REVIEW OF LITERATURE

Medicinal plants

The globe is rich in enormous diversity evolved during billions of years that plant inhabits a major part of the biota to serve mankind with various “plant derived natural products” being used in traditional medicinal systems all over the world. According to Schippmann et al. (2002, 2006) more than 50000 species are used for medicinal purposes worldwide, of which almost 13% are flowering plants. Over 8000 plant species are used in traditional and modern medicine in India (Planning Commission 2000), and 90-95% collection of medicinal plants is from the wild, of which more than 70% collection involves destructive and unscientific extraction. Over exploitation of trade species, destructive way of collection, vulnerability due to anthropogenic pressure are some of the major threats to medicinal plants. In order to achieve sustainable harvest of medicinal plants and other non-timber forest products a multi-disciplinary approach must be considered which include ecological, biological, socio-cultural and economical aspects of the species (Ghimire et al., 2004).

India’s diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific species). Our subcontinent is a vast repository of medicinal plants acquainted as one of the world’s 12 biodiversity centers with more than 45000 different plant species, among which 15000-20000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional indigenous systems such as Siddha (600), Ayurveda (700) and Amchi (600), Unani (700) and the modern allopathy using 30 plant species for ailments. Eastern Ghats, one of the nine Floristic Zones in India represents a wide range of diverse medicinal plants; in particular the Kolli hills located in the southern-eastern ghats harbours some of the rare and endemic plants (Samy et al., 2008).

*Cleome gynandra* Linn.

*Cleome gynandra* [syn. *C. pentaphylla* Linn., *Gynandropsis pentaphylla* DC., *G. gynandra* (Linn.) Briq] of Cleomaceae (Capparaceae) family is an annual herb, widely spread in many tropical and sub-tropical parts of the world. It is an erect glandular-pubescent annual herb, popularly used in the Ayurveda, Siddha, Folk and Tibetan systems of medicine. It is known as
Cat’s whiskers and Spider flower in English; Cararvella, varvar, surjavarta and arkapushpika in Sanskrit; Arkahuli, karaila, hulhul and churota in Hindi; and Velai keerai, neivayalla keerai and katte kadugu in Tamil. This wild leafy vegetable is indigenous to the tropical and pan tropical regions and plays an important role in agricultural and national system of these regions. (The Wealth of India, 1956; Chatterjee and Pakrashi, 1991; Chweya and Mnzava., 1997, and Heever et al., 2007).

The natural habitat of Cleome gynandra is in waste land and arable land with annual species as well as grasslands. Naidu et al (1980), Rajendrudu and Das (1982a, 1982b), Kumar et al (1984) and Rao and Rejendrudu (1989) have determined the species to have a C₄ photosynthetic pathway, an adaptational mechanism that enables it to survive in drier and hot environments. It grows well up to about 1000 m in semiarid, sub humid and humid climates, and is adapted to many soil types, but grows luxuriantly around rubbish dumps and soils supplied with organic manure.

Based on the research done by Narendhirakannan et al (2007) experimental rats suffering from arthritis were administered ethanolic spider plant leaf extracts at a dose of 150 mg/kg of body weight for 30 days. Analysis of enzymes involved in the expression of arthritis showed that the rats had recovered from the disease and their status was comparable to the healthy control rats. The control of the disease was related to substances present in the leaf extracts, including saponins, glycosides, lectins, steroids, flavonoids, tannins, triterpens, resins, phenolic compounds, and arthroquinones.

According to Mule et al (2008) and Narendhirakannan et al (2005) the Aqueous Extract of Gynandropsis pentaphylla (AEGP) showed a significant antinociceptive effect. Studies demonstrate that various flavonoids such as rutin, quercetin, luteolin, hesperidin and biflavonoids produced significant antinociceptive and anti-inflammatory activities. There are also a few reports on the role of tannins in antinociceptive and anti-inflammatory activities. The mechanisms of antinociceptive action of AEGP could be due to the presence of flavonoids and mediated through central and peripheral mechanisms. Most of the non-steroidal anti-inflammatory drugs (NSAIDs) have well balanced anti-inflammatory and ulcerogenic activities, which are considered to be due to PG (prostaglandins) synthetase inhibitor activity. The Flavonoids and tannins in AEGP are reported to inhibit PG synthesis.
Based on the studies of Anbazhagi et al (2009) they studied the pharmacognostical characters of the leaves of *Cleome gynandra* and microscopic characters of the plant has been discussed. This study also implies that dietary polyphenolic phytochemicals, especially the flavonoids, vitamin C and essential metal ions accumulated in leaves may supply substantial antioxidant activity, which in turn may inhibit, prevent or retard the development of several chronic diseases and thereby provide health-promoting effects.

Mondal et al (2010) studied the phytochemical examination of the roots of *Cleome rutidosperma* DC and isolated b-Sitosterol, Lupeol, and Betulinic acid for the first time from this plant. The chloroform extract of *C. rutidosperma* roots upon concentration under reduced pressure left a dark green sticky and oily residue (5.7%). In TLC examination of the residue showed number of spots (Solvent system: Benzene: Chloroform: Ethyl acetate. 1:3:1) on spraying with 5% alcoholic H₂SO₄ followed by heating. The chloroform residue was subjected to column chromatography over silica gel (200 mesh). The residue was subjected to column chromatography over silica gel afforded three compounds named C-01, C-02 and C-03. The compounds were characterized through chemical and spectral analysis and confirmed as b-Sitosterol (C-01) Lupeol (C-02) and Betulinic acid (C-03), respectively.

Based on the investigation done by Bala et al (2010) anticancer activity of methanol extract of *Cleome gynandra* (MECG) was evaluated in Swiss albino mice against Ehrlich Ascites Carcinoma (EAC) cell line at the doses of 200 and 400 mg/kg body weight intraperitoneally. MECG showed significant decrease in tumor volume, viable cell count, tumor weight and elevated the life span of EAC tumor bearing mice. Hematological profile such as RBC, hemoglobin, WBC and lymphocyte count reverted to normal level in MECG treated mice.

Mishra et al (2011) reviewed on the potent medicinal properties of *Cleome gynandra* and they compiled and documented all information on different aspects of *Cleome gynandra*. In this review article through various established facts regarding the medicinal applications of *cleome gynandra* has been cited regarding the Immunomodulator, Antioxidant, Anticarcinogenic, Analgesic properties etc.

Aparadh et al (2012) reviewed taxonomy and physiological studies in spider flower (cleome species). Cleomaceae is a small family of flowering plants in the order Brassicales, comprising more than 300 species belonging to 9 genera of which Cleome is the largest genus.
with about 180 - 200 species of medicinal, ethnobotanical, ecological importance. The present paper accounts for critical review of spider flower genus Cleome with special emphasis on its taxonomy, ecology, ethnobotany, cytology, biochemistry, physiology and pathology with a note on its importance. The review of the researches on Cleome will provide understanding the value of the species and guidelines for further researches with spider flower genus.

**Orthosiphon spiralis (Lour.) Murr.**

*Orthosiphon spiralis* (Lamiaceae), commonly called ‘Kidney Tea Plant’, is a medicinal plant widely used in the treatment of various kidney and urinary bladder diseases including nephro-cirrhosis and phosphaturia. The activity of the leaves is attributed to the presence of a bitter glycoside orthosiphonin (Wealth of India, 1966).

Based on a research done by Mariam *et al* (1996) they found that significant hypoglycaemic effect was observed in normal rats treated orally with 1.0 g / kg of body weight of the *Orthosiphon stamineus* extract. An addition to that, the hyperglycaemic effect induced by streptozotocin was also inhibited by the same dose of the same extract. From the results obtained from this research, it can be concluded that the aqueous extract of local Misai Kucing possessed some hypoglycaemic activities in both normal and streptozotocin-induced diabetic rats in lowering the blood sugar level.

The therapeutic effects of *Orthosiphon stamineus* have been ascribed mainly to its polyphenol, the most dominant constituent in the leaf which has been reported by Hollman & Katan (1999) to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems which could lead to some of the chronic diseases such as coronary heart disease and many more. This is proved by the research of Chew *et al* (2011) which among the different parts of plants studied, the leaves are reported to have the highest antioxidant properties whereby the phenolic fraction is the most active principle among the phytochemicals studied (Matkowski, 2008; Chew *et al.*, 2011; Pietta *et al.*, 1998).

According to Tezuka *et al* (2000), this herb contains several active chemical compounds such as terpenoids (diterpenes and triterpenes), polyphenol (lipophilic flavonoids and phenolic acids) and sterols. The antioxidant capabilities of the phenolic compounds are important for the human body to destroy the free radicals that exists in our body. The existence of free radicals in a large quantity in our body could have the ability to destroy the structure and the inner part of our
living cells, including genetic compounds (DNA) that could lead to cancer. Besides that, free radicals could also weaken the artery walls that will allow fat deposits to occur and leading to heart disease. In fact, the dried leaves and stem tips of *Orthosiphon spiralis* contains up to 12% minerals, inositol, phytosterols, saponins and up to 0.7% essential oils.

According to Medicinal Herb Index in Indonesia (1995), the scientific term of *Orthosiphon spiralis* have other synonyms such as *Orthosiphon stamineus*, *Orthosiphon aristatus*, *Orthosiphon grandiflorum* and *Orthosiphon spicatus*. This herb is also known by its vernacular names such as Java tea (English), Poonai meesai (Tamil) kumis kucing or misai kucing (Malaysia), This herb (commonly known as Java Tea) is distributed from India, Indo-China and Thailand through Malaysia to tropical Australia. Throughout Malaysia, it occurs as a wild plant. It is now grown in South East Asia, Africa, Georgia and Cuba. It grows well in wet soil and can be found in both temperate and tropical gardens.

Based on the studies by Akowuah *et al* (2004), the Rosmarinic Acid (RA) component is the main polyphenol compound in the leaves of java tea plant, which is the most polar component, compared to the three polymethoxylated flavones studied. The extract of the leaves of java tea using polar extracting solvents gave the highest activity of free radicals scavenging which is possibly due to the high concentration of caffeic acid derivatives, especially RA (Akowuah *et al*, 2005). Based on Sumaryono *et al* (1991), the derivatives of caffeic acid, including RA was reported to constitute 67% of total identified phenolics in aqueous methanol extract and about 94.6% in hot water extract.

