Secondary plant metabolites are the byproducts of primary metabolism and refer to compounds that get collected in specialized cells in absence of excretory systems. The secondary metabolites do not perform vital physiological functions for plant life as primary metabolites. Secondary metabolites are used in pharmaceuticals, agrochemicals as flavouring agent and also in aroma industries. The development of drugs from plants by drug companies encourages large scale pharmacological screening of herbs.

Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. Generally it is accepted that secondary metabolite production is associated with tissue differentiation. Biosynthesis of many important compounds needed some level of cellular or tissue differentiation such as roots, shoots, embryos, and other organs have produced markedly high products. So, the screening and selection of high producing cell lines and the optimization of growth and production media can be mentioned as common approaches. But, in some cases, undifferentiated cell cultures like callus and cell suspension can produce high amount of secondary metabolites as compare to that of the whole plant. Biotransformation, uses of bioreactor and hairy root cultures have also been shown large interest as tissue culture approaches. Among the techniques employed, optimization of nutrient media, optimization of culture conditions, identification of the most effective elicitors and the use of hairy root culture have been given considerable attention.
General introduction

Plant biotechnology includes not only modern but also age-old methods so as to manipulate the organisms for human needs. In this context, biotechnology can be defined as the applications of indigenous and scientific knowledge to the management of microorganisms or cells/tissues of higher organisms or their parts for the benefit of human beings (Verpoorte et al, 2002; Vanisree et al, 2004). Karl Ereky (1917) who coined the term biotechnology stated that all types of works are biotechnology by which products are produced from raw materials using living organisms (Edreva et al, 2008).

Plants are ‘The soul of life’:

Plants are essential to the balance of nature and our sustenance. Plants are the ultimate source of food and metabolic energy for nearly all animals, which cannot manufacture their own food. Besides foods like grains, fruits, vegetables and plant products are vital to humans. Plants provide wood, wood products, fibers, drugs, oils, latex, dyes and resins. Coal and petroleum are the fossil substances of plant origin. Thus, plants provide us not only sustenance but shelter, clothing, medicines, fuel and raw materials from which innumerable other products are made (Wickens, 2001; Rajshekharan, 2002; Sharma et al, 2011).

Human beings are directly or indirectly totally dependents on plants. Plants are inexhaustible source for herbal drugs. Today several distinct chemicals derived from plants are currently used as important drugs in one or more countries in the world (Vanisree et al, 2004). The oxygen in the air, comes from the photosynthesis of plants. The quality of air and water can be greatly influenced by plants; they protect the soil from erosion caused by heavy rains. Plants and plant communities provide the necessary habitat for wildlife and fish population. Therefore, plants and their products have always influenced human culture (Kapoor, 1990). Medicinal plants play a vital role in the maintenance of human health throughout the world. In fact, they are of critical importance in poor communities. Medicinal plants also play an important cultural role as well as an important economical role. Knowledge of their usage is well-known and their efficacy is believed, based on a long history of use (Chopra et al, 1956).

Medicinal plants: A source of secondary metabolites

In the recent era, herbal remedies and ayurvedic preparations are extensively used in the world. Plants also serve as the origin of modern medicine. Some of the most effective drugs such as aspirin derived from bark of cinchona and morphine derived from the opium poppy. Mostly used anticancer drugs like taxol, vincristine, vinblastine are derived from plant (Ravishankar and Ramachandra, 2000). There are hundreds of herbal supplements such as Ginkgo, Echinacea, Eucalyptus, Aloe, Withania etc (Kokate et al, 1997). Our ancient medicinal systems like
Ayurveda, Unani, Siddha are based on use of natural products/plants. The Indian scholars like Charaka, Sushruta and Bhagvatta have mentioned the significance of plants to cure of many human diseases (Patel and Jasrai, 2009). The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines, mostly plant drugs, for their primary healthcare needs. Also, modern pharmacopoeia contains at least 25% drugs derived from plants. Demand for medicinal plants are increasing in both developing nations due to growing recognition of natural products, being non-toxic, having no side-effects, easily available and that too at low-cost prices. United States and European Union are the key driver of the global herbal trade market (De Luca and St Pierre, 2000; Wink, 2008; Collin, 2001). The herbal medicine market was estimated over USD 23 billion in 2002, USD 60 billion in 2004 and reached up to 85 billion in 2007. This is expected to attain a value USD 5 trillion by 2050 (Patel and Jasrai, 2009).

