REVIEW OF LITERATURE

The relevant literatures concerning micropropagation, callus induction, hairy root induction, solasodine production with special reference to fungal elicitors, pharmacological aspects like antiaflatoxicosis and antimicrobial activity were mainly searched and collected for the family Solanaceae and the findings of different earlier scientific workers were summarized.

Wink, (1990) reported that plants produce an array of natural products, the so called secondary metabolite, which play a variety of roles such as defence molecules against attacks by animals and microorganisms. These substances are also important to man as a source of pharmaceuticals, fragrance, agrochemicals and food additives. Plants are still remaining the major source of many vital medicinal compounds. Kloucek et al., (2005) reported that plants are used in folk medicine in the treatment of skin diseases, veneral diseases, respiratory problems and nervous disorders.

Many species of the Solanaceae have been regenerated by shoot organogenesis using young leaf explants. Solanum surattense (Gupta and Handra, 1982), Solanum candidum, S.quitoense, Solanum sessiliflorum (Hendrix et al., 1987), Solanum melongena (Mukherjee et al., 1991) and Solanum commersonii (Cardi et al., 1993).

Micropropagation of Withania somnifera employing different explants, such as shoot tips (Sen and Sharma, 1991; Furmanowa et al., 2001; Ray and Jha, 2001), nodal segments (Tiwari and Singh, 1991), axillary meristems (Roja et al., 1991), leaves (Baburaj and Gunasekaran, 1995), and axillary leaves, axillary shoots, and hypocotyl and root segments (Rani and Grover, 1999) have been already reported.
Arulmozhi and Ramanujam (1997) conducted *in vitro* culture studies on *Solanum trilobatum* L. with foliar and stem explants on MS medium containing IAA, BAP and KIN combinations. Madhavan *et al.*, (1998) induced high frequency of shoot regeneration from mature seeds of *Solanum trilobatum* L. Callus was induced from root and shoot apical region and hypocotyls on MS medium supplemented with 2,4-D.

Abhyankar and Chinchanikar, (1996) showed direct shoot regeneration from leaf discs grown on MS medium supplemented with indole-3-acetic acid (IAA), 6-benzyladenine, and KIN in various combinations. Kulkarni *et al.*, (1996) determined direct shoot formation from leaf explants of *in vitro*-grown seedlings using MS medium containing IAA and BA in *Withania somnifera*.

Gresshoff, (1987) achieved rapid callus growth and organogenesis in *Solanum laciniatum*. The formation of shoots and roots was dependent on the relative concentrations of auxin and cytokinin. Baburaj and Thamizhchelvan, (1991) studied the regeneration potential of *Solanum surattense* Burm f. (*Solanum xanthocarpum* Schrad and Wendl) through leaf derived calli and organogenesis from stem explants has been reported. Recently, regeneration of an androgenically stable somoclonal variant through anther culture was reported (Prasad *et al.*, 1998). Other *Solanum* species well studied for *in vitro* morphogenetic responses are *Solanum nigrum* (Bhatt *et al.*, 1979), *Solanum elaegnifolium*, *Solanum viarum* (Kowalczyk, 1983), *Solanum dulcamara* (Emke and Eilert, 1986), *Solanum laciniatum* (Macek, 1989) and *S.khasianum* (Bhalsing and Maheswari, 1997).

Gita Rani and Avinash, (2003) observed callus induction from hypocotyls, root and cotyledonary leaf segments of *Withania somnifera* (L.) Dunal. grown on Murashige and Skoog medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and KIN. Maximum callusing (100%) was obtained from
root and cotyledonary leaf segments grown on MS medium supplemented with a combination 2,4-D and KIN. The calluses, when subcultured in the same medium, showed profused callusing. However, these calluses remained recalcitrant to regenerate regardless of the quality and combinations of plant growth regulators in the nutrient pool. When hypocotyl segments were used as explants, callus induction was noticed in 91% of cultures, which showed shoot regeneration on MS medium supplemented with 2,4-D and KIN. These shoots were transferred to fresh medium containing various concentrations and combinations of 6-benzyladenine (BA) and N6-(2-isopentenyl), adenosine (2-iP). Maximum shoot multiplication was observed after 60 d of the second subculture on MS medium containing BA. These shoots were rooted best (87%) on MS medium containing indole-3-butyric acid. The plantlets were transferred to the field after acclimatization and showed 60% survival.

Gita Rani and Avinash, S(2003) also suggested that the frequency of root formation was comparatively lower with higher concentrations of IBA alone or in combination with IAA or NAA and with all concentrations of IAA alone in Withania somnifera (L.) Dunal.

Karra et al., (1979); Jawahar et al., (1997) and Padmanabhan et al., (1973) suggested that the combination of auxin and cytokinin was found to be more effective for callus induction. Padmanaban et al., (1973) reported that the IAA and BAP combination provide optimum regeneration in Solanum trilobatum. Pawar et al., (2002) reported high frequency of adventitious shoots from a single explant of Solanum xanthocarum.

Lindsey et al., (1983); Banarjee et al., (1993) and Drewes et al., (1995) focused on the production of commercially important secondary metabolites from in vitro cultures, especially callus and cell suspension cultures of various plants. Alvarez et al., (1994) and Kittipongpatana et al., (1998) reported that plant tissue cultures have been suggested as a potential tool for the production of useful secondary metabolites.
Khanna et al., (1976) described that due to uncontrolled exploitation of the natural habitat and fluctuations in supply to raw materials, the production of secondary metabolite by plant cell cultures of Solanum species are known to harbour steroidal alkaloids. Guilietti et al., (1991) proposed, the genus Solanum is medicinally important for the presence of steroidal alkaloids mainly solasodine and other related glycosides.

Bhalsing et al., (2001) reported that the many plants in the Solanaceae family accumulate alkaloids based on a C27 cholestan skeleton, eg. Solasodine and tomatidine. These compounds are essentially nitrogen analogues of steroidal saponins, and they are usually present as glycosides, which have surface activity and hemolytic properties as do the saponins. Solanaceae family comprises a number of plants widely known for the presence of variety of natural products of medicinal significance mainly steroidal lactones, glycosides, alkaloids and flavanoids.

Rodrignez et al., (1979) reported that the synthesis of 16 dehydroepiandrosterone acetate from solasodine obtained from S. elegnifolium Cav. 16 DHPA is the key compound for the pharmaceutical industry for the production of steroidal compounds. Solanum glaucophyllum shown have the capability of the culture to produce steroids, sitosterol and the diosgenin and a steroid alkaloid, solasodine (Jain and Sahoo, 1981). Zenk and Meinhart, (1978) noticed that the several variant callus lines of S. laciniatum that have recently been isolated and yield higher levels of alkaloids than in usual cell culture. Chaudhry et al., 2010 developed a protocol for callus induction and regeneration in tomato (Lycopersicon esculentum) var. Moneymaker. using hypocotyl and leaf disc explants of tomato on MS medium having different concentrations of IAA, NAA, BAP and KIN.