According to Maheswari *et al* (2008) the methanol leaf extracts of *Orthosiphon stamineus* possessed hepatoprotective activity against paracetamol induced hepatotoxicity in rats. The hepatoprotective activity of the methanol extract was assessed in paracetamol induced hepatotoxic Rats. Paracetamol (2 g/kg) has enhanced the SGOT, SGPT, ALP and the Lipid peroxides in liver. Treatment of methanolic extract of *O. stamineus* leaves (200 mg/kg) has brought back the altered levels of biochemical markers to the near normal levels in the dose dependent manner.

Kannappan *et al* (2010) studied the preliminary phytochemical studies and Nephroprotective activity of *orthosiphon stamineus* in rats. These studies revealed the presence of flavonoids, tannins, saponins, phenols and terpenoids. The drug is found to be potent diuretic which causes excretion of sodium and potassium. The drug was administered intra peritonially at a
dose of 80 mg/kg weight for 9 days. Histopathological sections showed marked glomerular, peritubular and blood vessel congestion. These increased levels of serum creatinine, blood urea, urinary protein and extent of renal damage were decreased by the methanolic extract of Orthosiphon stamineus at both dose levels that is 100 and 200 mg/kg body weight in rats.

For centuries, Orthosiphon stamineus Benth, a native plant to Southeast Asia, has been extensively used as a source of medicinal agents due to its wide range of medicinal properties. It is usually consumed as an herbal tea and it was believed to be useful in the treatment of diabetes, fevers, high blood pressure and bone or muscular pain, hypertension, kidney stone as well as promoting health and well-being. The plant has also been reported to possess various novel secondary metabolites such as diterpenes, flavones and triterpene. Koay and Amir (2012) reviewed and summarized the phytochemical investigations and pharmacological properties of O. stamineus.

**Plant tissue culture**

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants (Rao and Ravishankar, 2002).

**Surface sterilization**

All living plant materials in nature were contaminated on their surface with dirt and microorganisms. Such microorganisms include viruses, bacteria, yeast, fungi, etc. (Omamor et al., 2007). These microbes compete adversely with plant tissue cultures for nutrients. Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to sterilize the tissues. These sterilization agents are also toxic to the plant tissues. During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009).

Contamination with microorganisms is considered to be the single most important reason for losses during in vitro culture of plants. The presence of these microbes usually result in
increased culture mortality but can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003).

The maintenance of aseptic (free from all microorganisms) or sterile conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explant itself must be sterilized. The importance is to keep the air, surface and floor free of dust. All operations should be carried out in laminar airflow sterile cabinet (Chawla, 2003).

The sterilizing agents widely used are Sodium hypochlorite calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide, silver nitrate and bromine water. Hypochlorite is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly. Calcium hypochlorite is used mostly in Europe and the concentration generally used is 3.25%, it may be less injurious to plant tissues than sodium hypochlorite. Mercuric chloride is used only as a last resort in the U.S. It is extremely toxic to both plants and humans and must be disposed of with care. Since mercury is so phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material (Oyebanji et al., 2009).

Ethanol is a powerful sterilizing agent but also extremely phyto-toxic. Therefore, the explant is typically exposed to it for only a few seconds or minutes. Ethanol, in general, is used prior to treatment with other compounds. The use of a two-step (two-source) sterilization procedure has proven beneficial with certain species. Ethanol is usually combined with hypochlorite for effectiveness, e.g. the use of 90 or 70% ethanol for 3 min and sodium hypochlorite (3.5%) for about 30 min. To enhance effectiveness in sterilization procedure, a surfactant like Tween 20 is frequently added to the sterilizing solution in general, the sterilizing solutions containing the explants are continuously stirred during the sterilization period (Oyebanji et al., 2009).

Oyebanji et al (2009) evaluated three different surface sterilization methods using seeds and excised embryos of cowpea, rice and sorghum as explants. Results showed that locally produced bleaching solution containing 3.5% Sodium hypochlorite at time intervals between 20 - 45 min produced the highest reduction in bacterial and fungal contamination (0%).
Dahab et al (2005) studied different sterilization methods using combination of chlorox solution at 30, 40, 50, 60 and 70 % and mercuric chloride (HgCl$_2$) at 0.0, 0.1, 0.2, and 0.4 % on Ruscus hypoglossum. The explants were dipped in ethanol 70% for 30 second before Chlorox and mercuric chloride treatments. One drop of Tween 20 (Polyoxyethlene sorbitan monolaurate) was used as a wetting agent per 100 ml of sterilizing solution. The best treatment which can be recommended to obtain free of contamination explants was 50 or 60% chlorox with 0.4% mercuric chloride.

Badoni and Chauhan (2009) standardized sterilization procedures for potato cultivar Kufri Himalini. Comparison was done between two important sterilant sodium hypochlorite and mercuric chloride with three time duration 2, 5 and 8 minutes. Result showed that amongst the two sterilants i.e. NaOCl and HgCl$_2$, NaOCl (1%) was found better for controlling the infection and it had not any adverse effect on explants even in long duration. Sodium hypochlorite (NaOCl) for 8 minute was selected for suitable sterilization chemical after 5 minute of savlon wash, 30-second dip in ethanol and at last washed with double distilled water.

Meghwal et al (2000) standardized the quick establishment of aseptic cultures in guava from mature field-grown stock plants for micropropagation through enhanced axillary branching technique. The maximum number of aseptic explants with shoot proliferation was obtained by a combination of surface sterilizing agents involving hydrogen peroxide (10%), silver nitrate (0.25%), and mercuric chloride (0.05%) treatment of explants one by one for five, six, and three minutes, respectively.

Colgecen et al (2011) analyzed the effects of sodium hypochlorite and Plant Preservative Mixture (PPM) on surface sterilization of Arnebia densiflora. The effects of the addition of an antibiotic-antimycotic solution to the culture medium had been studied on callus induction. Explants were initially sterilized with different concentrations of sodium hypochlorite and cultured on MS media containing kinetin (0.29 μM) and naphthalene acetic acid (NAA, 10 μM). No calluses were produced, either because of contamination of the explants, or loss of explants as a result of the high levels of sodium hypochlorite. The application of PPM and antibiotics at different concentrations reduced contamination and led to callus induction from shoot apexes and young root explants. The best callus responses were obtained using PPM at 1% - 2%, whereas callus induction on shoot apexes diminished at higher concentrations (4% PPM).
Plant Growth Regulators

Plant growth regulators (PGR) stimulate cell division and hence regulate the growth and differentiation of shoot and roots on explants and embryos in semisolid or in liquid medium cultures. The four major PGR used are Auxins, Cytokinins, Gibberellins and Abscissic acid. Addition of PGR is essential to the culture medium.

Elangomathavan et al (2003) studied the in vitro propagation of Orthosiphon spiralis using different concentrations of growth regulators. Multiple shoots were obtained on MS medium containing 2.22 mM BA, from nodal explants of a medicinally valuable plant Orthosiphon spiralis. The regenerated shoots were further subcultured and rooted on 1/2 strength MS medium supplemented with IBA 4.9 mM. 60–70 per cent of plants were successfully established in the field.

According to Leng and Keng (2004a) micropropagation method for Orthosiphon stamineus, using stem nodal segments has been established. The highest number of regenerated shoots was obtained on Murashige and Skoog (MS) medium supplemented with 6.7 mM benzyladenine with the formation of an average of 6.1 shoots per explant over a period of 4 weeks. The number of shoots increased with longer culture duration on proliferation medium. Multiple shoots which were maintained on the proliferation medium for 6 weeks had the highest proliferation rate. Separation of multiple shoots and culturing in larger flasks significantly promoted the growth and formation of plantlets. All the in vitro plantlets survived when transferred to the field and showed no significant morphological differences from the mother plants.

Ling et al (2009) reported adventitious roots of Orthosiphon stamineus were induced from different explants like leaf, root and stem by MS medium supplemented with Indoleacetic acid (IAA), indole-butyric acid (IBA) and naphthaleneacetic acid (NAA) each at the concentrations of 0, 1, 3, 5, and 7 mg/l. The best rooting ability was observed in leaf explants cultured on MS medium supplemented with 3 mg/l of IAA. Although IBA and NAA also managed to induce the adventitious roots from leaf explants, the rooting ability was relatively lower than IAA treatment. Root and stem explants were less efficient in inducing adventitious roots although managed to form some degree of adventitious roots.
According to Mungole et al. (2009) an efficient micropropogation protocol was developed for medicinal plant *Ipomoea obscura* (L.) by in-vitro culture of nodal part of mature plant. Callus of the leaves, node and bud of *Ipomoea obscura* (L) was initiated on MS basal media supplemented with various combinations of auxins 2, 4-D and NAA with cytokines kinetin and BAP. The MS media supplemented with 0.8 mg/l NAA with 0.8 mg/l kinetin induced three shoots per node in an average and was best for axillary bud proliferation.

The culture of undifferentiated mass of cell on agar media produced from an explant of a seedling or other plant part is called callus culture. Auxins and cytokinins are required for callus formation. Erisen et al. (2010) developed a callus induction and plant regeneration protocol from leaf and petiole explants of the endemic *Astragalus nezaketae*. Explants were cultured on Murashige and Skoog medium (MS) supplemented with different plant growth regulators (PGRs) (NAA, BA, 2, 4-D, Kin and TDZ). The combinations and concentrations of PGRs were shown significant variations for the frequency of callus formation, appearance of callus and the potential of callus differentiation. NAA x BA have been found highly effective in callusing and plant regeneration.

Thomas and Maseena (2006) developed a method for rapid micropropagation of *Cardiospermum helicacabum* through plant regeneration from leaf and nodal explant derived calli. The nodal and leaf segments were cultured on Murashige and Skoog (MS) medium supplemented with 2, 4-D; (0.5 – 9 mM) for callus induction. Callus production was highest at 5 mM 2, 4-D where 96 and 90% of cultured leaf and nodal cuttings produced callus, respectively. The viable calli were maintained at reduced concentration of 2, 4-D (2 mM). The addition of low concentrations of IAA into BA or kinetin containing medium significantly increased the frequency of shoot regeneration in both nodal cuttings and leaf-derived calli. The optimal result was observed on half-strength MS medium supplemented with 2.5 mM IBA, on which 91% of the regenerated shoots developed roots with an average of 4.2 roots per shoot within 45 days.

