity after the second subculture, as represented in Table 2.

**Table 2:** Estrogenic activities of the callus in proliferating media from both male and female species of Morus alba.

<table>
<thead>
<tr>
<th>Media</th>
<th>Estrogenic Activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS 1</td>
<td>306.29 ± 15.6</td>
</tr>
<tr>
<td>MS 2</td>
<td>142.22 ± 21.9</td>
</tr>
<tr>
<td>MS 7</td>
<td>161.1 ± 15.8</td>
</tr>
</tbody>
</table>
MS 1 medium produced callus with significantly higher estrogenic activity ($P = 0.0001$ at $\alpha = 0.05$) than MS 2 and MS 7. There was no significant difference between MS 2 and MS 7. Therefore these results suggest that MS 1 is a better medium for producing estrogenic activity in *in vitro* callus cultures of *M. alba*.

Callus cultures of *M. alba* from young stem explants were established in March, July, and September of 2007. Callus cultures for MS1, MS2 and MS7 were incubated or subcultured for three and half months. Callus extracts from March showed the highest estrogenic activity, significantly ($P = 0.0036$ at $\alpha = 0.05$) higher than July and September, seen in Figure 7.

![Figure 7](image-url)  

**Figure 7.** Estrogenic activities of the established calli in March, July and September. Yeast Culture = 26.66±15.2 MU, Estradiol = 4556.9±187.7MU and Genistein = 1492.19±88.7 MU. Each datum point represents the average of three experiments ± S.D.

As shown in earlier studies by Maier *et al.* in 1995 mulberry plant extracts had different estrogenic activities every month. The estrogenic activity was highest in March in that
study as well. The estrogenic activities of March, July and September were studied as

callus cultures could be established for these months only. The contamination rate for the

rest of the months was high and therefore the callus cultures could not be established.

Since the estrogenic activities of plant extracts were different in different months, care

was taken to collect the explants on the same day of the month for comparison. The

tissues did differentiate but the callus cultures did not differ morphologically.

New growth of stem explants were inoculated on March 23 in MS 1 medium. The

adult plant tissue extract was prepared the same day from both male and the female

specimens of *M. alba*. White meristematic friable callus was subcultured on the new

medium every other week.

The male callus extract (MCE), female callus extract (FCE) obtained from young

stems and corresponding tissue from adult male plant extract (MPE) and adult female

plant extract (FPE) were screened for estrogenic activity as seen in Figure 8.

![Figure 8](image_url)

**Figure 8.** Comparison of estrogenic activities of MCE, FCE, MPE and FPE and their

Solid phase (SP), F20 fractions (F20), and F80 fractions (F80). Yeast Culture = 28.51±

17.3 MU, Estradiol = 4159.13±211.1 MU, and Genistein = 1429.61±111.1 MU. Each
datum point represents the average of 3 experiments ± S.D.
The estrogenic activity of the crude plant and callus extracts showed that the FCE had a higher estrogenic activity than the corresponding MCE. MCE was significantly higher than MPE (P = 0.0061, α = 0.05). FCE was significantly higher than FPE (P = 0.00075, α = 0.05). The estrogenic activity of FPE was significantly higher than MPE (P = 0.00053, α = 0.05). The estrogenic activity of each extract studied is shown in Table 3.

Table 3: Comparison of estrogenic activities of callus with adult plant tissues.

<table>
<thead>
<tr>
<th>Tissue Extracts</th>
<th>Sampled Screened</th>
<th>Estrogenic activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE</td>
<td>Male Callus Extract</td>
<td>597.77 ± 58.1</td>
</tr>
<tr>
<td>MCE–F80</td>
<td>Male Callus Extract–F80 fraction</td>
<td>879.44 ± 124.3</td>
</tr>
<tr>
<td>MCE–HPLC7</td>
<td>Male Callus Extract–HPLC fraction # 7</td>
<td>1105.6 ± 82.2</td>
</tr>
<tr>
<td>FCE</td>
<td>Female Callus Extract</td>
<td>627.49 ± 57.3</td>
</tr>
<tr>
<td>FCE–F80</td>
<td>Female Callus Extract–F80 fraction</td>
<td>729.63 ± 67.4</td>
</tr>
<tr>
<td>FCE–HPLC9</td>
<td>Female Callus Extract–HPLC fraction # 9</td>
<td>918.61 ±23.2</td>
</tr>
<tr>
<td>MPE</td>
<td>Male Adult Plant Extract</td>
<td>152.5 ± 5.5</td>
</tr>
<tr>
<td>MPE–F80</td>
<td>Male Adult Plant Extract–F80 fraction</td>
<td>192.49 ± 6.8</td>
</tr>
<tr>
<td>MPE–HPLC</td>
<td>Male Adult Plant Extract–HPLC fraction # 7</td>
<td>375 ± 10.6</td>
</tr>
<tr>
<td>FPE</td>
<td>Female Adult Plant Extract</td>
<td>218.22 ± 6.8</td>
</tr>
<tr>
<td>FPE–F80</td>
<td>Female Adult Plant Extract–F80 fraction</td>
<td>249.44 ± 10.4</td>
</tr>
<tr>
<td>FPE–HPLC</td>
<td>Female Adult Plant Extract–HPLC fraction # 9</td>
<td>421.48 ± 21.8</td>
</tr>
</tbody>
</table>
These experiments established that both male and female callus cultures had significantly higher estrogenic activity than their corresponding plant extracts. The female tissue extracts obtained from FCE and FPE both had higher estrogenic activity than the corresponding MCE and MPE. This holds true with the earlier studies performed by Maier et al in 1995.

MCE, FCE, MPE and FPE were further screened for estrogenic activity after being passed through C$_{18}$ columns and SPE as tabulated in Table 4.