Vieira, (1989) noticed the solasodine content of Solanum mauritianum green fruits of natural populations growing on two different soils. A high content of solasodine was found in both populations of Solanum mauritianum.
El Badaoui et al., (1996) found those roots, fruits and occasionally the whole plant of *Solanum aculeatissmum* jacq. are used in medicine owing to presence of a steroidal glycoalkaloid solasodine. Solasodine shows close structural similarity of diosgenin and is efficient for the synthesis of cortisol and by products, diuretic drugs and corticosteroids.

Amir and kumar, (2004) have reported that various chemical constitutions are reported to be isolated from *Solanum* species, which include alkaloids, phenolics, flavanoids, sterols saponins and their glycosides. Alkaloids such as soladumlinidine and tomatidine were isolated from leaf, and stem of *Solanum* species.

The presence of glycoalkaloids in different parts (fruits, roots and shoots) of *Solanum* field plants are controversial (Zaki, 1963). This may be due to the great changeability of these species (Prelog and Jeger, 1953; Emke and Eilert,1986 reported that among the *Solanum* species studied *in vitro*,only 10 species were found to have the capacity to produce glycoalkaloids. Bhalsing et al., (2001) and Guilietti, (1991) have noticed that *Solanum surattense* contain steroidal alkaloid solasodine and closely related glycosides.

The steroidal alkaloids isolated from the roots of *C.betacea* include solasodine and tomatidenol (Schreiber, 1979) these compounds closely resemble the diosgnin which is used as precursors for the synthesis of corticosteroids and other contraceptive steroids (Roddick, 1996). The presence of these alkaloids in *C.betacea* may account for their ethanopharmacological uses in various areas (Bohs, 1989).

Lancaster and Mann, (1975) have analysed the steroidal alkaloids by using the colorimetric method. Subroto and Doran (1994) reported that possible trace quantities of Scopolamine in *Duboisia* shortly eratomas and crown galls detected using HPLC could not be confirmed by GC-MS analysis. The steroidal alkaloid content of *Solanum aviculare* shooty terotomas was also significantly reduced when compared with the intact plant.
Kuttipongpatana et al., (1998), studied an improved High Performance Liquid Chromatographic method for the qualification of solasodine. Balakrishnan et al., (1992), have estimated the total alkaloid content of the various parts of Solanum trilobatum. Ghazi and Mathees, (1989) reported that a method based on ion pairing - phase liquid chromatography was developed to separate and quantity the total alkaloid content in S.ptycanthum (Eastern black night shade).

Bhalsing (2000) reported that the isolation and characterization of solasodine from cultured cells of Solanum khasianum. The solasodine yield was two percent of dry weight of the callus. Sadykova et al., (1992) proposed a method for the quantitative determination of solasodine by HPLC on a Millikrom domestic micro column chromatograph. The amount of solasodine in various cell line of Solanum laciniatum has been determined.

Bhalsing (2000) reported the isolation of solasodine diosgenine, biotogenin, solasonin, tryogenin, isonuatigenin, tomatodenol, solanocpsine and tomatidine was carried out from non differentiating cultured cells induced from leaf explants and characterization of solasodine carried out by high performance liquid chromatography.

Bhatnagar et al., (2004) reported that highest solasodine content in Solanum laciniatum callus culture growing on the medium supplemented with L-arginine. The solasodine content in the shoots was higher, while the differentiated callus had a significantly lower amount of solasodine production. The shoots regenerated from callus yield 10 times more solasodine that undifferentiated callus culture.

Indrayanto et al., (1995) worked on the solasodine production in shoot cultures of Solanum laciniatum, with addition of different concentration of sucrose in the media. The modified MS medium increased the solasodine content in shoot culture of Solanum
laciniatum. A positive linear correlation between solasodine productivity and chlorophyll content occurred in these shoot cultures.

Ehmke and Eilert, (1986) reported that steroid glycoalkaloids in cell and shoot teratomas cultures of Solanum dulamera. Bhalsing and Maheswhari, (1997) reported that regeneration of Solanum khasianam from leaf explants regenerated on MS medium with 2, 4-D and KIN the yield of solasodine from 4-month old callus tissue was high at 2% of dry weight.

Indrayanto et al., (1995) reported the addition of L-arginine, caseinhydrolysate, banana powder or a reduction in the concentration of sucrose in the media increased the solasodine content in shoot cultures of Solanum laciniatum.

El-Badaouni et al., (1996) showed callus cultures of Solanum paludosum from roots, hypocotyls and cotyledons and leaves of plantlets cultured on a modified MS medium. Callus regenerated plants materials produced solamargine, the main steroid glycoalkaloid present in unripe fruits (El-Tayeb et al., 1997).

The major alkaloids identified in the alcoholic extracts of Solanum trilobatum L. are β-Solamarine (Purushothaman et al., 1987 and 1972; Balakrishnan et al., 1992) and Solasodine, (Balakrishnan 1992 and Krishnamurthy et al., 1996). Atta-ur-Rahman et al., (1998) reported that during the years (1996-97), new steroidal alkaloids are mainly isolated from the plants of the genera Solanum remain the largest sources of new basis.

Yu et al., (1996) studied that the growth and steroidal alkaloid production in Solanum aviculare hairy root culture as a function of different culture conditions (temperature, initial sucrose, exogenous solasodine concentration and osmolality of the medium).
Krishnamurthy et al., (1996) reported the isolation, identification and quantification of solasodine from various parts of field grown plants and unorganized callus cultures of different explants sources of *Solanum trilobatum* L.

Kaneko et al., (1976) reported that, a number of glycosides of 26-aminocholestane were isolated from the roots of *Solanum abutiloides* (Solanaceae). These alkamines are regarded as key intermediates in the biogenesis of steroidal alkaloids. The C-26 OH analogue (abutiloside c) was also isolated from the same plant.

Cipollini and Levey (1997) stated that highly rewarding solanum fruits may be able to maintain higher levels of glycoalkaloids because high nutrient content may offset the negative effects of glycoalkaloids on dispersers. Artificial fruit pulp media were used to measure how the mycelial growth rate of nine fungal species was affected by two glycoalkaloids, alph-solamargine both common in ripe the smallest leaves of *Solanum nigrum* L. and *Solanum incanum*. Many Solanaceous plants, known for the production of the glycoalkaloid solasodine, have been used for its exploitations from cell, tissue and organs (Jaggi and Kapoor, 1994).