Based on the research done by Mederos-Molina (2004) Stem and petiole explant of *Salvia canariensis* L. were cultured on MS and B5 culture media supplemented with BAP, NAA and IBA. Callus formation on the surface of stems and petioles and also high and low concentrations of NAA alone and combined with BAP, stimulated the callus induction and direct shoot formation, respectively. However, concentrations of IBA used here, inhibited shoot induction.
The shoots developed from both explants formed roots when transferred to half strength MS medium supplemented with IAA, NAA or IBA.

Safdari and Kazemitabar (2010) performed the experiment to optimize the medium for tissue culture of *Portulaca grandiflora*. Nodal segments of *Portulaca grandiflora* were cultured on media containing different levels of BAP or kinetin to direct shoot regeneration study. Leaf explants were cultured in different combination of BAP and NAA to examine callus formation. After the callus formation the formed calli were cultured on different combinations of BAP and NAA for shoot regeneration. BAP and NAA in all possible combinations were used for shoot regeneration from callus. The treatment containing 10 μM BAP was found to be the best one for shoot regeneration from nodal segments. The treatment with 10 μM NAA in combination with 10 or 5 μM BAP were found to be suitable treatments for callus production from leaf explants, as well. Moreover, 15 μM BAP alone or in combination with 5 μM NAA were found to be the best treatments for shoot regeneration from callus.

Sridhar and Naidu (2011) described an efficient protocol for rapid callus induction and plantlet regeneration from young leaves, internodal explants of *Solanum nigrum*. For *in vitro* callus induction auxins such as 2, 4-D, IAA and NAA in combination with cytokinin BAP were used. High frequency of green compact callus was obtained in leaf explants cultured on MS medium supplemented with 3.0 mg/l NAA+0.5 mg/l BAP. BAP or Kn alone or in combination with NAA and IAA was used for regeneration of plantlets from callus culture. High frequency and maximum number of multiple shoots were induced on MS medium supplemented with 3.0 mg/l BAP + 0.5 mg/l NAA. The best rooting response was observed on 0.5 mg/l IBA.

Zaidah and Nazri (2010) reported the effect of different growth regulators on shoot induction of *Orthosiphon aristatus*. Explants from stems were found to produce the best overall result with 0.2 mg/l BAP and 0.5 mg/l NAA but at a slower rate. The best result obtained from leaf explants was with 0.2 mg/l BAP which induces shoot faster than stem explants but at a smaller quantity. In comparison to the results from leaves explants, BAP with the same concentration using the stem explants gave a higher number of adventitious shoots. Stems are found to be a better explants source for regeneration of *O. aristatus*.

Shameer *et al* (2009) described an *in vitro* protocol for callus induction of *Beloperone plumbaginifolia* employing explants from node, internode, petiole, shootbud and leaf lamina. MS
medium supplemented with 5.37 µM NAA was better for calllogenesis from nodal and intermodal explant. MS medium with 5.37 µM NAA and 2.22 µM BA was found superior for shoot induction from nodal explants. Half- strength MS medium with 5.37 µM NAA induced adventitious roots and 85% plantlets survived when transferred in the field conditions.

Anburaj et al (2011a) reported that an efficient callus formation protocol was developed for medicinal plant *Cleome viscosa* by *in vitro* culture of leaf part of mature plant. The surface sterilization of explants (leaf) was observed from 3% hydrogen peroxide, 0.1%mercuric chloride and 70% ethanol. Callus formation was observed from five different culture media (B5, WPM, MS, SH, and Y3) devoid of plant growth regulators. Among those five culture media the MS medium was maximum of 59% callus response with an average of 84.1± 0.55 mg fresh mass was observed from leaf explants cultured on MS medium than others. Callus induction - Maximum regenerative callus biomass in the leaf explants was observed from IAA (2 mg/l).

Anburaj et al (2011b) established a protocol for the mass propagation of *Cleome viscosa* through in vitro organogenesis using leaf explants. IAA (2 mg/l) has a significant effect on the callus induction, callus index and callus physical appearance. The moderate response was observed with IBA (2 mg/l), and a low response observed with NAA (2 mg/l). 2 mg/l concentration of BAP significantly influenced the shoot proliferation. About 91% of rooting response was observed with NAA (0.1 mg/l).

Roy et al (2008) describes callus induction of the plant *Gymnema sylvestris* using internodal explants and the influence of different plant hormones like 2,4-D, kinetin, IAA, BAP on the growth of calli. Internodes were proved to be the best explant for culture, which were grown on MS basal medium (Murashige and Skoog, 1962) with different concentration of various growth regulators. The standard plant tissue culture protocol for callus culture and micropropagation was adopted. The highest efficiency of callus formation was observed in the medium containing different concentration of 2, 4-D and kinetin.

**Cell suspension culture**

The culture of tissues and cells cultured in a liquid nutrient medium produce a suspension of single cells and cell clumps, this is called suspension culture. Cell suspension culture in liquid medium is an important technique in plant tissue culture. It involves the large scale culture of isolated plant cells under conditions which induce them to synthesize the natural secondary
metabolites of the plants from which they were obtained. The techniques of callus culture and cell suspension culture have been reviewed in recent years, in particular from the viewpoint of studying biosynthesis of secondary metabolites. Biotechnologists are also trying to increase the synthesis of natural compounds or new compounds by higher plant cell culture as a result of mixing or feeding transformable precursors in the culture medium. Biotechnologists are also trying to augment the synthesis of medicinally important alkaloids in culture by means of a fungal elicitor (Dicosmo and Misawa, 1995).

There are numerous distinct advantages to producing a valuable secondary product in plant cell culture, rather than in vivo in the whole crop plant. Production can be more reliable, simpler, and more predictable. Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants. Compounds produced in vitro can directly parallel compounds in the whole plant. Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes. Secondary products in plant cell culture can be generated on a continuous basis there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather. In some cases, the yield per gram fresh weight may exceed that which is found in nature (Karuppusamy, 2009).

According to Mulabagal and Tsay (2004) they described the methods for callus and suspension culture for the production of bioactive secondary metabolites from medicinal plants. The evolving commercial importance of the secondary metabolites has in recent years resulted in a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted.

Leng and Keng, (2004b) studied successful callus induction from petiole, leaf and stem tissues of Orthosiphon stamineus cultured on MS medium containing different concentration of NAA (0 – 4 mg/l) and 2,4-D(0 – 2 mg/l). Highest fresh weight callus with best friability was obtained from leaf explants inoculated in MS medium supplemented with 1.0 mg/l 2,4-D plus 1.0 mg/l NAA. Cell suspension cultures were established from these cultures. The appropriate cell inoculum size was 0.75 g of cells in 20 ml culture medium. Cell suspension culture using MS medium supplemented with 1.0 mg/l 2, 4-D promoted the best cell growth with maximum biomass of 8.609 g fresh weight and 0.309 g dry weight 24 days after inoculation and the cells
grow in the suspension culture reached stationary phase in 15 days. MS medium supplemented with 1.0 mg/l 2, 4-D was considered as the maintenance medium for maintaining the optimum cell growth of *O. stamineus* in the cell suspension cultures with 2 week interval subculture.

Tan *et al* (2010) investigated the cell suspension culture response to different plant growth regulators (PRGs) for flavonoid production from elite cell line. Callus cultures were initiated from the leaf explants of *Centella asiatica* on Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-D, NAA, Dicamba, Picloram and IBA supplied singly and in combination with different concentrations of kinetin, BAP and TDZ. Callus induction was observed for all the plant growth regulators tested. The highest callus induction frequency (86.67%) was observed in MS medium containing 2.0 mg/l 2,4-D while the combination of 2.0 mg/l 2,4-D and 1 mg/l kinetin in MS medium gave the highest biomass yield (0.27 g dry weight culture/l). This combination was also found to be best for callus proliferation for all the accessions investigated. The flavonoids present in the four accessions were quercetin, kaempherol, luteolin and rutin based on High Performance Liquid Chromatography (HPLC) analysis. These results indicated that *C. asiatica* accession UPM03 was the potential elite cell line in mass production of flavonoid, especially luteolin.

Hakkim *et al* (2011) investigated the Rosmarinic acid accumulation in cell suspension cultures of *Ocimum sanctum* (L.). Callus was initiated from leaf explant on MS medium supplemented with 2, 4-D and kinetin. Suspension cultures were established by transferring friable callus to MS liquid medium supplemented with different growth regulators in different combinations. The highest Rosmarinic acid (104 mg/l) content and biomass growth (17.8 g/l) was observed in the cultures supplemented with 2, 4-D (1 mg/l) and kinetin (0.1 mg/l). It was twofold higher than that found in leaf callus induced on MS solid medium. RA formation was paralleled with cell growth. RA was quantified using Reverse Phase High Performance Liquid Chromatography (RPHPLC) with reference standard. RA was isolated from suspension harvested biomass and characterized by spectral analysis.

According to Cheng *et al* (2006) an efficient procedure has been developed for callus induction and cell suspension cultures of *Corydalis saxicola*. Leaf explants thus selected showed maximum response to callus induction (67.1%). Modified B5 medium supplemented with 0.5 mg/l 2,4-D plus 2 mg/l BA was the most favorable medium for callus formation with the highest induction rate (94.8%) and greatest fresh weight of callus (1.7 g per explant). Cell suspension
cultures were established by transferring 2 - 8 g fresh callus to 80 ml liquid B5 medium. An inoculum size of 8 g produced the greatest biomass accumulation, dehydrocavidine and berberine productions, which was 13.1 g/l, 8.0 mg/l and 4.1 mg/l, respectively.

Keng et al (2010) described the effect of different elicitor on cell biomass and alkaloid production in Eurycoma longifolia cells. Different concentration of chitosan, NaH$_2$PO$_4$, Na$_2$CO$_3$ and polyvinylpyrrolidone (PVP) were added as elicitor to optimize cell biomass and alkaloid production. MSBs medium supplemented with 100 mg/l chitosan induced significant increment in the cell biomass while higher amount of chitosan (150 mg/l) induced the highest production of 9-hydroxycanthin-6-one. The addition of 2 mg/l and 20 mg/l NaH$_2$PO$_4$ induced the highest increment in cell biomass and alkaloid production respectively. However, addition of different concentration Na$_2$CO$_3$ and PVP showed inhibitory effect on cell growth with no significantly increased in alkaloid production.