**Table 4:** Comparison of estrogenic activities of different fractions obtained with solid phase extraction.

<table>
<thead>
<tr>
<th>Tissue Extracts</th>
<th>Sample Screened</th>
<th>Estrogenic Activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE–SP</td>
<td>Male Callus Extract–Solid Phase</td>
<td>546.66 ± 58.09</td>
</tr>
<tr>
<td>MCE–F20</td>
<td>Male Callus Extract–F20 fraction</td>
<td>611 ± 56.71</td>
</tr>
<tr>
<td>MCE–F80</td>
<td>Male Callus Extract–F80 fraction</td>
<td>720 ± 124.29</td>
</tr>
<tr>
<td>FCE–SP</td>
<td>Female Callus Extract–Solid Phase</td>
<td>572.22 ± 57.29</td>
</tr>
<tr>
<td>FCE–F20</td>
<td>Female Callus Extract–F20 fraction</td>
<td>751.11 ± 77.89</td>
</tr>
<tr>
<td>FCE–F80</td>
<td>Female Callus Extract–F80 fraction</td>
<td>918.61 ± 67.35</td>
</tr>
<tr>
<td>MPE–SP</td>
<td>Male Adult Plant Extract–Solid Phase</td>
<td>156.66 ± 5.54</td>
</tr>
<tr>
<td>MPE–F20</td>
<td>Male Adult Plant Extract–F20 fraction</td>
<td>168.82 ± 18.79</td>
</tr>
<tr>
<td>MPE–F80</td>
<td>Male Adult Plant Extract–F80 fraction</td>
<td>198.88 ± 6.81</td>
</tr>
<tr>
<td>FPE–SP</td>
<td>Female Adult Plant Extract–Solid Phase</td>
<td>214 ± 6.78</td>
</tr>
<tr>
<td>FPE–F20</td>
<td>Female Adult Plant Extract–F20 fraction</td>
<td>235 ± 10.32</td>
</tr>
<tr>
<td>FPE–F80</td>
<td>Female Adult Plant Extract–F80 fraction</td>
<td>247.77 ± 10.36</td>
</tr>
</tbody>
</table>

Fraction SP or solid phase was obtained by passing the extracts through C$_{18}$ columns.

SPE fraction F20 was obtained by eluting the extracts with 20% methanol in C$_{18}$ columns.
SPE F80 fractions were obtained by eluting the extracts with 80% methanol. SPE fractions SP, F20 and F80 were tested for estrogenic activity. Of these SPE-F80 fractions had the highest estrogenic activity as seen in Figure 8. Estrogenic activity was higher after solid phase extraction.

The data in Table 3 shows estrogenic activity of MCE–F80 significantly higher than MPE–F80 (P = 0.0061, α = 0.05). The estrogenic activity of FCE–F80 was significantly higher than FPE–F80 (P = 0.00028, α = 0.05). There was significantly higher estrogenic activity of FPE–F80 than to MPE–F80 (P = 0.0026, α = 0.05).

The crude tissue extracts showed higher estrogenic activities of MCE–F80, FCE–F80, MPE–F80 and FPE–F80 than MCE, FCE, MPE and FPE. The female extracts had higher estrogenic activity than their corresponding male extracts in callus and adult plant tissues. Callus extracts had higher estrogenic activity than adult plant tissue extracts, both in male and female. Solid phase extraction helped in purifying the estrogen like compounds in *M. alba* extracts.

The active F80 fractions were eluted through the HPLC to purify and isolate the estrogeneically active peak. The fractions that had the highest estrogenic activity were selected for a comparison study in MCE, FCE, MPE and FPE. Fraction 7, referred as MCE–HPLC7, had the highest estrogenic activity in MCE-HPLC. Fraction 9, referred to as FCE–HPLC9, had the highest estrogenic activity in FCE–HPLC extract. Fraction 7, referred to as MPE–HPLC7, had the highest estrogenic activity in MPE–HPLC. Fraction 9, referred to as FPE–HPLC9, had the highest estrogenic activity in FPE–HPLC extract. The estrogenic activity of the highest fractions can be seen in Figure 9 and their activities
are displayed in Table 5.

**Figure 9.** Comparison of estrogenic activities of MCE, FCE, MPE and FPE and their fractionation after F80 and HPLC techniques. Yeast Culture = 28.51±15.3 MU, Estradiol = 4159.13±211.1 MU, and Genistein = 1429.61±153.3 MU. Each datum represents the average of minimum 3 experiments ± S.D.

**Table 5:** Estrogenic activities and retention times of estrogenically active fractions obtained from HPLC fractionation of MCE, MPE, FCE and FPE respectively.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Estrogenically active fractions</th>
<th>Retention Time (Minutes)</th>
<th>Estrogenic Activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE-HPLC7</td>
<td>Male Callus Extract-HPLC #7</td>
<td>13</td>
<td>1105.553</td>
</tr>
<tr>
<td>MCE-HPLC9</td>
<td>Male Callus Extract-HPLC #9</td>
<td>17</td>
<td>195.29</td>
</tr>
<tr>
<td>MCE-HPLC15</td>
<td>Male Callus Extract-HPLC #15</td>
<td>29</td>
<td>537.77</td>
</tr>
<tr>
<td>MCE-HPLC16</td>
<td>Male Callus Extract-HPLC #16</td>
<td>31</td>
<td>132.59</td>
</tr>
<tr>
<td>MPE-HPLC7</td>
<td>Male Adult Plant Extract-HPLC #7</td>
<td>13</td>
<td>374.99</td>
</tr>
<tr>
<td>MPE-HPLC9</td>
<td>Male Adult Plant Extract-HPLC #7</td>
<td>17</td>
<td>347.40</td>
</tr>
<tr>
<td>FCE-HPLC9</td>
<td>Female Callus Extract-HPLC #9</td>
<td>17</td>
<td>918.61</td>
</tr>
<tr>
<td>FCE-HPLC15</td>
<td>Female Callus Extract-HPLC #15</td>
<td>29</td>
<td>311.44</td>
</tr>
<tr>
<td>FPE-HPLC7</td>
<td>Female Callus Extract-HPLC #7</td>
<td>13</td>
<td>319.25</td>
</tr>
<tr>
<td>FPE-HPLC9</td>
<td>Female Callus Extract-HPLC #9</td>
<td>17</td>
<td>421.47</td>
</tr>
</tbody>
</table>
When comparing different HPLC tissue extracts, MCE–HPLC7 had higher estrogenic activity than FCE–HPLC (P = 0.016, α = 0.05). Male differentiated tissue extract was higher than adult plant extract MCE–HPLC7 (P = 0.0043, α = 0.05). Female differentiated tissue extract exceeded its corresponding adult plant extract; FCE–HPLC9 was higher than FPE–HPLC9 (P = 0.0007, α = 0.05); female adult plant extract was higher than male adult plant extracts, (FPE–HPLC9 higher than MPE–HPLC7, P = 0.0443, α = 0.05).

Each purification step of the extracts showed an increase in estrogenic activity as seen in Table 4. MCE–F80 had higher estrogenic activity than MCE (P = 0.014, α = 0.05). MCE–HPLC was higher than MCE–F80 (P = 0.034, α = 0.05). FCE–F80 was higher than FCE (P = 0.031, α = 0.05). FCE–HPLC was higher than FCE–F80 (P = 0.012, α = 0.01). MPE–F80 was higher than MPE (P = 0.00098, α = 0.05). MPE–HPLC was higher than MPE–F80 (P = 0.00059, α = 0.05). FPE–F80 was higher than FPE (P = 0.0039, α = 0.05). FPE–HPLC was higher than FPE–F80 (P = 0.001, α = 0.05).

Therefore each purification produced a higher concentration of estrogenically active material than the previous. Therefore we conclude that the attempts at purification were effective.