Dixit and Gupta, (1982) reported that solasodine; an alkaloidal constituent of the *Solanum xanthocarpum* has antiandrogenic activity. Steroidal alkaloids and glycosides from root of *S.torvum*, saponins, steroids from the root bark and unripe fruits of *Solanum macrocarpum* have reported (Fayez and Saleh, 1967). Cham, (1991) and Cham, (1994) reported that the mixtures of solasodine glycosides have been successfully used for the treatment of certain human skin carcinomas.

Steroidal glycoalkaloids such as solanine, solasodine, tomatillidine and dihydrotomatillidine were isolated in earlier studies from solanaceae (Bianchi et al., 2000).
Keeler et al., (1990) reported that the antifungal properties and a certain degree of toxicity of steroidal glycoalkaloids found in species of *Solanum*.

Motidome et al., (1970) noticed that the phytochemical studies showed that the most common glycosides are the triosides solasonine and solamargine. Kerber, (1993) and Mola et al., (1997) reported that the fruits of *S. lycocarpum* showed the presence of solasodine as a major component. The effects of these compounds mainly consist of membrane disruption and acetylcholinesterase inhibition (Roddick et al., 2001). Lewis, (1989) said that solasodine has been known to possess anti-inflammatory activity.

Mulchamdami et al., (1979); Gottlieb et al., (1987) and Putalun et al., (2004) reported that *Physalis minima* (L.) contains steroid compounds, physalin A-E, solasodine glycoside and withanolides, as active constituents solasodine glycoside has anti-neoplastic activity against Sarcoma 180 in mice (Cham and Wilson, 1987) and anti-human skin carcinoma activity (Cham et al., 1991) and can inhibit herpes simplex virus type I (HSV-1) (Ikeda et al., 2000).

Jacob and Malpathak (2004) reported that solasodine is recognized as a potential alternative to diosgonin. Hairy root lines of *Solanum khasianum* Clarke show enhancement of solasodine production when compared to non-transformed roots. *Agrobacterium rhizogenes*-mediated hairy root cultures of *S. khasianum* were raised with the objective of using them for solasodine production. During any transformation event the genes responsible for the hairy root syndrome, viz. rol A, rol B, and rol C, were transferred to the plant genome through the T-DNA (Schmulling et al., 1988).

Palazon et al., (1997) reported that the genetic changes induced by rolA, B, and C genes modified cell differentiation and favor root formation resulting in the stimulation of root-specific secondary metabolism. They also stated that the stimulation was positively correlated to the level of polypeptide encoded by the rol genes. rol B and rolC genes produce
b-glucosidases, which release active auxins and cytokinins from inactive conjugates, respectively (Estruch et al., 1991 a, b). Faiss et al., (1996) reported that A. rhizogenes has been used by a number of workers as a source of genes capable of altering hormone metabolism in plants.

Agrobacterium rhizogenes induced hairy roots have broadened the application of in vitro cultures of plants especially for production of secondary metabolites. The accumulation of steroidal-glycoalkaloid was achieved in transformed Solanum spp. roots. Jualang et al., (2002) reported the establishment of P. minima hairy root cultures for the production of physalins.

Porter, (1991) observed that Solanum spp. are highly susceptible to the infection by the hairy root-inducing bacterium, Agrobacterium rhizogenes. Purushothaman et al., (1969; 1972 and 1987) Mohanan et al., (1998) reported the presence of that β-solamarine and solasodine in Solanum trilobatum L. The glycoalkaloidal mixture from this plant, which contains β-solamarine as the major constituent has been shown antibacterial, antifungal, antimitotic and antitumour activities.

Subroto and Doran, (1994) reported that among the hairy roots obtained from several Solanum species, those of S. aviculare were reported to accumulate the highest level of secondary compounds.

Nadeem et al., (1997) used crude powder; ethanol and petroleum ether of Solanum nigrum L. obtained from a commercial source in India, revealed moderate to good hepatoprotective action against, CCL_4 and paracetamol - induced liver damage in rats as evidenced by enzymatic and histopathological examination. Patel et al., (2010) reported that compounds like soalsonine and solasodine from methanolic extracts from Solanum xanthocarpum berries was showed natriuretic activity antiurolithiatic activity on rodent.
Raju et al., (2000); Hernandez et al., (2004); Jin et al., (2004); Nartowska et al., (2004); Yang et al., (2004) and Trouillas et al., (2005) reported that steroidal saponin are a group of plant secondary metabolites that are glycosides. These molecules have long been thought to have pharmacological value, and researchers becoming increasingly interested in their potential pharmacological activities, especially as anticancer agents. Steroidal saponins have one or two sugar chains attached by glycoside.

Lampe et al., (1978); Allameh et al., (2000) and Decoudu et al., (1992) suggested that aflatoxins bind glutathione and deplete its accumulation in the body by causing extreme damage to the liver, where it is primarily synthesized. Recently, the views about the main mechanism of the various adverse effects of aflatoxins (Carcinogenic, Mutagenic and Teratogenic) gained momentum because of its ability to cause oxidative damage.

McQueen et al., (1982); Prabhu et al., (1989); Aboobaker et al., (1994) and Verma et al., (2001) noticed that the numerous in vitro and in vivo studies indicated that aflatoxins are cytotoxic compounds. Aflatoxins are toxic metabolites produced by Aspergillus flavus and A. parasiticus widespread contaminants of foods and feeds. Their high toxicity to both animals and humans makes aflatoxins the most dangerous known mycotoxins. Acute toxicity following consumption of high doses of aflatoxin was well documented (Wilson and Payne, 1994).

Southern and Clawson, (1979); Miller et al., (1981) and Harvey et al., (1988) reported that in pigs, it is characterized by feed refusal, reduced weight gain, changes in hematological and biochemical parameters, increased prevalence of infectious disease, and liver and kidney lesions.

The immunotoxic potential of aflatoxin was known in many species, including laboratory and domestic animals (Oswald and Comera, 1998; Bondy and Pestka, 2000). In
pigs, aflatoxin decreased the blastogenesis response to mitogen, reduces the complement titers, decreased the macrophage activation, depressed and delayed hypersensitivity (Miller et al., 1978; Silvotti et al., 1997 and Mocchegiani et al., 1998).

Marin et al., (2002) summarized that the mycotoxins are secondary metabolites of fungi that grow on a variety of feed and foodstuffs consumed by animals and humans. Among them, aflatoxins are the most abundant and toxic metabolites produced by Aspergillus molds.