**Plant Secondary metabolites: Important constituents of herbal medicines**

Chemical building up and destruction of the substances of a living organism constitutes metabolism. It is a dynamic process and represents the balance between synthesis and degradation. All organisms assimilated carbon and energy in protein, nucleic acids, lipids and carbohydrates. In plants a significant proportion of this energy is diverted to the synthesis of secondary product (Katare et al, 2009). Modern biology has explained the role of primary metabolites in relation to growth and development of plants. Higher plants are the source of a number of compounds derived from primary metabolite (polysaccharides, sugars, proteins and fats) pathways. Some compounds are present at a very much lower concentration. These are the secondary products of primary metabolism, known as plant secondary metabolites. Kossel was the first to give the concept of secondary metabolites. These compounds have a wide diversity in structure and size, found in exponentially throughout the plant kingdom (Collin, 2001).

Secondary plant metabolites are the byproducts of primary metabolism and refer to compounds that get collected in specialized cells in the absence of excretory systems. Plant secondary metabolites (PSMs) are synthesized as a means for plants to protect themselves against insects, mammals and other herbivores, against bacteria, fungi, viruses and even other competing plants. Some plants use PSMs in addition to attract pollinating and seed dispersing animals or for UV protection (Harborne, 1993; Wink, 1988; Wink, 1999; Wink, 2003, Wink, 2008). They are stored in specific cells and/or organs of the plant and often accumulate in vacuoles. Secondary metabolites are characterized by wide chemical diversity and every plant bears its own characteristic set (Verpoorte, 1998; De Luca and St Pierre, 2000). The secondary metabolites do not perform vital physiological roles for plant life as primary metabolites. Secondary metabolites are used in pharmaceuticals, agrochemicals as flavouring agent and also in aroma industries (Edreva et al, 2008). The development of drugs from plants by drug companies encourages large scale pharmacological screening of herbs.
Reasons for selection of experimental plants

There are numbers of plants having valuable medicinal properties. Among them Oroxylum indicum (L) Vent and Uraria picta (Jacq) Desv ex DC are valuable known root drugs. The whole plant and roots of both medicinal plants use as a drug for curing the various diseases like catarrhs, bleeding piles, scorpion sting, cough, chills, gonorrhea, heart troubles, fevers, diarrhea, dysentery, rheumatism, dropsy, flatulence, colic, tuberculosis, nasopharyngeal cancer (Hamid et al, 2004; Chauhan, 1999; Gohil et al, 2008; Tiwari et al, 2007; Warrier et al, 2001; Bhattacharje, 2000; Mao, 2002). Their roots were used to prepare variety of ayurvedic formulations such as Dashmularisht, Pancharisht, Chyavanprash, Syonaka putapaka, Syonaka siddamghrta, Brhatpancamulyadi kavath, Abana (Yasodha et al, 2004; Chen et al, 2003; Garg et al, 2012; Khanna et al, 1991). Both plants considered under the IUCN threat categories in India. The existence of O. indicum in natural population is highly threatened and has been categorized as vulnerable by the government of India (Ravikumar and Ved, 2000). Uraria picta is reported to be threatened in some regions of India (Nair and Joshi, 1979). The whole plant and roots of medicinal plants, Oroxylum indicum and Uraria picta are used as a drugs. Harvesting of roots ultimately destroys the whole plant. Moreover, harvesting the whole plant before seed setting has posed serious threat to the survival and accessibility of these highly useful plants. Certain limitations of conventional propagation methods, such as being labor-intensive, the demand for large areas for ex situ conservation, out-breeding, a long juvenile phase and seasonal dependence, make it necessary to apply in situ approaches.