Liang et al (2006) established the cell suspension cultures of Orthosiphon stamineus from friable calluses produced from leaf pieces of in vitro plantlets that were derived from nodal segments of the mother plants collected from three different geographical locations. All cell lines grew well in liquid MS medium supplemented with 4.5 mM 2, 4-D and 5.4 mM NAA. All cell lines exhibited the same growth pattern but produced different maximum cell biomass when cultured in this medium. The time of harvesting the plant cells from the culture medium and the geographical source of the original plant material were both found to affect the production of Rosmarinic acid (RA) in cell cultures. Two cell lines were successfully selected and identified to produce high amounts of RA. These cell lines were a fast-growing cell line from Air Itam, Penang and an intermediate-growing cell line from Relau Agriculture Research Centre, Penang which could produce 5% [(w/w) dry weight] and 4.5% [(w/w) dry weight] of RA, respectively.

Mehrabani et al (2005) investigated the callus culture of Echium amoenum and its major secondary metabolites. The callus culture of E. amoenum was initiated and established from seeds in MS media with three different ratios of plant growth regulators: kinetin, 2, 4-D and NAA. Methanolic extracts of freeze-dried calluses were compared by TLC and HPLC. The major secondary metabolite was separated by preparative HPLC and the structure of this pure compound was elucidated by UV, IR, one and two dimensional 1H and $^{13}$C-NMR and Mass spectroscopy. Rosmarinic acid was identified by various spectroscopic methods from callus culture of E.
Rosmarinic acid is widespread within the plant cell tissue culture of the Lamiaceae and Boraginaceae families, although in insignificant quantities.

Li et al (2011b) studied the effects of the time of addition and polysaccharide concentration on the growth and diosgenin accumulation in cell suspension culture of *D. zingiberensis*. Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), were prepared from the endophytic fungus *Fusarium oxysporium Dzf17* isolated from the rhizomes of *Dioscorea zingiberensis*. Among them, WPS was found to be the most effective polysaccharide. When WPS was added to the medium at 20 mg/l on the 25th day of culture, the cell dry weight was increased 1.34-fold, diosgenin content 2.85-fold, and diosgenin yield 3.83-fold in comparison to those of control.

Tang et al (2011) examined the effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*. Endophytic fungus and its elicitors were inoculated in cell suspension cultures of *C. roseus* respectively. The biochemical change of suspension cells was then tested. The results showed when the pH rises, the concentration of malonic dialdehyde increases as the activities of antioxidative enzymes (peroxidase and catalase) rises. The critical enzymes of alkaloid synthesis include phenylalanine ammonia-lyase and tryptophan decarboxylase in suspension cells were also found to increase, along with the alkaloid yield rises. The alkaloid yields reached 770.36 693.76 and 693.76 μg/gFW in the co-culture and elicitor-induced groups, which are 48 and 32% higher than the control group, respectively.

**Plant secondary metabolites**

According to Wyk and Wink, (2004) plants with secondary metabolites have been used by humans to treat infections, health disorders and illness since the early days of mankind. Many higher plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavor and aroma industries. On a global scale, medicinal plants are mainly used as crude drugs and extracts. Several of the more potent and active substances are employed as isolated compounds, including many alkaloids such as morphine (pain killer), codeine (antitussive), papaverine (phosphodiesterase inhibitor), ephedrine (stimulant), ajmaline (antirrhythmic), quinine (antimalarial), reserpine (antihypertensive), galanthamine (acetyicholine...
esterase inhibitor), scopolamine (travel sickness), berberine (psoriasis), caffeine (stimulant), capsaicin (rheumatic pains), colchicines (gout), yohimbine (aphrodisiac), pilocarpine (glaucoma), and various types of cardiac glycosides (heart insufficiency) (Wink et al., 2005).

Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolites found in plants. The techniques used to analyze the phytochemicals produced by plants were; Thin layer chromatography (TLC), Gel (column) chromatography, High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Mass spectrometry and Nuclear Magnetic Resonance (NMR).

Tsichritzi et al (1993) reported that an extract of the aerial parts of Cleome africana afforded in addition to known compounds two triterpenes of the dammarane type. The structures were elucidated using high field NMR spectroscopy.

Nagaya et al (1997) isolated eighteen dammarane-type triterpenes from the whole plant of Cleome africana by means of cytotoxic bioassay-directed fractionation. Twelve of them were novel compounds whose structures were elucidated by various spectroscopic methods. These dammarane triterpenes have significant cytotoxic activity against P388 leukaemia cells. Compounds 2, 4, 6, 10, 12 and particularly 13 exhibited potent activity; however, the relationship between structure and cytotoxic activity could not be deduced.

Das et al (1999) studied the Chemical examination of Cleome gynandra (whole plant excluding seeds) and isolated and identified a novel (20S, 24S)-epoxy-19, 25-dihydroxydammarane-3-one hemiketal (I). The structure of the new compound, designated as cleogynol, was determined using IR, $^1$H and $^{13}$C NMR spectral analysis and chemical methods.

Tezuka et al (2000) reported that they isolated, five new isopimarane-type diterpenes [orthosiphols F-J (1-5)] and two new diterpenes [staminols A (6) and B (7)] with a novel carbon framework, named as “staminane”, and three new highly-oxygenated staminane-type diterpenes [staminolactones A (8) and B (9) and norstaminol A (10)] from the MeOH extract of the aerial part of Vietnamese Orthosiphon stamineus. Together with these new diterpenes, sixteen known compounds were also isolated and identified as: 7,39,49-tri-O-methyluteolin (11), eupatorin (12), sinensetin (13), 5-hydroxy-6,7,39,49-tetramethoxyflavone (14), salvigenin (15), ladanein (16), tetramethylscutellarein (17), 6-hydroxy-5,7,49-trimethoxyflavone (18), vomifoliol
(19), aurantiamide acetate (20), Rosmarinic acid (21), caffeic acid (22), oleanolic acid (23), ursolic acid (24), Betulinic acid (25), and b-sitosterol (26). All the isolated compounds were tested for their cytotoxicity towards highly liver metastatic murine colon 26-L5 carcinoma cells, and the new diterpenes, except for 4, and flavonoids (11, 12, 16, and 18) showed cytotoxicity with an ED50 value between 10 and 90 mg/ml.

Awale et al (2001) reported five novel highly oxygenated diterpenes, orthosiphols K (1), L (2), M (3), and N (4) and norstaminone A (5), were isolated from the aerial part of Orthosiphon stamineus, together with three known diterpenes, orthosiphols A (6) and B (7) and neoorthosiphol A (8). All the isolated compounds showed mild to weak antiproliferative activities toward highly liver metastatic colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cell lines.

Awale et al (2002) reported nine highly oxygenated and structurally diverse diterpenes, named norstaminolacetone A (1), Norstaminols B and C (2 and 3), secoorthosiphols A-C (4-6) and orthosiphols R-T (7-9) have been isolated from the aerial part of Orthosiphon stamineus cultivated in Okinawa Prefecture, Japan.

Awale et al (2003) reported that they were isolated nine new highly-oxygenated isopimarane-type diterpenes [7-O-deacetylorthosiphol B (1), 6-hydroxyorthosiphol B (2), 3-O-deacetylorthosiphol I (3), 2-O-deacetylorthosiphol J (4), siphonols A—E (5—9)] together with nine known diterpenes [orthosiphols H (10), K (11), M (12) and N (13); staminols A (14) and B (15); neoorthosiphols A (16) and B (17); norstaminol A (18)] from the methanolic extract of Indonesian Orthosiphon stamineus.

Hossain and Ismail, (2005) reported that Orthosiphonic acid 1, a new lupine-type triterpene isolated from the leaves of Orthosiphon stamineus by thin layer chromatography, and it has been identified as 16β-hydroxybetulinic acid by GC coupled with mass spectrometry, UV, IR and 1H NMR spectroscopy.

Triterpenes belong to the biologically versatile group of terpenes, which are composed of isoprene subunits and consist of approximately 30,000 identified compounds (Dzubak et al 2006). In relation to the number of isoprenoid units, terpenes are subdivided into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterpenes (C25), triterpenes (C30), and tetraterpenes (C40), where the carbon skeleton is acyclic, or contains mono-, bi-, tri-, tetra-, and pentacyclic structures, respectively. Among these are included the pentacyclic lupane-type triterpenes which
are represented by a diverse assemblage of bioactive natural products. 3b-Hydroxy-lup-20(29)-en-28-oic acid (Betulinic acid), a C-28 carboxylic acid derivative of the ubiquitous triterpene betulin, is a member of the class of lupanetype triterpenes. However, unlike betulin, the oxidized derivative Betulinic acid possesses a number of intriguing pharmacological effects including anti-inflammatory, anticancer, and anti-HIV activities.

Fang et al (2006) isolated 9 different compounds from an ethyl acetate-soluble extract of the stem bark of Dichapetalum gelonioides. Using the LNCaP (hormone-dependent human prostate) cell line as a monitor, led to the purification of three dichapetalin-type triterpenoids [dichapetalins A (1), I (2), and J (3)], along with two dolabrane norditerpenoids (6, 7), and the additional triterpenoids zeylanol (8), 28-hydroxyzeylanol (9), and Betulinic acid. A recollection of the plant material was carried out, in order to obtain more of these compounds for additional biological testing. Two further phenylpyranotriterpenoids [dichapetalins K (4) and L (5)] were isolated from the recollected plant material. The structures of the new compounds 2–5 and 9 were determined based on spectroscopic data interpretation, and the relative configuration of 6 was confirmed using X-ray crystallography.

The phytochemical screening of Gynandropsis pentaphylla revealed the secondary metabolites which are of medicinal interest. G. pentaphylla is rich in tannin, sugar, essential oils, amino acid and phenolic group. However, in G. pentaphylla seeds extract (both benzene and ether solvents) contained tannins, alkaloids, flavones, sugar, phenolic group, saponin, amino acid and essential oil in plenty of amounts. Flavones and saponins were comparatively less in this species extracted using both benzene and ether solvents. The variance in the quantitative composition of the secondary metabolite establishes the fact that different plant parts are not likely to have the same medicinal potential. Leaf contained all the secondary metabolites screened (Borgio et al., 2008).

**Fungal endophytes**

Recent studies have shown that fungal endophytes are ubiquitous in plant species (Petrini et al., 1992; Espinosa-Garcia and Langenheim, 1990; Schulz et al., 1993; Fisher 1996; Faeth and Hammon, 1997). Endophytic fungi infect and inhabit predominantly the aerial tissues of the host plant without causing detectable symptoms. The relationships between endophytes and their host plant are thought to be symbiotic, such as that endophytes obtain nutrients and protection from the
host but contribute to effective host defense against pathogens, herbivores or abiotic stress (Saikkonen et al., 1998). Globally, there are at least one million species of endophytic fungi in all plants which can potentially provide a wide variety of structurally unique, bioactive natural products such as alkaloids, benzopyranones, chinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou, 2001).