The HPLC profiles of MCE and MPE are shown in Figure 10. The estrogenically active peaks have been marked with arrows on the chromatogram. The HPLC profiles of the adult plant extracts were not similar to HPLC profiles of callus extracts. The HPLC profiles of the male and female tissue explants were different. A greater number of estrogenically active peaks occurred in callus extracts than adult plant extracts as seen in
the different HPLC profiles of the plant tissue extracts. There were four estrogically active peaks in MCE. Two estrogically active peaks occurred in FCE, MPE and FPE. Three standards were used in this study, estradiol, genistein and biochanin A. Their HPLC chromatogram can be seen in Figure 11. The retention time of the three standards were 26 minutes [estradiol (E)], 19 minutes [genistein (G)] and 27 minutes [biochanin A (B)].
Figure 10. Reverse phase HPLC profiles of (A) MCE–F80, (B) MPE–F80, (C) FCE–F80 and (D) FPE–F80. A reverse-phase HPLC Alltima C18 (250 x 4.6mm; 5µm) column was used to separate SPE F80. Fractions at 1.5 min were collected using a Gilson FC 203B fraction collector. The solvent system was methanol (A) and water (B) with a gradient profile of 0-20 minutes from 40-90% A, 20-45 minutes from at 90% A, 45-46 minutes from 90-100% A, and 46-55 minutes at 100% A. The wavelength of detection was 280 nm (green line). The estrogenically active HPLC fractions are labeled on the chromatogram.
Figure 11. Reverse phase HPLC profiles of the standards estradiol (E), genistein (G) and biochanin A (B). A reverse-phase HPLC Alltima C18 (250 x 4.6mm; 5µm) column was used to separate SPE F80. Fractions were collected every 1.5 minutes using a Gilson FC 203B fraction collector. The solvent system was methanol (X) and water (Y) with a gradient profile of 0-20 minutes from 40-90% A, 20-45 minutes from at 90% A, 45-46 minutes from 90-100% A, and 46-55 minutes at 100% A. The wavelength of detection was 280 nm (green line). The estrogenically active HPLC fractions are labeled on the chromatogram.

Twenty-eight fractions were collected at an interval of 2.0 minutes each from MCE and 15 of these fractions were screened for estrogenically active compounds. Four fractions were active in MCE. In comparison FCE had two, MPE had two and FPE had two. The retention times of the four active peaks were 13 minutes (F5), 17 minutes (F7), 27 minutes (F15), and 31 (F16) minutes. The four active MCE peaks are represented as F5, F9, F15 and F16 in Figure 10A. Peak F5 had the highest estrogenic activity and F7 the lowest.

Twenty-eight fractions were collected at an interval of 2.0 minutes from MPE and screened for estrogenic activity. Two fractions were identified as active. The retention times of the two active peaks were 13 and 17 minutes. These two peaks were common with MCE and both the callus extracts. The peaks are labeled as F7 and F9 in figure 10B. The HPLC profile of MPE differs from the corresponding callus extract (MCE). Peak F7 had the highest estrogenic activity and F9 was lowest.

Twenty-eight fractions were collected at 2.0 minute intervals from FCE and fifteen of these fractions were screened for estrogenic activity. Three were estrogically active. The retention times of the active peaks were 13, 17 and 27 minutes. These peaks were labeled as F7, F9 and F15 as shown in Figure 10C. Peak F7 is common to MCE and
MPE. Peak F15 is common to MCE. The HPLC profile of FCE differs from its corresponding callus extract (MCE). Peak F7 has the highest activity and peak F15 the lowest.

Twenty-eight fractions were collected at 2.0 minute intervals from FPE and seventeen of these fractions were screened for estrogenic activity. Two fractions were estrogenically active. The retention times of the two active peaks were 13 minutes (F7) and 17 minutes (F9). These two peaks were common with MCE, FCE and MPE. These peaks are labeled as peaks F7 and F9 in Figure 10D. The chromatogram of FPE looks different from to FCE and MPE.

There was one common peak in all the samples i.e. MCE, MPE, FCE and FPE. The retention time of this common peak was 17 minutes. One common peak was observed in the callus extracts, MCE and FCE with a retention time of 27 minutes. Peak F16 from MCE was not detected in any other sample. It had a retention time of 31 minutes. The chromatograms of MCE, MPE, FCE and FPE do look different from one another but the tissue collected from the similar gender look more alike than the HPLC profiles of callus extracts i.e. there is more resemblance in the chromatograms of MCE and MPE, as well as FCE and FPE than with MCE to FCE or MPE to FPE.

The estrogenic activities of the fractions screened are shown in Figure 12. Fifteen fractions were screened for estrogenic activity from MCE as seen in Figure 12A. Fraction 7, retention time 13 minutes had the highest estrogenic activity of 1105.553 MU. It is represented as Peak F9 in Figure 10A. Fraction 9, retention time 17 minutes, had the lowest estrogenic activity of 195 MU. Fraction 15, retention time 27 minutes, had an
estrogenic activity of 531 MU. Fraction 16, retention time 31 minutes, was unique to MCE–HPLC and had an estrogenic activity of 500 MU. F7 had a significantly higher estrogenic activity compared to rest of the three active fractions (P = 0.002, α = 0.05). Fraction 16 was significantly higher than 9 (P = 0.0002, α = 0.05). There was no significant difference between the estrogenic values of Fractions 15 and 16.

Fifteen fractions from MPE–HPLC were screened for estrogenic activity as seen in Figure 12B. Fraction 7, retention time 13 minutes, had the highest estrogenic activity of 375 MU. Fraction 9, retention time 17 minutes, displayed 347 MU (Figure 10B) but was not significantly different from Fraction 7.

Fifteen fractions from FCE–HPLC were screened for estrogenic activity as shown in Figure 12C. Fraction 9, which had a retention time of 17 minutes, had an estrogenic activity of 918.61 MU. Fraction 9 is one of the common peaks found in all the HPLC samples of MCE, MPE, FCE and FPE. Fraction 15 corresponds to F7 in Figure 10C and had an estrogenic activity of 311 MU, significantly different from Fraction 9 347 MU (P = 0.00098, α = 0.05). FCE–HPLC had fractions common with the adult plant tissue (example F9) as well as the callus tissue (example F15).
Estrogenic Activity (MU)

A

B

3 4 7 8 9 10 11 12 13 14 15 16 17 18 20

#7 #9 #15 #16

Estrogenic Activity (MU)
Figure 12. Estrogenic activities of (A) MCE–HPLC fractions, (B) MPE–HPLC, (C) FCE–HPLC, and (D) FPE–HPLC fractions. HPLC fractions were collected every 2 minutes. Yeast Culture = 17.13±8.7 MU, Estradiol = 2792.19±154.4 MU and Genistein = 870.1±112.2 MU. Each datum point represents the average for 3 experiments ± S.D.
Seventeen fractions were screened for estrogenic activity from FPE–HPLC as shown in Figure 12D. Fraction 7, retention time 13 minutes, had the lowest activity of 319 MU. Fraction 7 is labeled as F7 in Figure 10D. Fraction 9, retention time 17 minutes, had the highest estrogenic activity of FCE–HPLC fractions, 421 MU (P = 0.015, α = 0.05).