Aflatoxins were first discovered and characterized in the early 1960s after more than 100000 turkey poult in England died of apparent poisoning from mould-contaminated peanut meal (Blout, 1961; Goldblatt, 1969). There are two general forms of the disease caused by exposure to aflatoxin, aflatoxicosis. Acute aflatoxicosis results in death. Chronic aflatoxicosis causes cancer, with the liver as the primary target organ, immune suppression, teratogenicity and other symptoms (Bennett and Klich, 2003). There was also some evidence that respiratory exposure to aflatoxin increased the occurrence of respiratory and other cancers (Dvorackova, 1990).

Robens and Cardwell, (2005) reported that economic costs of aflatoxin are difficult to estimate because such total estimates would need to include losses such as slow weight gain and immune suppression in farm animals, and even the loss of companion animals to aflatoxicosis.

Solasodine exhibited antinflammatory effects in arthritis caused by kaolin, similar to that of cortisone (Mueller-Dietz, 1972). Cruz, (1982) and Correa, (1984) reported that the fruits of Solanum lycocarpum St.-Hil (Solanaceae), popularly known as ‘jurubebão’, ‘beringela-do-cerrado’ or ‘lobeira’ (Wolf-fruit), have been widely employed in Brazilian Cerrado folk medicine for the management of diabetes and obesity and to decrease
cholesterol levels, and are reported to possess sedative, diuretic, antiepileptic and antispasmodic activity.

The dried white gum obtained from green fruits crushed in aqueous solution was commercialized under the name of ‘polvilho-de-lobeira’, and has been used for the treatment of diabetes and ulcers (Ortencio, 1994).

Genus *Solanum* has anti-inflammatory (Lin *et al.*, 1995), antihepatotoxic (Grace and Saleh, 1996) and hypotensive activities (Ibarrola *et al.*, 2000), besides inhibiting allergic reactions and histamine release (Kim and Lee, 1998). Additionally *Solanum americanum* was used to treat skin ulcerations caused by *Leishmania braziliensis* (Franca *et al.*, 1996). Toxicological studies gave demonstrated nephrotoxicity in mice treated with an ethanol extracts of *S. grandiflorum* (Pereira *et al.*, 1998).

Mckee *et al.*, (1959) have studied that several constituents of *Solanum* have been associated with antibiotic activity, mainly the steroidal alkaloids. Solanocapsine (from *S.pseudocapsicum*) was antibacterial and the drug solanine, found in great quantities in potatoes (*S.tuberosum*) is toxic to various species of fungi.

Alvarez *et al.*, (2001) reported a new antimycotic steroidal saponin Sc-1 was isolated form *Solanum chrysotrichum* leaves collected from Mexico, which was showed fungitoxic activity against dermatophyte, *Trichophyton mentagrophytes*.

Erdogrul, (2002) reported that the development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Kinghorn *et al.*, (1992) and Kone *et al.*, (2004) reported that many of the current drugs used to treat different types of diseases are from plant sources there is a great potential to diseases new substances to be used in the treatment of bacterial and fungal infections.
The steroidal alkaloids and their glycosides occurring in numerous species of *Solanum* genus have a variety of biological activities, including antifungal (Rowan *et al.*, 1983), molluscidial (Alzerreca and Hart, 1982), teratogenic and embryotoxic (Friedman, 1992). *Lochroma umbellatum* (*Solanaceae*) displayed great antimicrobial activity against *B.subtilis* and *S.aureus* (Rojas *et al.*, 2003). Chah *et al.*, (2000) and Wiart *et al.*, (2004) found that the fruits methanol ethanol extracts of *Solanum torvum* that exhibited antimicrobial activity against *B.subtilis* and *S.aureus*.

Edeoga *et al.*, (2005) reported that the medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds.

Kupena *et al.*, (1990) reported that, in addition to antimicrobial activity, some glycoalkaloids display antitumor effects as well. Beta solamine (isolated from 8-dulcamera) inhibits sarcoma 180 in mice and subsequent investigations have revealed that cytostatic activity of the steroidal glycoalkaloids such as solanine, which has been characterized as a mitotic poison.

Ajaiyeoba, (1999) reported *Solanum macrocarpum* and *S.torvum* leaf methanol extracts contained alkaloids, tannins and steroids. Both extracts exhibited concentration-dependent activities, slightly higher for *S.torvum* extracts. They observed antimicrobial properties provide a support to some of the traditional uses of both plants. *S.erianthium* (Makinde *et al.*, 1987) and *S.macrocarpum* have been evaluated for antimalarial properties. Giron *et al.*, (1998) demonstrated that aqueous macerated samples of *Solanum ligustrinum* inhibited the growth of *Candida albicans*, *Aspergillus niger* and *Achremonium falciforme*. 
The appearance of bacterial resistance to antimicrobial agents, found alternative antimicrobial components. It has been suggested that natural products are preferable to synthetic ones. Many of the drugs currently used to treat bacterial and other infections were first isolated from natural sources including ethno medicinal plants (Coe and Anderson, 1996).

The gram positive bacterium such as *Staphylococcus aureus* was mainly responsible for post operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte *et al*., 1996). *Bacillus subtilis* are rod shaped aerobic bacteria and are reported to have some pathogenic role (Gorden *et al*., 1973). The gram negative bacterium such as *Escherichia coli* was present in human intestine and causes lower urinary tract infection, coleocystis or septicemia (Levine, 1987 and Singh *et al*., 2004). *Pseudomonas* was an aerobic, nonfermentative, oxidase positive *Bacillus* which mainly causes urinary tract infection, wound or burn infection, chronic otitis media, septicemia etc. in human (Bodey, 1983) and also causes several diseases in fishes (Bullock *et al*., 1965). The effects of herbal compounds and phytochemicals on pathogenic and economically important bacteria have been well studied (Sato *et al*., 1996).

MATERIALS AND METHODS
Experimental material

Mature berries were collected from naturally grown plants of *Solanum melongena* L. var. *insanum* (L.) Prain. and sun dried. The healthy and viable seeds were collected from berries and used as an experimental material.

Explant source - Direct regeneration

The healthy young leaf explants of *Solanum melongena* L. var. *insanum* (L.) Prain. were obtained from 25 - 30 days old young seedlings (germinated in mud/plastic pots) were thoroughly washed with tap water for 15-20 minutes to remove any extraneous materials followed by immersion in detergent solution for five minutes. After washing with distilled water, leaf explants were again washed in 70% alcohol for few seconds and rinsed three times with distilled water. The leaves were brought to the inoculation chamber and surface sterilized with 0.1% HgCl₂ for 1-4 min and again washed with sterile distilled water for 5-7 times.

Culture medium

Basal MS (Murashige and Skoog, 1962) medium along with the various hormone composition were used. The pH of the medium was adjusted to 5.8 before adding agar (8.0 gm/L).