Oroxylum indicum (L) Vent: An endangered medicinal tree

Oroxylum indicum (L) Vent is an important medicinal plant distributed in India, Srilanka, Philippines and Indonesia (Jayaram and Prasad, 2008). In India, it is found mainly in ravine and moist places in the forests throughout the country up to an altitude of 1200 m (Bennet et al, 1992). Oroxylum indicum is derived from a combination of two words: Oroxylum means “mountain tree” and indicum means from India (McCann, 1954). Oroxylum indicum belonging to family bignoniaceae characterized with few branches. It is also known as Shivnak, Sonapatha, Shyonaka, Tetu or Midnight horror (Kumar et al, 2012). Oroxylum indicum is a small to medium sized deciduous tree with light grayish brown, soft, spongy bark and corky outside; Leaves are opposite, 3-pinnate near base, 2-pinnate about middle and simply pinnate towards apex. Leaf very large up to 150 cm in length, rachis stout and cylindrical, leaflets 2-4 paired, ovate or elliptic, acuminate, entire and glabrous with a rounded or cordate base; Inflorescence terminal erect racemes; Flowers are purple, deep scented, arranged on long sturdy peduncle coming out from the scantly canopy where they are pollinated by megachiroptera bats; Fruits flat capsule, about 80 cm long, sword shaped capsules full of many flat and papery winged seeds (Gokhale and Bansal, 2006; Gohil et al, 2008 and Dalal and Rai, 2004). Most parts of Oroxylum indicum tree are used in ayurvedic system of medicine. The roots are sweet, bitter, acrid showing astringent, refrigerant, expectorant, digestive, carminative, febrifuge, diuretic, anti-microbial,
anti-fungal and anti-inflammatory properties. It is a well-known tonic, useful in diarrhea, dysentery and rheumatism (Gohil et al, 2008; Tiwari et al, 2007). They are also useful for cure of vata, kafa, dropsy, flatulence and colic (Warrier et al, 2001). The roots are used for the treatment of tuberculosis and nasopharyngeal cancer (Bhattacharje, 2000; Mao, 2002).

Root and stem bark of *Oroxylum indicum* is used to prepare Dashmularisht and Chyavanprash. This plant is also one of the important ingredients in most commonly used ayurvedic formulations such as Syonaka putapaka, Syonaka siddamghrta, Brhatpancamulyadi kavath (Yasodha et al, 2004; Chen, 2003). Leaf decoction is given in treating stomachache, ulcers, rheumatic pain and enlarged spleen. Matured fruits are useful in treating cough, bronchitis and cholera. It has analgesic, anti-tussive and anti-inflammatory potencies (Yuan et al, 2008). Tender fruits are refreshing and stomachic and the seeds are purgative (Tiwari et al, 2007). Methanolic extract of seeds exhibits antimicrobial, analgesic, anti-tussive and anti-inflammatory properties. In China, seeds of *oroxylum indicum* are used as the crude drug ‘Mu Hu Die’ (Rasadah et al, 1998; Chen et al, 2005).
Phytochemical study revealed that the wood, wood bark, root, root bark contain iridoids. The crude extracts of all these parts studied were found to show antibacterial activities towards the gram-positive, gram-negative bacteria and yeast (Rasadah and Houghton, 1998). *Oroxylum indicum* contains valuable economic important active ingredients like flavonoids aglycone (mainly chrysin, baicalein) and certain glucosides of the flavonoids (eg baicalin) (Yuan et al, 2008). Baicalein is reported to possess an anti-inflammatory, anti-ulcer, anti-oxidant, hepatoprotective and immunomodulatory activity. While baicalein and chrysin both are reported to sustain anti-bacterial, anti-fungal, and anti-viral activity. Chrysin also used as toxic breast cancer drug (Zaveri et al, 2008). Baicalin has been shown to reduce the total cholesterol level and have detoxification and chemo-protective effects (Yuan et al, 2008).