Fraction 7, retention time 13 minutes, was common to HPLC extracts of MCE, MPE and FPE. Fraction 9, retention time 17 minutes, was common to HPLC extracts of MCE, MPE, FCE and FPE. Fraction 15 was common to HPLC extracts of MCE and FCE. Fraction 16 was found only HPLC extract MCE. The fractions that are common in all the samples could be similar compounds or different compounds with approximately the same structure but different binding affinities for the estrogen receptor in the yeast hybrid system. The estrogenic activity of male tissues obtained from callus and adult plant was lower than that of female tissues from callus and their corresponding adult plant tissue.

3.3 Spectrophotometry Analysis of MCE – HPLC15 compared to Biochanin A

UV spectrum analyses between 200 nm to 300 nm were carried out on MCE–HPLC15 (Figure 13A). The spectrophotometrical scan of MCE–HPLC15 showed a peak at 261 nm. The spectrophotometric scan of Biochanin A, one of the standards used, had a peak at 261 shown in figure 13B. The peak of Biochanin A and MCE–HPLC15 had similar absorbance. The concentration of MCE–HPLC15 seems lower than Biochanin A.
(1mg/l). The UV spectrum analysis of MCE–HPLC15, in addition to its HPLC profile indicates that MCE–HPLC15 could be Biochanin A.

Figure 13. Spectrophotometrical analyses of MCE–HPLC15 (A) and Biochanin A (B). The extract was scanned between 200–300 nm against ethanol blank.

3.4 Electrospray Mass Spectrometry (ESI–MS) of MCE – HPLC15 compared to Biochanin A

Fraction 15 was common in all callus cultures. The retention time of fraction 15 was similar to Biochanin A (one of the standards used in the study). This fraction could
be Biochanin A, one of the known phytoestrogens, therefore fraction 15 was selected for ESI–MS.

MCE–HPLC15 and FCE–HPLC15 were common in callus extracts and since MCE–HPLC15 had a higher estrogenic activity it was selected for the ESI–MS. MCE–HPLC15 was dissolved in ethanol and 1% acetic acid for analysis by ESI. Figure 15 shows the full scan of MCE–HPLC15 from 200 m/z to 300 m/z. This process is useful as it detects the presence of structurally similar compounds isolated by HPLC fractionation in the full scan MS mode. The uncommon peak in MCE–HPLC15 was at m/z 286 compared to the blank (ethanol). This peak was compared with the Biochanin A standard, as seen in Figure 16, and was further fragmented with MS/MS as depicted in Figure 15. Peaks found at m/z 270, 253, and 229 were obtained by Biochanin A MS fragmentation, as shown in Figure 17. The pattern of Biochanin A was similar to that from MCE–HPLC15. This indicates that MCE–HPLC15 does contain Biochanin A which exhibits a weak estrogenic activity compared to MCE–HPLC7.

The MS of Biochanin A, seen in Figure 16, was performed by isolating a molecular ion of interest which was at m/z 285 followed by resonance excitation resulting in collisional energy which was maintained at 35eV. Biochanin A has fragmentation ions at m/z 270, 253 and 229, as shown in Figure 17. The MS fragmentation of MCE HPLC15 does coincide with the standard Biochanin A pattern shown in Figures 15 and 17. This result, combined with the retention time of the fraction with HPLC, presents firm evidence that Biochanin A is present in MCE–HPLC15. This
part of the experimentation was done in Dr. Guido Verbeck’s lab in SRB at University of
North Texas, Denton.

Figure 14. ESI–MS chromatogram of MCE–HPLC15 in full scan mode. The peak which is different/unique in the MCE–HPLC15 is found at 284.33 m/z.
Figure 15. ESI–MS/MS of the peak obtained at 284 m/z. Fragmentation similar to Biochanin A. Fragmentation peaks found at 270 m/z, 253 m/z and 229 m/z respectively.

The sample MCE–HPLC15, when passed through UV spectroscopy displayed a peak at 260 nm which matches the standard biochanin A peak, confirming that male
callus extracts obtained from stem explants contain biochanin A.

**Figure 16.** ESI–MS ion trap chromatogram of biochanin A in full scan mode. The peak obtained at 285 m/z.
Figure 17. ESI–MS/MS of the peak obtained at 284 m/z. Fragmentation peaks found at 270 m/z, 253 m/z and 229 m/z respectively.
3.5 Results for Plant Tissue Extracts on Liquid Chromatography Mass Spectrometry (LC-MS)

Identification of the flavonoids was accomplished by using negative ion–LC–ESI–MS. Fraction FCE–HPLC 9 and fraction MCE–HPLC 16 were collected and analyzed in LC–MS. The LC–MS spectrum profiles can be seen in figures 18A, 18B and 19. Peaks that were observed in the spectrum profiles are noted in Table 5. The peaks in LC–MS in the range of 200–300 m/z were at m/z 227, 253, 255, 265, 281 and 282 and correspond to the compound presented in Table 5.
Figure 18. LC–MS in visible mode. (A) FCE–HPLC9 and (B) MCE–HPLC 16 shows the chromatograms obtained from the respective fractions.
Figure 19. LC–MS negative ion ESI–MS chromatograms of the MCE–HPLC16, FCE–HPLC9 and Ethanol as the blank.

Table 6. Peaks obtained in the LC–ESI–MS negative ion chromatograms with the suspected compounds from on the studies of Wang et al., 2003.

<table>
<thead>
<tr>
<th>HR Mass</th>
<th>tR</th>
<th>Elemental Composition</th>
<th>Tentative Identification of Compounds</th>
<th>Substance Class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>227</td>
<td>NO</td>
<td></td>
<td>Resververtrol</td>
<td>Stilbene</td>
<td></td>
</tr>
<tr>
<td>253</td>
<td>17.4</td>
<td>C_{15}H_{10}O_{4}</td>
<td>Diadzein</td>
<td>Isoflavone</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td>255</td>
<td>19.4</td>
<td>Isoliquiritigenin</td>
<td></td>
<td>Isoflavone</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td>265</td>
<td>NO</td>
<td>C_{16}H_{12}O_{4}</td>
<td>Formononetin</td>
<td>Flavonone</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td>281</td>
<td>19.5</td>
<td>C_{16}H_{10}O_{5}</td>
<td>Pseudobaptigenin</td>
<td>Isoflavone</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td>283</td>
<td>23</td>
<td>C_{16}H_{12}O_{5}</td>
<td>Biochanin A</td>
<td>Isoflavone</td>
<td>Wang et al., 2003</td>
</tr>
</tbody>
</table>
Plants add to their aesthetic value by constituting a major source of natural food for eating, air used for breathing, medicines to cure many illnesses. More studies are being performed to promote plants as a natural source of drugs. Plant secondary metabolism gives rise to commercially important compounds. Secondary metabolites typically are plant products and usually occur as terpenoids, glycosides and alkaloids. Secondary metabolites are commonly obtained commercially by extraction from intact plants. Secondary metabolites are produced in minute amounts within the plants making the extraction process difficult. Since supplying the raw materials for drug production can be erratic, it has become critical to develop an alternative source of important natural therapeutic products. Plant cell cultures provide an environmentally friendly, renewable alternative for these products. Plant cell culture has been useful in the production of numerous terpenoid and terpenoid-derived compounds, most notably high-value pharmaceutical products such as indole alkaloids.