Direct Plantlet regeneration / Micropropagation

For direct regeneration, the MS medium fortified with B5 vitamins containing KIN (1.0 to 5.0 mg/l) and BAP (1.0 to 5.0 mg/l) alone were used.

Callus induction
Explant source - Field grown stem

The healthy young stem explants of field grown plants Solanum melongena L. var. insanum (L.) Prain were thoroughly washed with tap water for 15-20 minutes to remove any extraneous materials followed by immersion in detergent solution for five minutes. After washing with distilled water, leaf explants were again washed in 70% alcohol for few seconds and rinsed three times with distilled water. The stem explants were brought to the inoculation chamber and surface sterilized with 0.1% HgCl₂ for 3-5 min and again washed with sterile distilled water for 5-7 times.

The young stem explants were used for callus induction. The callus induction medium consists of MS + B5 vitamins supplemented with varying concentrations of auxins like IAA (1.0 to 3.0 mg/l and 0.5 mg/l of cytokinin (KIN or BAP).

Root induction Medium

The root induction medium consists of MS half strength supplemented with IBA, IAA and NAA (0.5 to 2.0 mg/l).

Stock solution

Stock solutions of the ingredients were prepared and stored in stoppered brown bottles at refrigerated conditions.

Composition of MS medium (Murashige and Skoog, 1962) + B5 Vitamins (Gamborg et al., 1968)
<table>
<thead>
<tr>
<th>Essential Element</th>
<th>Concentration in medium (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro elements</strong></td>
<td></td>
</tr>
<tr>
<td>NH(_4) NO(_3)</td>
<td>1650</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl(_2) 2H(_2)O</td>
<td>440</td>
</tr>
<tr>
<td>Mg SO(_4) 7H(_2)O</td>
<td>370</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>170</td>
</tr>
<tr>
<td><strong>Micro elements</strong></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO(_4) 4H(_2)O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO(_4) 7H(_2)O</td>
<td>8.6</td>
</tr>
<tr>
<td>Na(_2) MoO(_4) 2H(_2)O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO(_4) 5H(_2)O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl(_2) 6H(_2)O</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Iron source</strong></td>
<td></td>
</tr>
<tr>
<td>FeSO(_4) 7H(_2)O</td>
<td>27.8</td>
</tr>
<tr>
<td>Na(_2) EDTA – 2H(_2)O</td>
<td>37.3</td>
</tr>
<tr>
<td><strong>Organic supplement</strong></td>
<td></td>
</tr>
<tr>
<td>Mynoinositol</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine – HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine – HCl</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>30000</td>
</tr>
<tr>
<td>Agar</td>
<td>80000</td>
</tr>
</tbody>
</table>

pH adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl.
## Preparation of Stock Solutions

<table>
<thead>
<tr>
<th>Name of the chemicals</th>
<th>StockSolution</th>
<th>M.S. Medium</th>
<th>ml/stock require to make desirable volume of the medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25 L</td>
</tr>
<tr>
<td><strong>Macro Elements MSA X 10x</strong></td>
<td>500ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_3$NO$_3$</td>
<td>16.5</td>
<td>16500</td>
<td>12.5</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>19.0</td>
<td>19000</td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>3.70</td>
<td>3700</td>
<td>12.5</td>
</tr>
<tr>
<td>CuCl$_2$ 2H$_2$O</td>
<td>4.40</td>
<td>1700</td>
<td></td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.70</td>
<td>4400</td>
<td></td>
</tr>
<tr>
<td><strong>Micro Elements MSB$_2$, X 100x</strong></td>
<td>500ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.0830</td>
<td>0.83</td>
<td>1.25</td>
</tr>
<tr>
<td>MSB$_2$ x 20x</td>
<td>500ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.124</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$ 4H$_2$O</td>
<td>0.446</td>
<td>22.3</td>
<td>6.25</td>
</tr>
<tr>
<td>ZnSO$_4$ 7H$_2$O</td>
<td>0.172</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Na$_2$MoO$_4$2H$_2$O</td>
<td>0.0050</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CuSO$_4$ 5H$_2$O</td>
<td>0.0005</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>CoCl$_2$ 6H$_2$O</td>
<td>0.0005</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td><strong>Iron Source MSC X 100x</strong></td>
<td>500ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$ 7H$_2$O</td>
<td>2.78</td>
<td>27.85</td>
<td>1.25</td>
</tr>
<tr>
<td>15.0Na$_2$EDTA 2H$_2$O</td>
<td>3.73</td>
<td>37.25</td>
<td></td>
</tr>
<tr>
<td><strong>Organic Supplement B, Vitamins</strong></td>
<td>100 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myoinositol</td>
<td>2.0</td>
<td>100</td>
<td>1.25</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.02</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine – HCL</td>
<td>0.02</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Thiamine – HCL</td>
<td>0.20</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>
Sterilization of culture media

The sterilized of MS medium with various concentrations of growth hormones were dispensed in culture tubes (20) ml. The culture tubes were then plugged with cotton and autoclaved at 121°C for 15 min. After autoclaving, the culture tubes were left undisturbed until the medium was solidified. Then culture tubes were transferred to the inoculation chamber after inoculation.

Preparation of growth hormones

IAA (Indole acetic acid) 100 gm was taken and made into paste using 0.1N NaOH and diluted up to 100 ml with distilled water. The cytokinins like KIN and BAP (Benzyl amino purine) were prepared using 0.1N HCl with distilled water. The hormone solution were kept in brown bottles and stored in the refrigerator. The prepared stock solutions have been used within 30 days to avoid contamination or precipitation of ingredients.

Inoculation

Before starting inoculation, all the required equipment’s /materials (sterilized forceps, petriplates, sterile blade, sterile distilled water and spirit lamp) were transferred to laminar air flow chamber. The surface and two sides of the chamber was wiped with alcohol and the door was tightly closed. Then the UV light was switched on for 15 min. After that, the equipment’s were sterilized by dipping in 95% alcohol followed by flaming and cooling.

Before starting the inoculation, hands were cleaned with alcohol and the inoculation was carried out in vicinity of the flame. The sterilized explants were placed on the medium at the centre of culture tubes.
Calculation

The experimental results were calculated as follows:

\[
\text{Percentage of response} = \frac{\text{Number of explants responded}}{\text{Number of explants inoculated}} \times 100
\]

The fresh and dry weights were measured individually using accurate balance. Fresh weight was determined by placing the callus on filter paper, without any traces of agar and the dry weight was measured for the same callus placed in an oven at 60°C for overnight and weighed.