**Uraria picta** (Jacq) Desv ex DC: A valued medicinal herb

*Uraria picta* (Jacq) Desv ex DC known as *Prithakparni* in Sanskrit belongs to the family fabaceae. It is distributed throughout Africa, Asia and Australia. Among Asia, it is found in Pakistan, Sri Lanka, India, Nepal, Sikkim, Bhutan, Bangladesh, Myanmar, Thailand, Brunei, Indonesia, Malaya, Philippines, Papua New Guinea, Sabah, Cambodia, Vietnam, Southern China and Taiwan (Ohashi and Lokawa, 2007). It is found in dry grasslands, waste places, open deciduous forests and in all plains of India (Hooker, 1973). The genus *Uraria* has more than 11 species known from India, of which *Uraria picta* is well known for its medicinal properties (Jain and Defilipps, 1991). *Uraria picta* is 1-15 m tall, apprised pubescent, suffructicose sparingly branched herbs; Stems with short, rough hairs; Leaves imparipinnate with 5-9 leaflets (lowermost leaves often 1-3 foliolate), leaflets are narrowly lanceolate, 7-25 cm long, lowermost smaller, variegated, shiny, hairless above and rough hairy below; leaflets margins is entire. Inflorescence is up to 55 cm long, terminal, densely many flowered spike-loke raceme, covered with long whitish hairs. Flowers are pink, bluish or reddish, complete. Fruit pods are 5-9 mm long, folded into 3-6 segments, brown to black, turning grayish-white when old (Okusanya et al, 1991; Gurav et al, 2008).

It is beneficial in catarrhs, bleeding piles and for scorpion sting (Hamid et al, 2004). Roots are aphrodisiac in nature, had fracture healing properties which are essentially credited to its property of accumulation of phosphorous and deposition of calcium (Anonymous, 1976). The decoction of their roots is useful in cure of cough, chills and fevers. The pulverized leaves of this flora are employed medicinally in Southern Nigeria as a remedy for gonorrhea. In Ghana, the plant is used in heart troubles (Chauhan, 1999). In India, the plant is used as an antidote against the bites of certain vipers (Allen and Allen, 1981). Its use in *Dasamula*, an ayurvedic medicine has shown significant improvement in patients of primary neurological disorder (Garg et al, 2012). It is also used in the preparation of *Abana*, an ayurvedic drug remedy useful in the treatment of hypertension, tachycardia and angina (Khanna et al, 1991). It contains flavonoid, exhibiting a range of biological activities like anti-inflammatory, anti-thrombotic, hepatoprotection properties due to the free radical scavenging ability (Patwardan, 2005).
Previous phytochemical studies of *Uraria picta* led to the identification of two Isoflavanones, (1) 5,7-dihydroxy-2’-methoxy-3’-4’-methylenedioxy-isoflavanone and (2) 4’-5-dihydroxy-2’-3’-diethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone and six known compounds including isoflavanones, steroids and triterpenes were isolated from the roots (Rahman et al, 2007). Earlier experiments estimated that *Uraria picta* exhibits acaricidal properties (Igboechi et al, 1989), antimicrobial activity (Rahman et al, 2007; Ahire et al, 2011) and showed nephrotoxicity in mammals (Kale et al, 2012).