Primary metabolism is an important source of precursors for the synthesis of secondary phenolic metabolites, which have a range of functions in metabolism,
signalling, and defence against abiotic and biotic stress. Central metabolism requires high levels of limited plant resources and during intense growth the synthesis of phenolic metabolites may be substrate- and/or energy limited. On the other hand, either abiotic or biotic stresses divert substantial amounts of substrates from primary metabolism into secondary defensive product formation and this could lead to constraints on growth. Plants, in fact, have limited resources to support their physiological processes, hence all requirements cannot be met simultaneously and there is a constant switch between growth and defense (Coley et al., 1985; Herms and Mattson, 1992). Therefore, a principal feature of plant metabolism is the flexibility to accommodate developmental changes and to respond to the environment.

4.1 Callus Culture Establishment

Tissue culture is a technique to enhance the application of biotechnological approaches to crop plants. Induction and growth of callus tissues depends on medium composition and concentrations of hormones (Brisson et al., 1988). The conventional approach to establishing or improving callus cultures has been to test a range of media and explants (Trione et al., 1968; O’Hara and Street 1978; Pierik, 1997; Jauhar, 2006). Modifications involve qualitative and quantitative change in micronutrient elements, sources of carbon and growth regulators responsible for particular hormonal balance in the growth system. Other factors involved in tissue culture are light and temperature during the growth conditions.
Plant growth regulators have been shown to influence biomass and cell differentiation (Suri and Ramawat 1995). Reduction in biomass is always correlated with increased accumulation of secondary metabolites (Ramawat and Mathur 2007). Plant growth regulators are known to influence markedly the production of secondary metabolites in *in vivo* (Haque *et al.* 2007) and *in vitro* cultures (Taguchi *et al.*, 2001).

*Morus alba* callus initiation and growth were studied on a number of MS media by supplementing them with different concentrations of hormones (auxins and cytokinins) and vitamins. Among the different hormonal concentrations screened, MS medium supplemented with 1 mg/l of NAA and 0.5 mg/l of BAP (MS 1) was best for *Morus alba*. Numerous reports indicate that it is difficult to select a medium that would ensure good production of the metabolites of interest and at the same time provide fast, continuous growth of callus. The reason is that the pathways of primary and secondary metabolism often compete for nutrients and precursors (Luczkiewicz and Glod, 2003). This study established MS 1 is an effective media for mulberry callus culture initiation and maintenance. Earlier researchers have supplemented MS or other medium with varying concentrations of NAA (0, 0.5, 1.0, or 2.0 mg/l) and BAP (0, 0.5, 1.0, 1.5, or 2.0 mg/l) alone or in combination, to obtain the callus cultures for *Morus* species (Jain *et al.*, 1996). They were not as effective as our results with MS 1.

For instance, callus cultures of *Genista* plants grown on Schenk and Hilderbrandt modified medium accumulated phytoestrogens, derivatives of Genistein, Daidzein, and Formononetin, in concentrations many times higher than intact plants (Schenk and Hilderbandt, 1972). Studies in *Cicer arietinum* suspension culture showed biosynthesis of
free and glycosylated biochanin A and formononetin by stimulation with the auxin 2,4-D.
The auxin 2,4-D has also been shown to induce production of Daidzein in callus cultures of five species of *Psolarea* genus. Leguay and Jounneau in 2005 showed that 2,4-D acts as a blocker of the key enzyme in the phenylpropanoid pathway, chalcone synthase. Cytokinins like BAP have been shown to induce biosynthesis of isoflavonoids. Clearly medium components, or phytohormones, play an important role on the biosynthesis of isoflavones for *in vitro* cultures.

Young green stem and petiole explants from mature plants were the best explants for the initiation of callus in white mulberry used. Callus initiation and proliferation period of stem and petiole explants were approximately 20 days.

Exposure to light is not required for the initiation isoflavonoid biosynthesis, as indicated by their considerable concentration in the roots of intact plants and in *in vitro* cultures that are kept in complete darkness. The modification of light conditions in callus cultures of several *Genista* plants indicate the presence or absence of light seems to have a selective effect on the accumulation of ester derivatives of Genistein, which are considered to be a storage form of phytoalexins. In the environment UV stress increased isoflavonoid synthesis which serves to protect *Phaseolus vulgaris* (Brenda, 2002).

The mulberry callus cultures were soft, friable and greenish-white even after the third subculture. Earlier studies by Katarina et al., 1998 indicated that callus cultures differ in anatomy, cell size and friability and growth potential with every species. It is evident that the mulberry callus exuded more phenolics with every subculture because the color changed from green to brown. The proliferation of callus cultures was slow due to
the possible accumulation of phenols which are known to interfere with callus proliferation (Nosov, 1994). For future studies, leaching of phenolics could be reduced by using activated charcoal in the medium. Rhizogenesis was seen in the subcultures after two months indicating the presence of an elevated level of endogenous auxins, which are responsible for the initiation of roots (Muller, 2000).

Comparable or larger concentrations of phytoestrogens were obtained from in vitro cultures of species that, in their intact form, were characterized by a rich isoflavonoid metabolism. These included cultures of Glycine max, Maackia amurensis and Cicer arietinum, and Lupinus, Psoralea and Genista species. Synthesis and metabolism of isoflavonoids are stimulated in vitro cultures by using both traditional as well as more advanced biotechnological strategies (Farag et al., 2008).