Statistical analysis

Each experiment/treatments was repeated thrice. A completely randomized design was used in all the experiments. Wherever appropriate standard deviation and mean separation was carried out using Dungan Multiple Range Test (DMRT).

Hairy root induction

Plant material

The in vitro raised shoots were used for hairy root induction (Infection with Agrobacterium rhizogenes ATCC 15834)

Bacterial strain

Wild type strain of Agrobacterium rhizogenes (ATCC 15834), obtained from Microbial Type Culture Collection, Institute of Microbial Technology Chandigarh, India (MTTC), were used for infecting the plant material. Nutrient broth (NB) and nutrient agar used for growth and maintenance of the bacterial culture. The strain showed better growth
response in nutrient broth. Therefore, nutrient broth was used for culturing of *Agrobacterium rhizogenes*

**Plant tissue culture medium**

MS basal medium was used for maintenance and growth of hairy roots in slants.

**Induction and establishment of hairy root culture**

For hairy root induction, 48-hrs old culture of *Agrobacterium rhizogenes* was first centrifuged at 3000 rpm for 5 min and the resultant cell suspension was resuspended in 5 ml of sterile MS medium. This suspension was used for infecting the plant material.

**Co-cultivation**

The *in vitro* grown shoots were placed on glass petridishes. Bottom of the shoots were purposely wounded using sterile needle. A 10 μl aliquot of prepared suspension was applied at a base of shoot and placed in to the culture tubes containing the MS basal medium. The infected shoots were subjected to co cultivation for 48 hrs.

**Proliferation**

After 48 hrs of co-cultivation of shoots and bacterial cells, the shoots were removed and basal portion of shoots were washed thoroughly with sterile MS medium containing 250 mg /l cefotaxime. Washed shoots were blot dried with sterile filter paper and placed on proliferation medium. Cultures were maintained at 25±2°C with 16/8 hr photoperiod 3000-lux.till inductions of hairy roots. After initiation of the hairy roots the fast growing root tips were sub cultured and maintained on solid MS medium. The hairy root cultures were also maintained in MS liquid medium on a rotary shaker (80 rpm) in complete darkness. Various growth characters of established root clones were observed.
PCR analysis of hairy roots

Isolation of genomic DNA

Genomic DNA was extracted using CTAB method (Doyle and Doyle, 1987) from each of the hairy root lines as well as from control (non-transformed roots).

Confirmation of transformation-PCR

PCR primers specific for the amplification of the 780 bp fragments of the rol B genes were used. A 50 µl PCR mix contained 200mg of DNA, 10 pmoles primers, 200 µM dNTP mix, 1U of Taq DNA polymerase, 1X PCR buffer and 2 mM MgCl₂. PCR conditions were 94°C for 5 min and 72°C for 10 min. The sequences of the primers used in the PCR are as follows.
1. 5’ ATGGATCCCAAATTGCTATTCCCCACGA3’ and
2. 5’TAGGCTTCTTTCATTGATCCAAATTGCTATTCCCCACGA3’

Solasodine production

Field grown whole plants of Solanum melongena L. var. insanum (L.) Prain. were collected. The whole plants were washed with tap water and shade tried. Then the plants were separated into leaf, stem, root, pericarp, and seed. All the separated parts were subjected to shade dry during for 10-15 days. Then the dried parts were ground into powder and solasodine was estimated using HPLC.

Production of solasodine by adding fungal elicitors

The dried powder of the pericarp, stem and stem callus were taken for solasodine production using HPLC. Four fungal cultures like Penicillium purpurogenum, Penicillium citrinum, Aspergillus flavus and Aspergillus nidulans cultures were used for solasodine production.
Maintenance and preparation of fungal cultures

*Penicillium purpurogenum, Penicillium citrinum, Aspergillus flavus and Aspergillus nidulans* cultures were obtained from Centre for Advanced Study in Marine Biology, Parangipettai, Annamalai University, Chidambaram, and Tamilnadu. All the fungal cultures were maintained in soubourad dextrose agar slants. PDA broth was used for preparation of fungal elicitors.

**Composition of SDA**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological peptone</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>-</td>
</tr>
<tr>
<td>Agar</td>
<td>-</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Potato dextrose broth**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>-</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>-</td>
</tr>
</tbody>
</table>

One ml of *Penicillium purpurogenum, Penicillium citrinum, Aspergillus flavus and Aspergillus nidulans* were added individually with 31 ml of distilled water containing 1g of
the dry powdered samples of stem, stem callus and pericarp of *Solanum melongena* L. var. *insanum* (L.) Prain. were incubated for 240 hours at room temperature (25-28°C) at 200 rpm. Two ml of the sample were collected at 240 hrs, and then extracted with 10 ml methanol. The extract was analyzed by using HPLC. The solasodine content in the samples were compared with standard solasodine.

**HPLC analysis**

HPLC instrument used was JASCO PU-980 intelligent HPLC pump with JASCO UV-970 intelligent UV/Vis is detector including JASCO 807-IT integrator. The column was Bondapak C$_18$ (300x3.9 mm). The infra Red spectrophotometer (IR) used was Bio Rad FTS 3000 Excalibur Series. Standard Solasodine was obtained from Latoxan, rue, Leon Blum, 26000 Valence - France.

One gm of each plant sample was added to 5 ml of ethanol free chloroform, mixed and lower phase was removed and evaporated to dryness and dissolved in the mobile phase methanol: Tris buffer 0.01 M = 75:25 up to 5 mL. Twenty micro liter of the sample solution was injected in the HPLC with conditions as follows. Ambient temperature, flow rate 1.00 mL min$^{-1}$, mobile phase: Methanol: Tris buffer 0.01 M (pH 7.0) = 75: 25, detector UV/Vis wavelength: 205 nm.

**Calculation**

Solasodine content of leaf, stem, root, pericarp, seed and stem callus were calculated as follows.
<table>
<thead>
<tr>
<th>Sample area</th>
<th>Standard weight</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>--------------</td>
<td>-----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Standard area</td>
<td>Dilution</td>
<td>Sample weight</td>
</tr>
</tbody>
</table>

Pharmacological studies

Aflatoxin production

Aflatoxin for experimental induction of aflatoxicosis was produced on rice culture medium (Shotwell et al., 1996). Fifty grams of rice was taken in 250 ml erlenmeyer flasks and soaked in 25 ml of water for two hours with frequent shaking. The flasks were autoclaved, cooled and inoculated with Aspergillus flavus. The flasks were kept at room temperature and shaked several times a day. After 48 hours of inoculation, mould growth was seen as white spots on the surface of the rice, later turning to bright yellow color. Subsequently, in about a week’s time the color changed from bright yellow to brown, on the tenth day, mould rice was briefly steamed for ten minutes to kill the fungus and dried in hot air oven overnight at 60°C after which it was ground to fine powder. The powdered mould rice was analyzed for its aflatoxin content by following the method of modified Romer.