According to Nalini et al (2005) fungal endophytes were isolated from Crataeva magna, a medicinal plant growing along the streams and rivers, constituting riparian vegetation in Karnataka, southern India. Fresh bark and twig pieces were used for the isolation using standard methods. Ninety-six endophytic fungal isolates were isolated from 800 bark and twig segments. Mitosporic fungi represented as a major group (85%) followed by zygomycetes (10%) and ascomycetes (5%). Bark samples contained more endophytes than twig samples. Verticillium, Nigrospora oryzae and Fusarium verticilloides were the dominant fungal endophytes.

Raviraja (2005) reported on the fungal endophytes in five medicinal plant species from Kudremukh Range, Western Ghats of India. Nearly eighteen species of endophytic fungi were isolated from bark, stem and leaf segments of five medicinal plant. The dominant species were Curvularia clavata, C. lunata, C. pallescens and Fusarium oxysporum. The highest species richness as well as frequency of colonization of endophytic fungi was found in the leaf segments, rather than the stem and bark segments, of the host plant species. The greatest number of endophytic fungal species were found within Callicarpa tomentosa (11 species), whereas Lobelia nicotinifolia harbored the lowest number of fungal endophytes (5 species). The study provides evidence that fungal endophytes are host and tissue specific.

Gangadevi and Muthumary (2007) studied on endophytic fungal diversity in young, old and senescent leaves of Ocimum basilicum L., a medicinal plant. This study provides the first report on diversity of endophytic fungi of medicinal plants from Chennai city, Southern India. Added to it, one of the isolates, Phyllosticta sp.6, was found to produce taxol in artificial culture media. The endophytic fungus is thus expected to be a potential source of natural bioactive agent.

Fungal elicitor prepared from pathogenic microorganism can be added into plant cell suspension to enhance target substances. For example, Candida albicans and Staphylococcus aureus elicitors increased the production of bilobalide and ginkgolides in Ginkgo biloba cell suspension cultures (Kang et al., 2009).
All plants in natural ecosystems appear to be symbiotic with fungal endophytes. This highly diverse group of fungi can have profound impacts on plant communities through increasing fitness by conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption, or decreasing fitness by altering resource allocation. Historically, two endophytic groups (Clavicipitaceae (C) and Nonclavicipitaceae (NC)) have been discriminated based on phylogeny and life history traits. Here, Rodriguez et al (2009) showed that NC-endophytes represent three distinct functional groups based on host colonization and transmission, *in planta* biodiversity and fitness benefits conferred to hosts.

Endophytic fungi isolates from foliage and sapwood of *Hevea brasiliensis* were studied to determine the total diversity of endophytes inhabiting leaves and sapwood, and differences between respective endophyte communities found in leaves and sapwood. Endophytes were recovered from 72% (161) of the 225 samples, with a total of 175 isolates. Ascomycota was dominant, representing almost 97% of the isolates. In contrast, Basidiomycota and ‘Zygomycota’ were represented by 1% and 2%, respectively. Among the genera isolated *Penicillium*, *Pestalotiopsis* and *Trichoderma* were the most frequently isolated. A greater diversity of endophytes was found in sapwood than in leaves. However, endophytic colonization frequency was greater in leaves than in sapwood. Comparisons between leaves and sapwood demonstrated a spatial heterogeneity in endophyte assemblages among plant parts and sites (Gazis and Chaverri, 2010).

Both pathogenic and non-pathogenic fungi have been employed as the preparation sources of fungal elicitors. Fungal endophytes, being non-pathogenic fungi, have attracted more and more attention, because of protective effects towards pathogens and herbivores of their host plants (Zhao et al., 2010). Fungal elicitor has become one of the most important and successful strategies to improve secondary metabolite production in plant cell culture. Gao et al (2011) reported that endophytic fungal elicitor promotes terpenoids biosynthesis and biomass of plant cultures.Treating *Euphorbia pekinensis* suspension cultures with endophytic fungal elicitor from *Fusarium* sp. E5 resulted in accumulation of isoeuphipenensin and euphol, activated defense-related enzymes, and increased biomass. The elicitor was prepared from extract of fungal endophyte *Fusarium* sp. E5 mycelium, and added into 21 day-old cell suspension cultures. The results showed that the biomass of culture after elicitor treatment was increased by 19.35%,
whereas the isoephupkensin and euphol contents were 5.81 and 3.56 times greater than those of the control.

Based on the research done by Tong et al (2011) a total of 72 endophytic fungal isolates including 48 from leaves, 14 from stems, 6 from roots and 4 from flowers were obtained from medicinal herb, Orthosiphon stamineus by adding host plant materials in isolation medium. Sixty-six (92%) of the 72 isolates exhibited significant inhibitory activity on at least one of the test microorganism. The endophytic fungi were most prevalent (67%) in the leaves and were least prevalent in the flowers. Beside thin cuticle layer, mature leaves growing to about 12 cm in length and about 7 cm in width, provide a large surface area for fungal penetration. The least number of isolates from flowers was thought to be due to wilting of flowers within a few days, therefore it received lower amount of inoculum.

Li et al (2011b) studied the effects of the time of addition and polysaccharide concentration on the growth and diosgenin accumulation in cell suspension culture of Dioscorea zingiberensis. Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), were prepared from the endophytic fungus Fusarium oxysporium Dzf17 isolated from the rhizomes of Dioscorea zingiberensis. Among them, WPS was found to be the most effective polysaccharide.

**Elicitor induced production of secondary metabolites**

Compounds that induce the production and accumulation of secondary metabolite in plants are known as elicitors. Elicitation is characterized by enhancement of secondary metabolite production with the elicitors which are classified as abiotic or biotic, depending on their origin. Abiotic elicitors include ultraviolet irradiation, salts of heavy metals and other chemicals, while biotic elicitors refer to the substances obtained mainly from plants or microorganisms (Zhao et al., 2010). These elicitors when added to medium in low concentration enhance the metabolite production. Nowadays, employment of fungal preparations as elicitors has become one of the most important and successful measures to enhance secondary metabolite production in plant cell cultures (Schulz et al., 2002).

Ogata et al (2004) studied the drastic increase in Rosmarinic acid (RA) accumulation, induced in cultured cells of Lithospermum erythrorhizon after their exposure to yeast extract or methyl jasmonate. Addition of the elicitors to the cell cultures only slightly enhanced the activity
of Rosmarinic acid synthase, which catalyzes formation of 4-coumaroyl-4-hydroxyphenyllactic acid (CHPL), the first reaction specific to RA biosynthesis. In contrast, the two-step cytochrome P450-catalyzed hydroxylations of CHPL to form RA were dramatically up-regulated by the elicitor treatments, indicating that these hydroxylation activities are likely to play a key regulatory role in elicitation of RA biosynthesis.

Wang et al (2004) studied the induction by Methyl Jasmonate (MJ) and salicylic acid (SA) of taxol and relevant taxane biosynthesis in suspension cultures of Taxus chinensis var. mairei both theoretically and experimentally. The theoretical model shows that the apparent number of elicitor molecules binding with hypothetical receptor molecules for MJ in inducing taxol biosynthesis is about 75% lower than that for salicylic acid. The apparent binding constant between the elicitor and hypothetical receptor molecules for MJ is 10 orders of magnitude higher than that for SA. MJ increased taxol production more significantly than did SA as observed in our experiments. The induction model is able to predict induction efficiency of an elicitor. SA might apparently increase taxol production by blocking the biosynthesis pathway from baccatin III to cephalomannine, based on the observation that SA promotes cephalomannine production.

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao et al., 2005). Therefore, the treatment of plant cells with biotic and abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extract, Methyl Jasmonate and chitosan (Karuppusamy, 2009).

The effect of different concentrations of Methyl Jasmonate (MJ) was studied on embryogenic cell growth of Eleutherococcus senticosus. The accumulation of eleutherosides and chlorogenic was tested in and results revealed that addition of 200 µM MJ was suitable for optimum accumulation of eleutheroside B, E, E1 (7.3 fold increase) and chlorogenic acid (3.9 fold increase). However, addition of MJ at higher concentration (above 100 µM) was detrimental for biomass accumulation (Shohael et al., 2007).

According to Taha et al (2009) Catharanthus roseus is still the only source for the powerful antitumor drugs vinblastine and vincristine. Calli Subculture had been done on MS-medium containing 1 mg/l kin. Effect of mannitol as abiotic stress at the concentrations 0, 2000,
4000 or 8000 ppm or *Aspergillus niger* as biotic stress at the concentrations 0, 0.05, 0.15 and 0.25% on calli growth parameters, achievement and production of vinblastine and vincristine was investigated. Supplementation of MS medium with 8000 ppm mannitol or 0.25% of *Aspergillus niger* resulted the highest value of total alkaloids, vinblastine and vincristine production. The best results of calli growth parameters as well as enhancement the biosynthesis is of indole alkaloids were recorded with leaf stem and root calli cultures, respectively.

Kuzma *et al* (2009) studied effect on diterpenoid Methyl Jasmonate (MJ) accumulation in *Salvia sclarea* hairy root culture in shake flasks and sprinkle bioreactor. MJ concentration and exposure time to the elicitor were factors that strongly affected the diterpenoid production. The highest diterpenoid accumulation (67.5±7.1 mg/g dry weight, calculated as a sum of ferruginol, salvipisone, aethiopinone and 1-oxoaethiopinone) without reduction of biomass was achieved, when the 23-day-old hairy roots in bioreactor culture were exposed to 125 µM MJ for 7 days. The roots produced 9 and 3.8 times as much aethiopinone (40 ± 5.9 mg/g dry weight) and salvipisone (12.6 ± 0.4 mg/g dry weight), respectively, as roots cultured in shake flasks.

Fan *et al* (2011) Carbon and nitrogen status, as well as triterpenoid production were investigated in cell suspension cultures of *Betula platyphylla* Suk. treated with fungal elicitor. The fungal elicitor was observed to inhibit the cell growth and enhanced triterpenoid production. The maximum triterpenoid production was 123.82 mg/l DW in the 1-day elicitated cells, which was twofold higher than that of the control. The response of carbon and nitrogen status to the fungal elicitor was 18.62% decreased sucrose, 307.79% decreased soluble proteins, 146.93% increased total soluble sugar, 484.54% increased glucose, slightly increased total amino acids and 906.96% (about 9-fold) increased C/N ratio in the 1-day elicitation, which slowly equals the control with increasing incubation time. These results indicate that changes in carbon and nitrogen status were activated or were partially directed towards triterpenoid production, especially C/N ratio.