4.2 Estrogenic Activity of Callus Extracts

Khafagi et al., in 2003 showed that desired phytochemicals could be produced in cell cultures. Indeed, by using tissue culture technology, it is possible to achieve the synthesis of a wide range of compounds such as alkaloids, flavonoids, terpenes, steroids, glycosides, etc (Sarin R, 2005). It is also possible to identify cell lines that produce higher levels of compounds of interest than the mature plants. Furthermore, new molecules that have not been found previously in plants can be produced by cell cultures. Delle Monache et al. in 1995 working on Maclura pomifera callus described the accumulation pattern of flavonoids, which was quantitatively and qualitatively different in callus culture than in the parent plant. Triterpenes and flavonoids were isolated from callus and
cell cultures. However, xanthones and stilbenes, which were reported in whole plant, were not found in *Maclura pomifera* tissue culture. New compounds were isolated from callus culture, notably the rare 2,4,2',4'-tetrahydroxy-3'-(3-methyl-2-butenyl) chalcone, a prenylated chalcone and a flavonone (Delle Monache *et al.*, 1995). Thus, tissue culture constitutes a genuinely effective means of achieving production of novel metabolites.

There is great interest in the medical fields in developing alternatives to the intact plants for the production of plant secondary metabolites. Phytoestrogens appear to possess potential preventive effects against cardiovascular diseases, osteoporosis and age related diseases (Barnes, 1998; Le Bail *et al.*, 1998). Studies have shown that fresh leaves produce the highest content of flavonoids, air dried leaves are second and oven dried leaves least. The order may be explained by decomposition of flavonoids over time. However there is a possibility that the matrix changed to make it less extractable (Zhishen *et al.*, 1999). In this study, callus cultures were established using young stems and petioles as they yielded high estrogenic activity.

In previous studies, mulberry extracts from adult plants were screened for estrogenic activity and it was found that female mulberry extracts had higher activity than males (Maier *et al.*, 1995). In this study, white mulberry callus extracts had significantly higher activity than adult plants. Significantly higher activity occurred in both male and female callus extracts than adult plants. This indicates that the MS 1 medium used for the initiation and proliferation of the callus extract yielded higher estrogenic activity.

MS 1 medium could be made even more effective by adding activated charcoal or other agents that would increase the estrogenic activity of callus by removing
phenolics. Studies carried out by Luczkiewicz in 2008 showed that SH medium enhanced the production of isoflavonoids in *Genista* species. For further studies white mulberry callus could be established on SH medium to study its ability to increase estrogenic activity.

Ethrel, potent plant growth regulator that is converted to ethylene, treatments are known to increase the accumulation of isoflavonoids such as Genistein in cell cultures (Goyal and Ramawat, 2007). MS media can be modified in nitrogen content to increase the accumulation of isoflavoids in cell cultures (Goyal and Ramawat, 2007). The biosynthesis and metabolism of secondary metabolites, including isoflavonoids, can be regulated by using biotic and abiotic elicitors (Tebayashi et al., 2001). Significantly higher estrogenic activity occurred in male and female callus extracts (1105.6MU; 918.61 MU) than in corresponding adult plants (375 MU; 421.48 MU).

The MS 1 medium used for initiation and proliferation of mulberry explants also proved to be suitable for enhancing estrogenic activity in callus extracts. In future work, for the purpose of enhancing the production of secondary metabolites in calli of mulberry species, calli should be challenged with different chemicals, especially those providing a better source of nitrogen which might be helpful in enhancing the production of secondary metabolites. Earlier studies used of kinetin and IAA (Indole-acetic acid) in varying concentrations, to obtain callus from stem explants (Seki *et al.*, 1971; Thomas, 2003).

A difference in estrogenic activity was noted with different seasons or months of the year. The comparative concentration of estrogenic activity in July, October and
March were studied. In adult mulberry plants higher estrogenic activity was seen during the flowering season in mulberry callus as well as intact plant. A study by Maier et al., (1995), found highest estrogenic activity in female adult plant extract for the month of March. The estrogenic activity of callus extract from male white mulberry was significantly higher than that from female white mulberry. This could be due to the fact that accumulation of secondary metabolites is related to the degree of morphological and biochemical differentiation of explant tissues used (Maraschin et al., 2002). The callus cultures were obtained from the stems and petioles of mature plants. For future studies callus cultures could be differentiated into male and female plants. Callus cultures could be obtained from the differentiated male and female plants to study the difference in estrogenic activity between the sexes from *in vitro* cultures. Differentiation of callus may be responsible for the increase in production of the phytoestrogens.

An increase in estrogenic activity was noticed with increased callus age in earlier studies done during my masters thesis. This could be due to injury of callus, its adaptation to the nutrition medium, or the developmental age of the explant. Secondary metabolite production levels often vary from one subculture to another (Bourgaurd et al., 2001). Other studies showed that subcultured callus exhibited higher amounts of flavonoid compounds in *Chrysosplenium americanum*, thus indicating *de novo* synthesis (Brisson et al., 1988).
4.3 Chromatographic Techniques

The varied chemical structures of metabolites in plants necessitates the use of different analytical techniques to cover the wide range of polarities within the cell. Systematic identification of the metabolites occurring in species is particularly important, given the wealth of natural products being produced. Higher transcriptional activity was induced by SPE F80 fractions of callus extract than in crude callus extracts. SPE has proved to be an efficient method for fractionation and concentration of phytochemicals with estrogenic activity. The resulting SPE fractions separated according to their polar–non polar properties, as follows: SM and F20 fractions contained water soluble compounds, while the flavonoids were retained in the SPE F80 fractions (Klejdus et al., 2000).

It is important to note that there was significantly higher estrogenic activity in MCE–HPLC 7 than in FCE–HPLC9. Callus cultures could alter the metabolic pathway to cause higher estrogenic activity in male callus. It might also be possible that there is a new compound being formed in the MCE which, on purification, yields a significantly higher estrogenic activity. Callus cultures had higher estrogenic activity than adult plants whether male or female. In intact plants estrogenic activity was always higher in females than males.

Estrogenic activity decreases with the concentration of methanol indicating adsorption and hydrophobic interactions with the C18 column. The greater the number of hydroxyl groups, the stronger the retention and the greater number of saccharides the
smaller the retention (Graham 2001). The C_{18} column was chosen because it was suitable
and highly reproducible for phytoestrogen profiling in *Morus alba*.

HPLC analyses showed different active fractions for the four extracts screened. The HPLC profiles of male and female adult plant and callus extracts were similar but
different from profiles of corresponding callus extracts. These results indicates similar
but different estrogenic compounds *in vitro* and *in vivo* mulberry extracts.

The HPLC profiles of male callus extract had four active estrogenic fractions,
whereas the female callus extract had only three. The HPLC profiles of male and female
adult plant tissues had two active fractions. It is evident that calli obtained from callus
tissues have chemically different phytoestrogens than those obtained from adult tissues. It
may also be possible that callus cultures produce different and or novel compounds as
indicated earlier by Delle Monache *et al.*, for *Maclura pomifera* (1995). When the
estrogenic activities were compared among the different extracts, HPLC fractions of all
four extracts had higher estrogenic activity than SPE.