Extraction and estimation of aflatoxin

Aflatoxin content in the mouldy rice powder was estimated by the modified Romers method (1975). 10 g of the samples was taken and added with 40 ml of distilled water. Beat it in the mixie for two minutes.

After the addition of 60 ml of acetone added and again beat it for two minutes. Contents were slightly being heated up. High temperature should be avoided. The contents
were filtered through fluted whatman No 1 filter paper. 30 ml of the filtrate was taken and 0.6 g of cupric carbonate added approximately.

Ferric gel was prepared by adding 6 ml of ferric chloride (0.41M) to 34 ml of 0.2 M NaOH and the contents were swirled. The ferric gel was immediately transferred to the flask containing the extract and contents were mixed thoroughly and allowed to stand for two minutes with occasional swirling. It was then filtered through whatman No.1 filter paper. Then 40 ml of the filtrate was taken in 250 ml separating funnel then mixed with 40 ml of (0.03%) H₂SO₄ and extraction was done by add 10 ml of chloroform then mixed it slowly.

The chloroform layer was collected in a 100 ml beaker, to which 10 ml of chloroform was added again mixed thoroughly, and allowed to settle and collecting the chloroform in the same 100 ml beaker. In a second funnel, 40 ml of 0.02M KOH and 1% KCL mixture were taken. To which 20 ml of the collected chloroform extract was added. Mixed slowly and the layer was collected through anhydrous sodium sulphate bed by drop to remove any traces of moisture. The chloroform extract was kept in an oven at 50°C till it becomes dried. The dry aflatoxin film was rediluted with 0.2 ml of chloroform and spotted on the TLC plate taking exactly 5μl, 10 μl, 20μl and 40μl besides the standard spots of 5μl and 10μl.

**Preparation of aflatoxin standard**

The given aflatoxin in a suitable standard flask with Benzene: Acetonitrile (98+2) mixture to give a concentration of 10 μg/ml was prepared. The concentration of the stock solution was standardized using spectrophotometer (vide AOAC 1990 15th edition Ch: 49 pp 1185-1186). From the stock solution, prepare aflatoxin solution in benzene: acetonitrile-containing 41 g/ml was prepared in a suitable standard flask, which is the working standard.
After spotting the standards and sample, the spots in an unsaturated developing tank containing chloroform: acetone: water in the ratio 88:12:1 was developed. After developing three fourth of the plate, the plate was carefully removed from the tank, dried well and viewed in a UV cabinet viewer using long wavelength (364 nm).

**Administration of toxin to the rats**

After estimating the aflatoxin, the mouldy rice powder was mixed with the commercial rat feed (AFB\textsubscript{1} free) obtained from Hindustan Lever to get a 6-ppm concentration.

**Extraction of bioactive compounds**

20 to 30 gms of dried powder of leaves, stem, root, pericarp, seed and stem callus were loaded on soxhlet apparatus, ethanol: water (4:1) used as solvent at 42\textdegree C for 4 hrs. After extraction, ethanol was evaporated by vacuum filter.

**Animals**

Albino rats weighing about 150-200g, used in the present study were maintained under normal temperature (25\textdegree C) and relative humidity (50-60%). Care was taken in maintaining the animals as per the rules and regulations given by the animal ethical committee.

**Experimental protocol**

**Treatment of animals**

After acclimatization period for three weeks the animals divided in to groups namely group A (normal rats 6 nos) and B experimental group which contain 84 animals. The rats in
group B were fed with the 6 ppm aflatoxin-contaminated feed for 3 weeks to induce the aflatoxicosis followed by a progression period for 3 weeks with normal diet. The intoxicated rats were grouped into 14 (group 2-15) for treatment with the ethanol extract from leaf, stem, root, pericarp, seed and stem callus of *Solanum melongena* L. var. *insanum* (L.) Prain. were suspended in physiological saline and administered orally.

**Group 1:** Normal animals received the AFB$_1$ free normal feed and water *ad libitum* throughout the study period.

**Group 2:** Control animals fed with normal feed and water throughout the study period after the aflatoxin intoxication of about 3 weeks period.

**Group 3:** Aflatoxin intoxicated animals treated with standard drug silymarin 25 mg/kg b.wt for three weeks daily.

**Group 4:** The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain leaf extract at a dose of 25 mg/kg b.wt for three weeks daily.

**Group 5:** The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain stem extract at a dose of 50 mg/kg b.wt for three weeks daily.

**Group 6:** The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain root extract at a dose of 25 mg/kg b.wt for three weeks daily.

**Group 7:** The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain pericarp extract at a dose of 50 mg/kg b.wt for three weeks daily.

**Group 8:** The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain seed extract at a dose of 25 mg/kg b.wt for three weeks daily.
Group 9: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. root extract at a dose of 50 mg/kg b.wt for three weeks daily.

Group 10: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. pericarp extract at a dose of 25 mg/kg b.wt for three weeks daily.

Group 11: The intoxicated animals treated with *Solanum melongena* L. var *insanum* (L.) Prain pericarp extract at a dose of 50 mg/kg b.wt for three weeks daily.

Group 12: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. seed extract at a dose of 25 mg/kg b.wt for three weeks daily.

Group 13: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. seed extract at a dose of 50 mg/kg b.wt for three weeks daily.

Group 14: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. stem callus (IAA with BAP) extract at a dose of 25 mg/kg b.wt for three weeks daily.

Group 15: The intoxicated animals treated with *Solanum melongena* L. var. *insanum* (L.) Prain. stem callus (IAA with KIN) extract at a dose of 25 mg/kg b.wt for three weeks daily.