**Needs for the in vitro approaches:**

The estimated demand of *Oroxylum indicum* in Southern India is 500 kg per annum (Jayaram and Prasad, 2008). In India, twigs of the tree are trading at a low price of Rs 9/kg but its extract in international market is believed to fetch Rs 500,000/kg (Gokhale and Bansal, 2006). The existence of *O. indicum* in natural population is highly threatened and has been categorized as
vulnerable by the government of India (Ravikumar and Ved, 2000). It is naturally propagated by seeds. However, the seed setting is poor and seed viability is low (Tiwari et al, 2007). Destructive and non-sustainable collection methods coupled with low regeneration and habitat destruction have posed serious threat to the survival and availability of this highly useful tree (Yasodha et al, 2004). *Uraria picta* is reported to be endangered in some parts of India (Nair and Joshi, 1979). It is naturally propagated by seeds. However, the seed set is poor and seed viability and percentage of germination is low (Gurav et al, 2008). It is a seasonal herb, so only available in winter.

The whole plant and roots of medicinal plants, *Oroxylum indicum* and *Uraria picta* are used as a drug. Harvesting of roots ultimately destroys the whole plant. Moreover, harvesting the whole plant before seed setting has posed serious threat to the survival and availability of these highly useful plants. There are certain limitations of conventional propagation methods such as a long juvenile phase, being labour-intensive and seasonal dependence. The need for large areas for *ex situ* conservation, out-breeding and industrial demand make it necessary to apply *in situ* approaches. However, with the growing need for medicinal plants, especially those that are rare and endangered in nature, large-scale production of these species are a prerequisite to meet the pharmaceutical needs and also for the effective conservation of these valuable medicinal plants. Tissue culture techniques can be applied to conservation efforts, especially for those species in which either the underground parts or the whole plant is used in drug preparation (Jasrai et al, 2013).

**Plant Tissue Culture: A tool for production of secondary metabolites**

Biotechnology is a rapidly developing area, which still remains underexploited. During the past two decades, plant cell biotechnology has evolved as a new area of biotechnology as a tool for production of plant secondary metabolites without plant sacrifice (Verpoorte et al, 1994). Plant cell culture technologies were introduced at the end of the 1960s as a tool for both studying and producing plant secondary metabolites. Many studies have been undertaken with the objective of improving the *in vitro* production of plant secondary compounds (Patel and Jasrai, 2009). Among the various approaches of plant biotechnology, plant tissue culture has widely been accepted as an essential tool to conserve medicinal plants and improve secondary metabolite production (Moyo et al, 2011).

Plant tissue culture technology shows promise for the large-scale production of valuable plant products; however, the commercial use of plant cell cultures is not routine because of difficulties in achieving acceptable, reproducible product levels in reasonable periods of time (Roberts and Shuler, 1997). The production of secondary metabolites using plant cells has been the subject of extended research. It was expected that the biosynthetic capacity of plants could be exploited *in vitro* using plant cells and cell tissue systems analogous to microbial cells in fermentation processes (Dornenburg and Knorr, 1995). Plant tissue culture has explored possibilities to
produce valuable drugs using various in vitro systems involves optimizing the various chemicals, physical factors necessary for growth and development of plant. Over the last 50 years, many tissue culture techniques including micropropagation, bioreactors, callus culture, suspension cell cultures, specialized organ cultures (root and hairy root cultures) and even large scale fermentation of suspended cells were successfully established for the production of secondary metabolites (Bairu and Kane, 2011; Chattopadhyay et al, 2002; Bourgaud et al, 2001; Collin, 2001; Verpoorte et al, 2002). The production of pharmaceuticals by plant tissue culture offers a number of advantages such as control of supply of product independent of the availability of plant itself, cultivation under controlled and optimized conditions, possibility of synthesizing novel compound not present in nature, by feeding of compounds analogous to natural substrates, no dependence on climate and geographical location etc (Chattopadhyay et al, 2002).