When the estrogenic activities of the four extracts were compared, it was evident
that callus had significantly higher estrogenic activity than adult plants. The female adult
plant tissue extract had significantly higher concentrations of estrogenic activity than the
male. Conversely, in callus cultures the differentiated tissues from male had higher
estrogenic activity than female. Earlier studies by Maier *et al.*, 1997 showed that female
adult plant tissues have a higher estrogenic activity than the male adult plant. The
chromatograms of MCE, MPE, FCE and FPE do look different from one another but the
tissue collected from the similar gender look a lot more alike than the HPLC profiles of
callus extracts, i.e. there is more resemblance in the chromatograms of MCE and MPE, as well as FCE and FPE than with MCE to FCE or MPE to FPE.

When estrogenic activities were compared among callus extracts, SPE and HPLC fractions of the mulberry species, MCE-HPLC extracts showed higher estrogenic activity than FCE–HPLC extracts. The FCE and FCE-SPE fractions had higher estrogenic activities than the corresponding, MCE and MCE-SPE fractions. The higher activity of MCE, MCE-SPE fractions may be due to the fact that the estrogens in callus were inhibited by the presence of other chemicals in the crude extract, chemicals which were removed during HPLC fractionation.

The sample that eluted out at 17 minutes was common to all from HPLC fractions. The common fraction had different levels of estrogenic activity in separate extracts. The sample that eluted out at 27 minutes was common to both callus extracts, namely MCE and FCE–HPLC. Fractions that were eluted out at 14 and 17 minutes were present in both the male extracts, differentiated tissue as well as in adult plant. In both female extracts, common retention times occurred for fractions that were eluted at 17 minutes. In both callus extracts, MCE and FCE, retention times were common for fractions that were eluted at 27 minutes. The fraction obtained from MCE–HPLC eluted at 29 minutes was not found in any other sample and thus could be a novel compound.

No single wavelength is ideal for monitoring all classes of known phytoestrogens since they display absorbance maxima at different wavelengths. Most of the phytoestrogens contain benzoic acid and hydroxycinnamic acid derivatives in their structure. Most benzoic acid derivatives have an absorption peak at 240-285 nm which is
referred to as band II and hydroxycinnamic acid derivatives have a peak between 300-400 nm, referred to as band I (Mabry et al., 1970). The UV-visible spectrophotometrical analyses of callus extracts and their SPE F80 fractions absorbed in both bands I and II. The HPLC fractions did not. HPLC fractions are considered to be pure compounds. Based on spectrum profiles of estradiol and Genistein standards, the phytoestrogens in male callus extract HPLC fraction 15 (F5) could be biochanin A.

The principles of electrospray separation and mass analysis of electrospray technique, together with its sensitivity and resolving power, greatly expand the range of metabolic profiling. Profiling schemes for Arabidopsis and other plants have been developed in recent years (Roessner et al., 2000, 2001; Fiehn et al., 2000). The main focus of these mostly gas chromatography mass spectrometry based approaches have been primary metabolites such as sugars, amino acids, organic acids, or sugar alcohols. Several hundred compounds can be robustly and reliably detected. Liquid chromatography mass spectrometry allows a more comprehensive profiling of metabolites (Roessner et al., 2000). The coupling of electrospray ionization (ESI) MS with capillary (Cap) electrophoresis (Soga et al., 2002) and hydrophilic interaction chromatography (Tolstikov and Fiehn, 2002) has been successfully applied in the identification of metabolites. MCE–HPLC 15 was analyzed by ESI–MS. This had a peak at 285m/z. A literature search of natural compounds identified the compound as biochanin A. Biochanin A was identified was with the help of mass spectra from total ion chromatogram and mass and intensity of the peak. The fragmentation of the unknown compound confirms the identity of biochanin A. Peaks from fragmentation was then
compared with the standard biochanin A obtained from Sigma Aldrich. Fragmentation peaks obtained at m/z, 270, 253 and 229 showed the loss of a water molecule, methyl group and a ketone group from the structure of biochanin A.

Previous studies by Alventosa et al., 2008 showed that no aglycones were detected in ethanolic extracts from triguero asparagus but acid hydrolysis confirmed the presence of three different aglycones, Quercetin, Isorhamnetin and Kaempferol. Electrospray coupled with MS provides a qualitative analysis of natural compound mixtures such as isoflavones, with high reproducibility and sensitivity.

Biochanin A exhibited an [M–H] ion peak at 284 m/z as the base peak at -35V, however raising the extraction cone voltage produced new peaks at m/z 270 ([M–H–CH3]-, m/z 253 [M–H–CH2–COH]-) and m/z 229 ([M–H–CH3–COH–CO]-). In general, compounds with a methoxy group (biochanin A) exhibit one fragment ion at m/z [M–H–CH3] when a high extraction cone voltage is used.

Other compounds could be analyzed by building a library of compounds in the phenylpropanoid pathway. The presence of biochanin A existence of isoflavonoid pathway in white mulberry. Therefore the precursors of biochanin A are present in the Mulberry. Constructing a library would help us analyze the different compounds. This could be considered the first step in analyzing the different metabolites present in mulberry. In-depth literature study obtained as much information as possible on metabolites previously detected in mulberry plants.

Isoflavonoids are natural products produced almost exclusively by plant species belonging to the legume family. They play an important role in the interaction with
environmental micro-organisms, both as phytoalexins and as signals for symbiotic nitrogen-fixing bacteria. Isoflavonoids are antioxidants and can, in addition, have phytoestrogenic activity. Their inclusion in the human diet, mainly as soybean products, is linked to various health-promoting effects of soy products such as reduced incidence of breast and prostate cancers, and prevention of osteoporosis. Consequently, isoflavonoid preparations are popular as dietary supplements.

The current status of metabolomics can be viewed as being in some ways equivalent to the situation of sequencing programs such as the Human Genome Project around 1990 (Sumner et al., 2003). The enormous potential of comprehensive biochemical phenotyping for the functional analysis of biological systems is realized and numerous projects have been initiated. However, major technological limitations need to be overcome. For instance, the chemical diversity of the metabolome necessitates the use of different analytical techniques to cover the wide range of polarities found among the metabolites occurring in a cell. Metabolomic approaches aim at monitoring the biochemical status of an organism by simultaneously measuring as many metabolites as possible. A robust and reproducible analysis that provides qualitative and quantitative data and allows high sample throughput is desired. Equally important at this stage is to contribute to cataloging the metabolome of an organism. No metabolome is completely known as yet. Systematic identification of the metabolites occurring in a species is particularly relevant for plants, given the wealth of natural products they produce. Sequence data indicate that *Arabidopsis* expresses a large number of enzymes for which substrates and products are unknown (The *Arabidopsis* Genome Initiative 2000).
Most likely, the numbers will be significantly higher once the analysis is extended to other tissues such as flowers (Chen et al., 2003), to different developmental stages, and to plants exposed to environmental stimuli. Increases in the number of detectable mass signals can be achieved by extending the analysis to the negative mode, which is less effective yet allows the measuring of metabolites not seen in positive mode. MCE–HPLC 9 and MCE–HPLC 16 were analyzed by LCMS. A number of peaks were detected in the samples mentioned above. The peaks seen in LCMS were at m/z 227, 253, 255, 265, 281 and 282. The retention times of the peaks at m/z 253, 255, 281 and 282 were 17.4, 19.4, 19.5 and 23 minutes. The compounds observed in the samples could be diadzein, isoliquirtegenin (precursors of biochanin A), pseudobaptigenin, biochanin A. The order of elution in reverse phase chromatography is same as the order of the compounds that are being eluted. More hydrophobic compounds are eluted later from the C18 column. The order of elution could further be compared by running the standards through LCMS.