Li *et al* (2011a) investigated the effects of the oligosaccharides from the endophytic fungus *Fusarium oxysporum* Dzf17 as elicitors on diosgenin production in cell suspension cultures of its host *Dioscorea zingiberensis*. Three oligosaccharides, DP4, DP7 and DP10, were purified from the oligosaccharide fractions DP2-5, DP5-8 and DP8-12, respectively, which were prepared from the water-extracted mycelial polysaccharide of the endophytic fungus *F. oxysporum* Dzf17. DP7 at 6 mg/l was found to significantly enhance diosgenin production, with a yield of 3.202 mg/l, which was 8.27-fold of control. When the cell cultures were treated with DP7
twice on days 24 and 26, and harvested on day 30, both diosgenin content and yield were significantly increased and reached the maximums of 1.159 mg/g dw and 4.843 mg/l, both of which were higher than those of single elicitation, and were 9.19 and 12.38-fold of control, respectively.

**Purification of secondary metabolites**

High performance liquid chromatography protocols have been developed to allow the purification and analysis of a very wide range of soluble aromatic secondary metabolites in unfractionated biological extracts. High resolution of metabolites is achieved in 25 minutes by chromatography on a reverse phase C18 column in a gradient of 0 to 55% acetonitrile in water at pH 3. For example, near-baseline resolution of over 20 phenylpropanoid metabolites and 18 naturally occurring metabolites of indole-3-acetic acid can be obtained. The methods can be applied directly to whole tissue extracts without prepurification or enrichment. Moreover, the simplicity and sensitivity of the protocols allow their application to a large number of very small tissue samples, such as those encountered in research on host-microbe interactions. Such profiles allow one to monitor simultaneously the various alternative metabolic fates of a complex array of molecules. Examples of the application of the protocols to a number of plant and microbial secondary product investigations and to screening for flavonoid mutants of *Arabidopsis thaliana* (L.) Heynh. have been studied by Graham (1991).

A preparative HPLC procedure to isolate and purify picrocrocin, the compound responsible for the taste of saffron and precursor of the aromatic safranal, and the mixture of yellow pigments from a saffron hydroalcoholic extract has been developed. A reversed-phase C18 column was employed as the stationary phase. The best separation was obtained with 45% methanol, plus a 90% acetonitrile pulse 3 min after starting the run, as mobile phase. To obtain the highest yield from the system, sample size was increased up to 2 ml of 200 mg/ml saffron extract; under such conditions a good resolution was obtained and picrocrocin and saffron pigments were separated with a high purification yield and purity (Castellar *et al.*, 1993).

Secondary metabolites include a very wide variety of compounds with different structures and chemical properties. In order to obtain an overview of the secondary metabolite content of a plant species such as *Lotus japonicus*, a profiling technique coupling sequential extraction with different chromatographic methods (GC-MS, HPLA-DAD/MS) was established. This method
allows the qualitative analysis of ionic (charged) compounds such as alkaloids and non-ionic (neutral) compounds such as terpenoids and phenolic compounds. This technique can also be used for the isolation of new compounds (Rispail et al., 2005).

Centrifugal liquid-liquid partition chromatography presents significant advantages for the separation and purification of plant metabolites owing to the short operational time of the process and the elimination of possible irreversible adsorption of compounds. The crude chloroform extract from roots of Plumbago auriculata was analysed by countercurrent chromatography using hexane: ethyl acetate: methanol: water (40:10:10:2, v/v) as solvent system. The isolation of the naphthoquinones plumbagin and epi-isoshinanolone, the steroids sitosterol and 3-O-glucosylsitosterol, plumbagic and palmitic acids was easily achieved. Naphthoquinones are typical components of Plumbago species and they show interesting biological activities (De Paiva et al., 2005).

Hussain et al (2012) developed a simple and rapid method for the separation and determination of Betulinic acid from a complex matrix, extracts of Orthosiphon stamineus, using a combination of the two techniques. A few studies are reported about using the combination of TLC and RP-HPLC for the separation and determination of analyte(s) from a complex matrix. The samples having higher contents of the analyte and fewer interfering species were prepared using TLC. The samples were then eluted through C18 column using isocratic solvent system comprising acetonitrile, methanol and acetic acid acidified water of pH 2.8 in a ratio of 70:20:10 (v/v/v), respectively, and detection was carried out at 210 nm. The method was validated and applied successfully to quantify Betulinic acid in various types of extracts of the plant.

Bandoniene et al (2005) separated and identified Rosmarinic acid on the basis of high performance liquid chromatography (HPLC)–UV–mass spectrometry data in 80% methanol in water extracts from the leaves of Salvia species (S. officinalis, S. glutinosa, S. aethiopis, S. sclarea, and Borago officinalis) as a dominant radical scavenger towards the 2, 2’- diphenyl-1-picrylhydrazyl (DPPH) stable radical in HPLC–DPPH system. The content of Rosmarinic acid in the plants is calibrated and quantitated from chromatograms obtained by UV detection at 280 nm. The concentration ranges from 13.3 to 47.3 mg of the phenolic acid per gram dried leaves of all plants is tested. S. glutinosa and S. sclarea have the highest concentration of Rosmarinic acid.
Taralkar and Chattopadhyay (2012) developed a method for determination of Ursolic acid and Betulinic acid from their methanol extract of *Vitex negundo* Linn leaves. Analysis was carried out using Waters’ symmetry C-18 column with acetonitrile: methanol (80:20) as isocratic elution mode with UV detection (λ=210nm). The method is pretty linear for ursolic acid in the range of 0.01-0.1 mg/ml (R² = 0.9961) and for Betulinic acid in the range of 0.003-0.018 mg/ml (R² = 0.999). The method was validated by mixing these acids standards in methanol and found that is accurate, sensitive and has a good reproducibility.

Hossain and Ismail (2009) performed on extraction of *Orthosiphon stamineus*, Benth by using different solvent for the identification and quantification of the caffeic acid derivatives such as caffeic acid and Rosmarinic acid which confers to the leaves of this plant with remarkable pharmaceutical properties. High Performance Thin-Layer Chromatographic (HPTLC) allows the identification and the quantification of more than 20 samples in the same chromatographic run. The analysis of the samples requires 15-30 min compared with more than 2 h using a typical HPLC method. Using the techniques of the HPTLC and the UV-VIS spectra we have found that the extraction of this herb plant contain, the caffeic acid and Rosmarinic acid ranging between 0.029% up to 0.506% and up to 0.24% to 2.24% respectively.

Mukherjee *et al* (2010) established a simple, sensitive, reliable, rapid and validated high-performance thin-layer chromatography method for estimation of Betulinic acid in hydro-alcoholic extract of *Nelumbo nucifera* (Nymphaeaceae) rhizome. The system was found to produce a compact spot for Betulinic acid (R(f) = 0.30). A good linear precision relationship between the concentrations (2-10 μg) and peak areas were obtained with the correlation coefficient (r) of 0.99698. This validated HPTLC method provides a new and powerful approach to estimate Betulinic acid as phytomarker in the extract.

Ondrejovic *et al* (2012) studied evaluation of the solid-phase extraction for elimination of interference compounds from lemon balm extracts aimed for photometric determination of Rosmarinic acid. The results indicated that interfered compounds were eliminated. The lemon balm extracts should be pretreated by adsorption on normal stationary phase (silica gel) in ratio sample volume to silica gel weight 1:1 (v/w), elution by mobile phase diethyl ether: acetic acid (9:1; v/v) – volume - 40 times of crude extract volume with flow rate 5 ml/min. After selection of Solid-phase extraction conditions, the method was validated with comparison to HPLC analysis.
The results suggest that this method may be useable for determination of Rosmarinic acid by photometric measurement based on the complexation of Fe$^{2+}$ ions with Rosmarinic acid.

**Betulinic acid and its biological activity**

Betulinic acid is a novel anticancer drug and induces apoptosis and hence differs from “Classical” anticancer agents such as doxorubicin (Fulda et al., 1997). Betulinic acid is a prototype cytotoxic agent that triggers apoptosis by a direct effect on mitochondria (Fulda et al., 1998). In isolated mitochondria, Betulinic acid directly induces a loss of transmembrane potential independent of a benzyloxy carbonyl-Val-Ala-Aspfluoromethyl ketone inhibitable caspase. These finding show that the induction of mitochondrial PT alone is sufficient to trigger the full apoptosis program and that Betulinic acid may induce apoptosis via a direct effect on mitochondria.

The lupane-type triterpene Betulinic acid is found widely throughout the plant kingdom. Hundreds of published reports have described the occurrence of Betulinic acid across a multitude of taxonomically diverse genera. Furthermore, given the widespread occurrence of the structurally related precursor betulin among plants, it is conceivable that the distribution of Betulinic acid is even much greater. One of the most widely reported sources of Betulinic acid is the birch tree (*Betula* sp., Betulaceae) where both Betulinic acid and Betulin can be obtained in substantial quantities (Cole et al., 1991 and Galgon et al., 1999). Other known sources of Betulinic acid include *Ziziphus* sp. (Rhamnaceae), *Syzygium* sp. (Myrtaceae), *Diospyros* sp. (Ebenaceae), and *Paeonia* sp. (Paeoniaceae). A multitude of extraction and isolation schemes have been used for the procurement of Betulinic acid and other related triterpenoids (Chang et al., 1999).

Selzer et al (2000) studied the effect of Betulinic acid alone and in combination with irradiation in human melanoma cells. Betulinic acid strongly and consistently suppressed the growth and colony forming ability of all human melanoma cell lines. In combination with ionizing radiation, the effect of Betulinic acid on growth inhibition was additive in colony-forming assays. Betulinic acid also induced apoptosis in human melanoma cells as demonstrated by Annexin V binding and by the emergence of cells with apoptotic characteristics and was more pronounced in human melanoma cell lines than in normal human melanocytes.

Betulinic acid is a white crystalline solid that exhibits limited solubility in organic alcohols such as MeOH and EtOH, CHCl$_3$, and ether. Betulinic acid has low solubility in H$_2$O,
petroleum ether, DMF, DMSO, and benzene. However, Betulinic acid is highly soluble in pyridine and acetic acid (Cichewicz and Kouzi, 2004).