**Hematological studies (Barbara and Brown, 1980)**

At the end of 3 weeks, rats were killed by cervical dislocation under mild ether anesthesia and collected blood for analyzing the following hematological parameters.

a) **Hemoglobin (Hg)**
Procedure

The concentration of hemoglobin was measured by the standard procedure using Shali’s haemometer. Blood sample was drawn into the pipette upto the mark and transferred to the rectangular cell containing a little amount of N/10 Hcl placed in haemometer (Hellige Shali’s haemometer No. 304-B, Helliage, USA). After 5 min, a colour comparison was made with standard colour prim of haemometer. If the colour of the solution was high, distilled water was added to this solution and mixed with a sterile until a good colour match was obtained. The final reading of the solution in the tube was noted. From the cuvette reading, hemoglobin in g/100 ml of blood or its percentage was calculated.

b) Packed cell volume (PCV)

Using a Pasteur pipette, the Wintrobe tube was filled with blood, starting at its bottom and withdrawing the pipette as the tube is filled from below upwards. The blood was column was brought to the ‘O’ mark. Air bubbles, if any were removed from the top of the column of blood so that it stands exactly at ‘O’. The tube was centrifuged for about 20 min at 2500 rpm. The reading of the packed cells was taken, the tube again centrifuged for 5 minutes and the reading was noted. The procedure was repeated if there is a difference. Final reading was recorded when three consecutive readings were identical i.e., when the red cells have been fully packed.
C) Total Red blood cells (RBC)

Procedure

Blood was taken up to 0.5 marks in the RBC pipette and excess blood was wiped off from the tip. The pipette was then filled to 101 marks with RBC diluting fluid. The RBC pipette was horizontally shaken and a drop of resultant mixture was discharged under the cover glass of a Naubauer counting chamber (Naubauer, Feinoptic, Germany). Number of erythrocytes in 80 small squares was counted under the light microscope. The number of cells in 1 mm$^3$ undiluted blood was calculated using standard formula.

d) Total white blood cells (WBC)

Procedure

Blood was drawn up to 0.5 mark in the WBC pipette, diluted with WBC diluting fluid up to 11 mark and mixed properly. The resultant mixture was charged under the cover slip in the Naubauer chamber and the number of cells in four corner block (each block is sub divided in to 16 squares) was counted. The total leucocytes count per 1 mm$^3$ of blood was calculated by multiplying the average number of cells in the four blocks by 200.

e) Clotting time

Procedure

About 4cc of blood collected and 1cc is placed in each of four small test tubes. As soon as blood enters the barrel syringe, stop watch is started. The test tubes are placed in water bath at
37°C. The test tubes are tilted at every 30 sec. The time when the blood does not move on tilting is being noted down.

f) Erythrocyte sedimentation rate (ESR)

The Wintrobe pipette was filled half full with heparinised blood collected after sacrificing the animals. The long needle of the pipette was put in the Wintrobe tube so that the needle touched the bottom of the tube. The bulb of the pipette was then pressed continuously but gently to insert blood in the wintrobe tube and at the same time the needle was withdrawn slowly and gradually. The above procedure was continued until the wintrobe was filled to the zero mark. Adequate care was then set in the wintrobe rack in an exactly vertical position (if the windrobe tube is not exactly vertical, the cells will pile up on the wall of the tube and fall faster and hence the reading will be erogenously high). The sedimentation was then read at the first hour and second hour interval from the scale on the left side of the wintrobe tube.

Antimicrobial activity

Extract of bioactive compounds

The plant extract preparation employed in this study had been mentioned in the pharmacological studies page no 44. Plant extracts were screened against following Gram-positive and Gram-negative bacteria was obtained from the Microbial Type Culture Collection Centre, Chandigarh, India (MTCC).
**Bacteria**

*Echerichia coli*

*Vibrio cholerae*

*Salmonella typhi*

*Staphylococcus epidermidis*

*Pseudomonas aeruginosa*

*Klebsiella pneumoniae*

*Shigella dysentriae*

*Staphylococcus aureus*

*Streptococcus sp*

*Bacillus subtilis*

**Antibacterial assay**

Bacterial culture was maintained in Nutrient agar slants (Hi-media Laboratories Pvt.Ltd., Mumbai) at 4°C.
Preparation of nutrient agar

13 g of nutrient agar were suspended in 1000 ml distilled water and heat to dissolve the agar completely and sterilized by autoclaving at 15 lbs (121°C) 15 minutes. This culture medium used for the cultivation and maintained of bacteria in slants at 4°C.

Composition of nutrient agar

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.0±0.2</td>
</tr>
</tbody>
</table>

Preparation of Inoculums for bacteria

Active cultures of experiments were prepared by transferring one loop of culture of bacterial strains from the stock cultures to the sterilized nutrient broth and incubated for 24 hours at 37°C on a rotary shaker.
Nutrient broth composition

**Ingredients**  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/ L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.0±0.2</td>
</tr>
</tbody>
</table>

Weighed ingredients were suspended in 1000 ml-distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 mts. This broth was used for cultivation of bacteria.

**Agar well diffusion method**

Antibacterial activity of ethanolic extracts of leaf, stem, root, pericarp, seed and stem callus were carried by Agar well diffusion method using Mueller Hinton agar medium.

**Composition of Mueller Hinton Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/ L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion</td>
<td>30.00</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>17.50</td>
</tr>
<tr>
<td>Starch</td>
<td>1.50</td>
</tr>
<tr>
<td>Agar</td>
<td>17.00</td>
</tr>
</tbody>
</table>
pH (25°C) - 7.0±0.2

**Preparation of well**

Mueller-Hinton agar medium were suspended in 1000 ml of distilled water and boiled to dissolve the agar completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The sterilized media was poured into petriplates. The medium was allowed for solidification. The wells (7 mm) were punched on the solid Mueller Hinton agar using pipette tips. The different concentrations of extracts (25, 50, 75 and 100 µg/ml) were screened against four bacterial cultures. Ethanol was used as a control.

**Antifungal assay**

Ethanolic extracts like leaf, stem, root, pericarp, seed and stem callus were screened against following fungal pathogens by using agar well diffusion method.

**Fungi**

*Epidermophyton floccosum*

*Trychophyton mentagrophyte*

*Trychophyton tonsurans*

*Trychophyton rubrum*

*Matura mycetoma*

*Aspergillus flavus*
The fungal strains were maintained on potato dextrose agar slants at Department of Microbiology, J J College of Arts and Science, Pudukkottai, Tamilnadu, India. (Hi-media Laboratories Pvt. Ltd., Mumbai).

Composition of SDA

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological peptone</td>
<td>-10.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>-40.00</td>
</tr>
<tr>
<td>Agar</td>
<td>-15.00</td>
</tr>
<tr>
<td>pH</td>
<td>-5.6±0.2</td>
</tr>
</tbody>
</table>

Preparation of SDA

65.0 g of SDA agar were suspended in 1000 ml distilled water and heat to dissolve the agar completely and sterilized by autoclaving at 15 lbs (121°C) 15 minutes. This culture medium used for the maintenance of fungi in slants at 4°C.

Well diffusion method

Preparation of well

Sabouraud Dextrose agar medium were suspended in 1000 ml of distilled water and boiled to dissolve the agar completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The sterilized media was poured into petriplates. The medium was allowed for solidification. The wells (7 mm) were punched on the solid Mueller Hinton agar
using pipette tips. The different concentrations of extracts (25, 50, 75 and 100 μg/ml) were screened against four bacterial cultures. Ethanol was used as a control.