The identification of compounds, in particular secondary metabolites, through a metabolomic profiling approach encounters some major difficulties. The number of commercially available standards of secondary metabolites reported in a specific plant species or tissue is low. In an automated online separation, PDA detection, MS measurement, and/or MS/MS fragmentation of mass signals, it is difficult to meet optimized levels for all eluting compounds. Due to overlapping compounds, low intensity mass signals, or difficulties in the isolation of the mass signal for MS/MS fragmentation, the extraction of usable information for identification purposes can be complicated. The lack of dedicated software and databases that integrate spectroscopic and MS data limits
the identification procedure to a manual level. Comparing the ESI results with HPLC and spectrophotometer analysis proves the presence of biochanin A in white mulberry. HPLC15, the compound isolated was compared with the commercial standard of biochanin A obtained from Sigma Aldrich. This compound has been previously described in other plant tissues, such as soy, populous and eucalyptus. The results for fractionation, spectrophotometrical analysis and chromatographic techniques all indicate the presence of the Biochanin A in the callus cultures of *Morus alba*. Biochanin A is almost exclusively produced in Fabaceae. Biochanin A is present in the isoflavone pathway. The precursor to biochanin A is genistein or diadzein. Biochanin A is produced by the replacement of a hydroxyl group to a methoxy group from Genistein in the A ring.

Revisiting the Goals and Objectives:

- This study established a tissue culture medium that yields significantly higher estrogenic activity. The medium established is MS + NAA (1g/l) +BAP (0.5g/l). *In vitro* cultures obtained from the differentiated tissues of both male and female explants that were established had significantly higher estrogenic activity than the adult plant tissues.

- The estrogenic activity of *in vitro* cultures was also tested for different months. March samples had the highest estrogenic activity. *In vitro* cultures had similar trends in increase and decrease of estrogenic activity with different seasons as was seen in adult plants.
- Female adult tissue had significantly higher estrogenic activity than the male adult tissue. The callus cultures differentiated from female explants had higher estrogenic activity in the adult plant extracts.
- After fractionation female adult tissue still had higher estrogenic activity than male adult tissue. After fractionation callus culture extracts from differentiated male explants had higher estrogenic activity.
- SPE–F80 had the highest estrogenic activity among the different fractions.
- Four estrogenically active fractions were seen in MCE. Three estrogenically active fractions were seen in FCE. Two estrogenically active fractions were seen in MPE and FPE.
- MCE–HPLC15 had a retention time 27 minutes, similar to biochanin A HPLC and therefore was selected for further analysis.
- Spectrophotometerical analysis had an absorption peak at 261 nm similar to biochanin A.
- ESI–MS peak for MCE–HPLC15 was at 284 m/z, similar to biochanin A.
- Fragmentation pattern of biochanin A was similar to the sample MCE–HPLC15.
- Our experiments proved biochanin A to be present in mulberry callus extract obtained from the male differentiated tissue.
4.4 Significance of the Study

Establishing a tissue culture system for mulberry and Osage-orange is very important for continuing phytoestrogen studies. Callus cultures produce soft friable callus that can be further used for obtaining suspension and protoplast cultures. Callus and cell cultures are useful not only for the study of the phytochemicals of interest, but also for biotransformation and regeneration of entire plants. Tissue culture offers a unique environment for manipulating sexual development of the regenerated plants by supplementing media with different hormones. Thus, tissue culture provides an insight into the molecular and genetic mechanisms of sex development in dioecious plants. Also, the hairy root cultures obtained through this work could be used in the future for karotype analysis and gender determination (since it takes 8-12 years for the mulberry life cycle to determine tree gender) and biotransformation studies.

The potential of tissue culture techniques for the production of several secondary metabolites has been known for many years. Evidently tissue cultures stimulate the production or induce the biosynthesis of novel compounds not found in the mature plant. The development of callus culture could provide an alternative supply of phytochemicals such as novel phytoestrogens to be used in medicine. Phytoestrogens in *Morus* and tissue cultures may prove to be medically beneficial for diseases such as cancers, cardiovascular diseases, and osteoporosis.

In addition, identifying phytoestrogens in tissue culture will help us understand their functions in plants. Since *Morus* species are dioecious (Harrar and Harrar, 1962), tissue culture studies may prove that cultures obtained from male explants contain
different phytochemicals than the cultures obtained from female explants. Comparison of the biochemical composition of adult male and female tissues may provide insight in the development of sexes in the plants under study. Earlier studies on Cucumber plants showed that treatment with 2-chloroethylphosphonic acid influenced sex determination only at stamen primordial differentiation stage in both monoecious and gynoecious cucumbers (Kopcewicz, 1972). Cucumber plants (Cucurbita pepo L.) with induced femaleness had higher endogenous amounts of estrogens than those which were not purposely induced (Kopcewicz, 1972). The capability of controlling or inducing female sex in crop plants may allow beneficial crop yield increases for an exponentially growing human population. Identifying the chemicals present in an organism would provide a greater understanding of a plant’s metabolism. Since the genome of mulberry has not been sequenced and is not known the metabolic pathways present in are still in question.
REFERENCES


Datta A and Srivastav PS (1997) Variation in vinblastine production by *Catharanthus roseus* during *in vivo* and *in vitro* differentiation. Phytochemistry **46**:135-137


Ercisli S and Orhan E (2006) Chemical composition of white (Morus alba), red (Morus rubra) and black (Morus nigra) mulberry fruits. Food Chemistry 103: 1380-1384


Gonzalez JM, Friero E, Jouve N (2001) Influence of genotype and culture medium on callus formation and plant regeneration from immature embryos of Triticum turgidum Desf. cultivars. Plant Breeding 120:513-517


Miller JM (1972) Assays for β-galactosidase, in Miller JM, Eds., Expression In Molecular Genetics, Cold Spring Harbor, New York


Suri SS and Ramawat KG (1996) Effect of *Calotropis* latex on laticifers differentiation in callus cultures of *Calotropis procera*. Biologia Plantarum 38: 185-190