**Tissue culture approaches to improve production of secondary metabolites:**

Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. Generally it is accepted that secondary metabolite production is associated with tissue differentiation. Biosynthesis of many important compounds needed some level of cellular or tissue differentiation such as roots, shoots, embryos, and other organs have produced markedly high products (Endress, 1994; Lindsey and Yeomam, 1983; Benjamin et al, 1987). So, the screening and selection of high producing cell lines and the optimization of growth and production media can be mentioned as common approaches. But, in some cases, undifferentiated cell cultures like callus and cell suspension can produce high amount of secondary metabolites as compared to that of the whole plant (Bourgaud et al, 2001). Biotransformation, uses of bioreactor and hairy root cultures have also been shown large interest as tissue culture approaches. Among the techniques employed, optimization of nutrient media, optimization of culture conditions, identification of the most effective elicitors and the use of hairy root culture have been given considerable attention (Verpoorte et al, 1994).

Some important tissue culture techniques are described below.

**Micropropagation:** Multiplication of genetically identical plants by asexual methods known as in vitro clonal propagation. In 1960, Morel initiated this method for orchid propagation. Since then protocols for several trees, fruits, medicinal and crop species have been developed through this method. Micropropagation of plants achieved by forced proliferation of shoots from axillary or apical buds, production of adventitious buds and somatic embryogenesis (Jasrai et al, 2003; Razdan, 1993). Establishment of micropropagation protocol allows fast and continuous commercial propagation of valuable medicinal plant and in vitro propagation ultimately protects this type of rare and endangered plant by does not destroy the mother plant. Established micropropagation protocol serve as an essential tool to conserve medicinal plants and improve secondary metabolite production.
**Callus culture:** Establishing dedifferentiated cultures from the proliferating cells of the parent tissue known as a callus culture. A callus is an amorphous mass of loosely arranged parenchyma cells formed as a result of wounding, at the cut end of explant (Yeoman, 1970; Yeoman and Macleod, 1977). Successful callus induction for secondary metabolite production has been reported by several researchers (Maurya and Singh, 2010; Takeda and Katoh, 1981; Beutelmann and Bauer, 1977; Peterson, 2003). Through callus culture also produced embryo and subsequently plantlets. Callus culture extensively used for the establishment a protocol for cell suspension culture and subsequent production of secondary metabolite.

**Cell suspension culture:** A cell suspension culture consists of cell aggregates dispersed and growing in moving liquid media under controlled conditions. *In vitro* generated pieces of calli transferred in liquid media for the establishment of cell suspension culture (Chawla, 2002; Sharma et al, 2011). Cell suspension cultures have provided an alternative for the production of a number of high-value secondary metabolites.

**Root culture:** Root tips of either primary or lateral roots used as explants for establishing the root culture. As roots are indeterminate organs and its growth is unlimited. So, establishment of a protocol for root cultures was one of the great achievements of modern plant tissue culture (Slater et al, 2008).

**Biotransformations:** The conversion of a small part of a chemical molecule by means of biological systems is termed biotransformation. Implementation of specific enzyme present in cultured cells via catalyst, glycoxylation, methylation, reduction, hydroxylation, esterification and epoxidation for increase the biosynthesis of particular compounds in cell culture.

**Hairy root cultures:** Transformation of plants with *Agrobacterium rhizogenes* is one of the plant tissue culture technique which used for increased production of secondary metabolites. These soil bacteria are capable of infecting plant cells and cause the proliferating growth as roots, the so-called ‘hairy roots’. Hairy roots can grow without growth hormones in the medium. Hairy roots have similar production profiles as normal roots.

**Biotechnological production:** Genetically engineered plant cell culture has great potential for altering the metabolic profile of plants. Cell suspension cultures from transgenic plants can be generated for obtaining the particular plant secondary metabolites.

**Objectives of present study:**

The present studies on *Oroxylum indicum* and *Uraria picta* were undertaken for developing protocols for the production of important plant pharmaceuticals through-

1. Culture of differentiated cells, including micropropagation.
2. Optimization of growth medium for callus proliferation and their ability to produce secondary metabolites through changes in physical factors and nutrients levels.

3. Introduction of precursors to *in vitro* culture for increased the secondary metabolite production.

4. Isolation/extraction and quantification of phytochemicals (HPTLC analysis).