Yogeeswari and Sriram (2005) reviewed the biological properties of Betulinic acid and its derivatives. Betulinic acid is a naturally occurring pentacyclic triterpenoid and has been shown to exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties.

White birch bark, *Betula alba* (which contains Betulinic acid) has been used by Native Americans as a folk remedy. They used it in tea and other beverages to treat stomach and intestinal problems such as diarrhea and dysentry. In Russia, it has been reportedly used since 1834. In 1994, scientists at the University of North Carolina reported that chemicals found in white birch bark slowed the growth of Human Immunodeficiency Virus (HIV). A researcher at the University of Illinois reported that Betulinic acid killed melanoma cells in mice. Since then, a number of researchers have conducted laboratory tests on Betulinic acid to determine antitumor properties, especially with respect to melanoma cells with some promising results which may warrant future study. Betulinic acid has recently been selected by the National Cancer Institute for addition into the RAID (Rapid Access to Intervention in Development) program (Pisha *et al*., 1995).

Smith *et al* (2007) studied the Safety, Virologic Effect, and Pharmacokinetics of Single-Dose 3-O-(3,3-Dimethylsuccinyl) Betulinic Acid (Bevirimat) against Human Immunodeficiency Virus Infection. Bevirimat [3-O-(3,3-dimethylsuccinyl) Betulinic acid] is the first in a new class of anti-human immunodeficiency virus (HIV) drugs that inhibit viral maturation by specifically blocking cleavage of the Gag capsid (CA) precursor, CA-SP1, to mature CA protein, resulting in defective core condensation and release of immature noninfectious virions. This disruption to Gag protein processing results in defective core condensation and the release of noninfectious virus particles and it blocks the spread of the infection to new cells. Bevirimat has demonstrated potent *in vitro* and in vivo activities, with an *in vitro* 90% inhibitory concentration of 22.1 ng/ml (37.8 nM), and retains activity against viruses that are resistant to other classes of antiretrovirals.

Theo *et al* (2009) identified Betulinic acid from the ethyl acetate fraction of *Peltophorum africanum* (fabaceae). The stem bark of *P. africanum* has been traditionally employed to treat diarrhea, dysentery, sore throat, wounds, HIV/AIDS, venereal diseases and infertility. The ethyl
acetate fractions which contains Betulinic acid, showed the highest selective index. Butanol and ethyl acetate extracts of the stem bark were screened for their inhibitory activities against HIV-1 using MAGI CCR5+ cells, which are derived from HeLa cervical cancer cells. Extracts of the stem bark of *Peltophorum africanum* showed inhibitory activity against HIV-1, CXCR4 (X4) and CCR5 (R5) tropic viruses. From these studies they reported that Betulinic acid is the predominant anti-HIV-1 constituent of *Peltophorum africanum*.

**Rosmarinic acid and its biological activities**

Park *et al* (2008) reviewed the possible biotechnological ways to produce Rosmarinic acid, thus will help the scientists to take action for future study in this discipline. Rosmarinic acid, an important phenolic compound, is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae. Rosmarinic acid has a number of interesting biological activities, e.g. antiviral, antibacterial, anti-inflammatory, and antioxidant. The presence of Rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects. The biosynthesis of Rosmarinic acid starts with the amino acids phenylalanine and tyrosine. Plant cell cultures, e.g. from *Coleus blumei* or *Salvia officinalis*, accumulate Rosmarinic acid in amounts much higher than in the plant itself (up to 36% of the cell dry weight). Similarly some other biotechnological researches for production of Rosmarinic acid were done from shoot culture, producing hairy root, using bioreactor, and the treatment of elicitors.

According to Akowuah *et al* (2004) More than twenty phenolic compounds were isolated from *Orthosiphon stamineus* including lipophilic flavones, flavonol glycosides and caffeic acid derivatives such as Rosmarinic acid and 2, 3-dicaffeoyltartaric acid, were identified and quantified by HPLC. Recently reported a rapid, quantitative and simultaneous HPLC-based determination of major phytochemicals from the extract of *O. stamineus* leaves and reconfirmed its strong antioxidant potency and total phenolic content.

Toth *et al* (2003) developed a simple HPLC method to determine the content of lemon balm (*Melissa officinalis* L.) Rosmarinic acid is known for its antiviral and antioxidative properties. The influence of the plant development phase at harvest time on the content of Rosmarinic acid was studied in *Melissa* leaf samples of Slovak origin. Only slight variability of Rosmarinic acid content was observed. Maximal values in the respective leaf drug samples were found in the plant development phase of full flowering (3.91%).
Mehrabani et al (2005) reported that Rosmarinic acid was isolated from the Iranian medicinal plant *Echium amoenum*. Concentrated methanolic extract of the grounded dried petals of *Echium amoenum* was fractionated by column chromatography and the fractions were purified by preparative HPLC. The structure of main pure component which was characterized by UV, IR, one and two dimensional $^1$H and $^{13}$C-NMR and Mass spectroscopy was found to be Rosmarinic acid which is widespread in the plants of the Lamiaceae and Boraginaceae families in insignificant quantities. Rosmarinic acid has an antimicrobial, antiviral, and anti-inflammatory effect, which makes it a valuable product for the pharmaceutical and cosmetic industries.

Hossain et al (2009) reported that Rosmarinic acid and caffeic acid were identified and quantified from *Orthosiphon stamineus* extract by HPTLC method. High performance thin-layer chromatography (HPTLC) allows the identification and quantification of more than 20 samples in the same chromatographic run. The analysis of the sample requires 15-30 min compared with more than 2 h using a typical HPLC method. Using the techniques of the HPTLC and UV-VIS spectra found that the extract of the plant contains the caffeic acid and Rosmarinic acid ranging between 0.029% up to 0.24% to 2.24% respectively.

**Anticancer activity**

Bala et al (2010) studied the anticancer activity of methanol extract of *Cleome gynandra* (MECG) in Swiss albino mice against Ehrlich Ascites Carcinoma (EAC) cell line at the doses of 200 and 400 mg/kg body weight intraperitoneally. MECG was administered for nine consecutive days. Twenty-four hours of last dose and 18 h of fasting, the mice were sacrificed and antitumor effect of MECG assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight and hematological parameters of EAC bearing host. MECG showed significant decrease in ($p < 0.01$) tumor volume, viable cell count, tumor weight and elevated the life span of EAC tumor bearing mice. Hematological profile such as RBC, hemoglobin, WBC and lymphocyte count reverted to normal level in MECG treated mice. Results showed that the extract has potent dose dependent anticancer activity and that is comparable to that of 5-fluorouracil.

Sini et al., 2012 reported the cytotoxic activity of *Orthosiphon thymiflorus*. Different concentrations of the methanolic extract of leaves of the plant *Orthosiphon thymiflorus* were subjected to cytotoxic activity study against Dalton Lymphoma Ascites (DLA) cells using the MTT assay. Percentage cell viability of cell lines were carried out by using Trypan blue dye
exclusion technique MTT assay was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extract. Cell viability was inhibited to different extents by different concentrations of the extract.

Based on the research done by Abdelwahab et al (2011) antiapoptotic and antioxidant activities of aqueous-methanolic extract (CAME) of Orthosiphon stamineus Benth (OS), and its hexane (HF), chloroform (CF), n-butanol (NBF), ethyl acetate (EAF) and water (WF) fractions were investigated. Antioxidant properties were evaluated using the assays of Folin-Ciocalteu, aluminiumtrichloride, β-carotene bleaching and DPPH. The role of OS against hydrogen peroxide induced apoptosis on MDA-M231 epithelial cells was examined using MTT assay, phase contrast microscope, colorimetric assay of caspase-3, western blot and quantitative real-time PCR. OS reduced the oxidation of β-carotene by hydroperoxides. Cell death was dose-dependently inhibited by pretreatment with OS. Caspase-3 and distinct morphological features suggest the anti-apoptotic activities of O. stamineus. This plant not only increased the expression of Bcl-2, but also decreased Bax expression, and ultimately reduced H2O2-induced apoptosis.

Mullauer et al (2009) reported the anticancer activity of Betulin (BE) (precursor of Betulinic acid) which is combined with cholesterol against A549 and HeLa cell lines. Betulinic Acid (BetA) and its derivatives have been extensively studied in the past for their anti-tumor effects. BE induces apoptosis utilizing a similar mechanism as BetA and is prevented by cyclosporin A (CsA). BE induces cell death more rapidly as compared to BetA, but to achieve similar amounts of cell death a considerably higher concentration of BE is needed. The cholesterol sensitized cells to BE-induced apoptosis, while there was no effect of cholesterol when combined with BetA. Despite the significantly enhanced cytotoxicity, the mode of cell death was not changed as CsA completely abrogated cell death. These results indicate that BE has potent anti-tumor activity especially in combination with cholesterol.

Faujan et al (2010) studied the cytotoxic effect of Betulinic acid (BA), isolated from Melaleuca cajuput a Malaysian plant and its four synthetic derivatives against various cell line or peripheral blood mononuclear cells (PBMC) by MTT assay. Betulinic acid acetate (BAAC) was most effective than other Betulinic acid derivatives. It had most active cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC), suggesting its action is specific for tumor cells. BA and
BAAC inhibit HL-60 cell line at low concentration with IC$_{50}$ values at 2.60 and 1.38µg/ml, respectively, after 72 h. DNA fragmentation analysis showed ladder formation in the 100 – 1500 bp region in HL-60 cell lines after 24 h of treatment with IC$_{50}$ values. The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. BA and BAAC have been shown to induce a time dependent increase in the sub G1 peak indicating apoptotic phenomenon as obtained from the DNA content histogram analysis. Thus, Betulinic acid isolated from the plant showed good potential as an anti-cancer compound with less toxicity to human normal cells.

Alitheen et al (2010) evaluated the toxicity of damnacanthal, nordamnacanthal, Betulinic acid and zerumbone isolated from local medicinal plants towards leukemia cell lines and immune cells by using MTT assay and flow cytometry cell cycle analysis. The results showed that damnacanthal significantly inhibited HL-60 cells, CEM-SS and WEHI-3B. Nordamnacanthal and Betulinic acid shows stronger inhibition towards CEM-SS and HL-60 cells. Zerumbone was demonstrated to be more toxic towards those leukemia cells.

Sharma et al (2011) evaluated the in-vitro cytotoxicity activity of methanolic extract of Glochidion zeylanicum (Gaertn.) root. In this study the extract was tested using human cancer cell lines HepG2, HT-29 and PC-3 for its effects on cell viability, growth inhibition and cell morphology. Cell viability, inhibition were determined by XTT-assay. Morphology was studied by using DAPI staining technique. The results showed decreased cell viability and increased growth inhibition in a concentration dependent manner and also altered the cell morphology after treatment with the plant extract. The data demonstrated that methanolic extract of roots of Glochidion zeylanicum (Gaertn.) has a potential cytotoxicity activity on HepG2, HT29 and PC3 cell lines but the effect was more significant on PC3 cell lines.

**Antioxidant activity**

Kumaran et al (2005) reported an activity-directed fractionation and purification process which is used to identify the DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging components of Coleus aromaticus Benth. The ethyl acetate phase showed strong DPPH radical-scavenging activity *in vitro*, when compared with water and hexane phases. The ethyl acetate fraction was then subjected to separation and purification using Sephadex LH-20 chromatography. Three compounds showing strong DPPH radical-scavenging activity were shown, by spectral methods (1H NMR, 13C NMR, and MS) and by comparison with literature
values, to be Rosmarinic acid, chlorogenic acid and caffeic acid. In addition, HPLC identification and quantification of isolated compounds were also performed. Rosmarinic acid was found as a major component and principally responsible for the radical-scavenging activity of *C. aromaticus*.

Muchuweti *et al* (2007) studied the total phenolics content and antioxidant activity from the methanolic exytact of *Cleome gynandra* using DPPH and reducing power assays, a β-carotene linoleic acid model system and the inhibition of lipid peroxidation in rat brain. Phenolic compounds were also quantified using HPLC. Total phenolic compounds for *Cleome gynandra* was 1327.333±1.658 mg g⁻¹ dry mass. The extract contained vanillin, caffeic acid, p-coumaric acid and ferulic acid. Extract showed a time dependent decrease in radical scavenging of DPPH and β-carotene.

Based on the research done by Matkowski (2008) he found that *Orthosiphon stamineus* shows more antioxidant activity than *Glechoma hederacea*. Aqueous methanol extracts were prepared by reflux extraction from *O. spiralis* and *G. hederacea*. The extracts were fractionated using liquid–liquid extraction (LLE) with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA), and n–butanol (BuOH). All extracts and fractions were studied for their antioxidant activity using spectrophotometric assays: DPPH scavenging, phosphomolybdenum reduction, and deoxyribose degradation assay. Polyphenol content was studied by the Folin–Ciocalteu method, Arnow reaction, and Lopez–Arnaldos assay. These plants can provide efficient antioxidant protection by complementary mechanisms such as free radical scavenging and metal ion reduction. However, *O. spiralis* is more effective as an integral extract due to the synergistic action of its components, while *G. hederacea* needs fortification by fractioning the crude extract into polar solvents to increase the proportion of Rosmarinic acid, the most active agent in this herb.

Das *et al* (2009) investigated *Berberis aristata* DC (Fam: Berberidaceae) against human colon cancer cell line (HT29) to explore its anticancer potential. In addition, a phytochemical screening of the methanolic extracts was also done. The effect of *Berberis aristata* methanolic extract on proliferation of HT29 cancer cell line was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) microculture tetrazolium viability assay. The cells were exposed to different concentrations (100, 50, 25, 12.5, 6.25, 3.125 and 1.5 μg/ml) of *Berberis aristata* methanolic extract or vehicle for 72 h. Cisplatin (5, 2.5 and 1.25 μg/ml) acted as positive control and vehicle (DMSO) as negative control. Following treatment, the cells were exposed to
Tetrazolium dye (5 mg/ml) for 4 h. The formation of the purple coloured formazan complex was dissolved by adding DMSO (100 μl) and read at 490nm using ELISA microtiter plate reader to determine the inhibitory concentration, IC. About 40% increment in cell killing was seen when the dose of *Berberis aristata* methanolic extract was 50, increased from 1.5 to 25 μg/ml. At a concentration of 100 μg/ml, 54.89% cytotoxicity was recorded. The IC50 value of *Berberis aristata* methanolic extract was 1.8964 μg/ml after 72 h of incubation. In this study, it was observed that *Berberis aristata* methanolic extract induces a concentration dependent inhibition of HT29 cells, with an IC value of 1.8964 μg/ml after 72 h of incubation. The result of the phytochemical screening of the 50 investigated methanolic extract of stems of *Berberis aristata* showed the presence of alkaloids as active chemical constituents.

Hoong Ho *et al* (2010) Extracts of the *Orthosiphon stamineus* plant were tested for antimicrobial and antioxidant activities. Whole *O. stamineus* plants (powdered) were extracted using various concentrations of methanol. The inhibition observed with these *O. stamineus* extracts was comparable to the inhibition seen with the natural food preservative 5% lactic acid; this is likely due to the high concentration of Rosmarinic acid found in the *O. stamineus* extracts. This study showed that the highest concentration of Rosmarinic acid had the best antibacterial and free radical scavenging activities. This suggests that Rosmarinic acid content is closely associated with antibacterial and free radical scavenging activities of *O. stamineus* extracts.

Yam *et al* (2010) studied the anti-inflammatory activity of *Orthosiphon stamineus* leaf extracts and identification of the active compounds contributing to its anti-inflammatory activity using a developed HPLC method. Active chloroform extract of *O. stamineus* was fractionated into three fractions using a dry flash column chromatography method. These three fractions were investigated for anti-peritoneal capillary permeability, *in vitro* nitric oxide scavenging activity, anti-inflammatory and nitric oxide (NO) inhibition using carrageenan-induced hind paw edema method. These results suggest that the anti-inflammatory properties of these CF2 may possibly be due to the presence of flavonoid compounds capable of affecting the NO pathway.

Maizura *et al* (2011) reported the total phenolic content antioxidant activity of the Herb and spices namely kesum (*Polygonum minus*), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*). Herb and spices were extracted by using juice extractor without the additional of solvent. These herb and spices were determined for moisture content and the extracts were analyzed for Total Phenolic Content (TPC) and antioxidant activity (DPPH radical scavenging
assay and FRAP Ferric-Reducing Antioxidant Power assay). Kesum extract had the highest total phenolic content followed by ginger and turmeric extract. A significant and positive high Pearson’s correlations between TPC and DPPH assay (r = 0.86) and between TPC and FRAP assay (r = 0.91), respectively, was observed for all plants extracts. This indicated that phenolic compounds were the main contributor of antioxidant activity in plants.

Sundarammal et al (2012) evaluated the antioxidant activity of Orthosiphon thymiflorus essential oil isolated from leaves. *O. thymiflorus* is an aromatic plant widely distributed in the Western Ghats of South India. In vitro antioxidant activity of the isolated oil showed dose dependent free radical scavenging activity against DPPH (IC50 437.12±0.02 μg/ml) and hydroxyl radicals (IC50 317.88±0.02μg/ml). Metal chelating and prevention of deoxyribose degradation activities of the oil was observed with the IC50 of 339.78±0.01μg/ml and 158.33±0.02μg/ml respectively. Antioxidant activity of the oil was low when compared with the positive controls such as BHT and ascorbic acid. The present study shows various chemical components present in the *O. thymiflorus* essential oil and with its significant antioxidant activity thus has great potential to be used as natural food supplement.

**Apoptotic DNA fragmentation**

Studies have determined the *in vitro* cytotoxic properties of Betulinic Acid (BA) towards the human mammary carcinoma cell line MDA-MB-231 and the human promyelocytic leukaemia cell line HL-60 and the mode of the induced cell death. The cytotoxicity and mode of cell death of BA were determined using the MTT assay and DNA fragmentation analysis, respectively. The compound was cytotoxic to MDA-MB-231 and HL-60 cells with IC50 values of 58 μg/ml and 134 μg/ml, respectively. Cells treated with high concentrations of BA exhibited features characteristic of apoptosis such as blebbing, shrinking and a number of small cytoplasm body masses when viewed under an inverted light microscope after 24 h. The incidence of apoptosis in MDA-MB-231 was further confirmed by the DNA fragmentation analysis, with the formation of DNA fragments of oligonucleosomal size (180-200 base pairs), giving a ladder-like pattern on agarose gel electrophoresis. BA was more cytotoxic towards MDA-MB-231 than HL-60 cells, and induced apoptosis in MDA-MB-231 cells (Yazan *et al.*, 2009).

The host-selective AAL toxins secreted by *Alternaria alternata* f sp *lycopersici* are primary chemical determinants in the Alternaria stem canker disease of tomato. They are
sphinganine analog mycotoxins that cause cell death in both animals and plants that they report to be the hallmarks of apoptosis during cell death induced by these toxins in tomato. DNA ladders were observed during cell death in toxin-treated tomato protoplasts and leaflets. The intensity of the DNA ladders was enhanced by Ca$^{2+}$ and inhibited by Zn$^{2+}$. In situ analysis of cells dying during development in both onion root caps and tomato leaf tracheary elements revealed DNA fragmentation localized to the dying cells as well as the additional formation of apoptotic-like bodies in sloughing root cap cells. Hence, these sphinganine analog mycotoxins may be used to characterize further signaling pathways leading to apoptosis in plants (Wang et al., 1996).

Natural products from plants are rich sources of chemical diversity and most of the pharmacologically active principles currently used as drugs, including anticancer agents are plants products. The study evaluated some Egyptian flora as anticancer agents. The materials used were leaves of Luffa aegyptiaca (sponge gourd), Solenostemma arghel (argel), Cassia italica (Senegal senna), Ocimum basillcum (basil), Colocasia antiquorum (taro), Beta vulgaris (beet) and fruit of Capsicum frutescens (chili pepper). Antioxidant activity was assayed using the 2,2'diphenylpicrylhydrazyl (DPPH) radical method. Anticancer activity was assayed in vitro against acute myeloid leukemia (AML) and acute lymphocyte leukemia (ALL); and in vivo against Ehrlich ascites carcinoma cells (EACC). The results showed significant antioxidant activity of most of extracts in DPPH assay. Solenostemma arghel hot water extract significantly reduced EACC induced tumor growth and delayed animal death (with EACC) by 29 days. Among all the extracts S. arghel showed high cytotoxicity (66 - 90%) on ALL and AML cells from patients. DNA fragmentation patterns showed cytotoxicity may due to the induction of apoptosis. In conclusion, some natural products from Egyptian flora have potential for use as therapeutics for diseases such as cancer (Nassr-Allah et al., 2009).