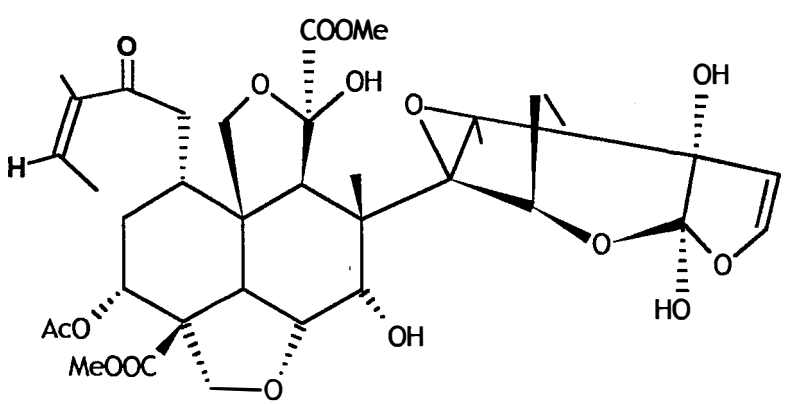
2 The secondary metabolites selected, their significance and objectives of the present study

CHAPTER 2

THE SECONDARY METABOLITES SELECTED, THEIR SIGNIFICANCE AND OBJECTIVES OF THE PRESENT STUDY

The literature review on Neem indicated that biocidal and medicinal properties of neem are attributed mainly to its triterpenoid group and phenylpropanoid group of compounds. Hence three main triterpenoids (Azadirachtin-A, Nimbin and Salannin) and two flavonoids (Quercetin and Kaempferol) were selected for the present study and their accumulation in callus, cell suspension and during the event of direct and indirect somatic embryogenesis in neem were analysed. Characteristic features of each of the compounds selected are given below:

Azadirachtin-A ($C_{35}H_{44}O_{16}$ MW:720)



Azadirachtins, supermolecules of insect control, is obtained from the seed kernels have the greatest potential to be developed as

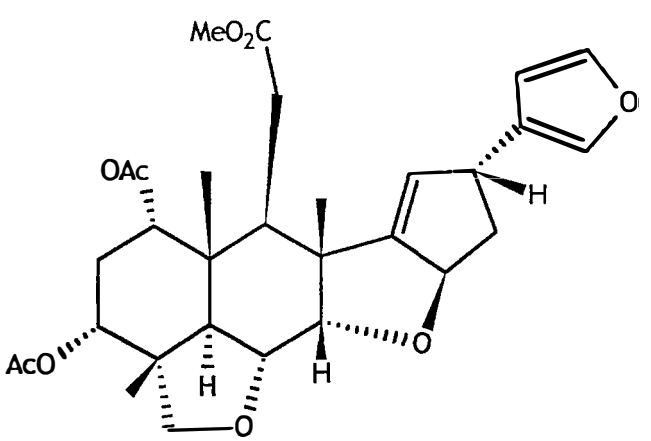
a bioactive agent against the insects and microorganisms than any other chemicals from neem (Govindachari, 1992; Govindachari and Gopalakrishnan, 1998). Chemically azadirachtin is a tetranortriterpenoid with a decalin segment and a modified furan segment that are joined by a single bond between C-8 and C-14 (Govindachari, 2002). This compound not only serves as models for the development of new synthetic alternative but also offer the prospect of leading to the invention of new class of pesticides (Singh *et al.*, 1996). Azadirachtin is a complex and highly oxidized triterpene having many functional groups (Govindachari *et al.*, 1992b). Its biosynthesis is thought to involve a steroid intermediate, tirucallol (Schmutterer, 1995). Azadirachtin has no less than 16 positional analogues (isoforms) and two important isoforms are Azadirachtin-A and B which constitute about 99% of the component which is high in the *A. indica*, compared to other species like *A. excelsa* and *A. integrifolia* (Govindachari *et al.*, 1994; Kabaleeswaran *et al.*, 1994). The remaining 1% comprise of Azadirachtin-C, -D, -E, -F, -G, -H, -I and -K (Govindachari *et al.*, 1991; 1992a; 1992c; 1995; 1996a; 1999b).

Azadirachtin exists as yellowish solid powder having a melting point of 160 -180°C. It has to be stored below $^{-}$20°C (Govindachari and Gopalakrishnan, 1998). Despite recent success in the chemical synthesis of the furan and the decalin moieties,

chemists are still faced with the challenge of combining the two fragments to produce the end product, azadirachtin. Environmental toxicity data of azadirachtin and its formulated products are inadequate, sketchy at best and sometimes confusing. Its biochemical effects at the cellular level remain unsolved at present.

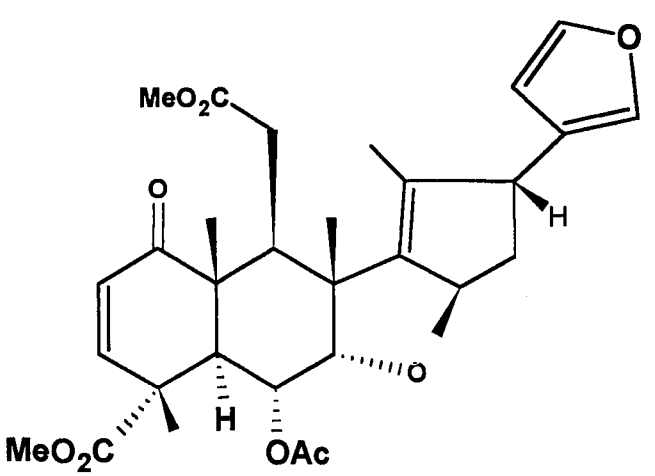
The issues of azadirachtin and its transformation products, metabolism and bioactivity, degradation, transport and fate in the environment are yet to be addressed and characterized. Progress has been made on resolving many technical difficulties involving the quality and the standard of azadirachtin derived from neem extracts. The manufacturing of the formulated products based on the refined azadirachtin is forging ahead in many countries. To date, data gaps exists on the impact of azadirachtin and its transformation products not only on non-target aquatic organisms, especially on the invertebrates and salmonid fish, but also on terrestrial organisms (Anonymous, 2004).

Salannin ($C_{34}H_{44}O_9$ MW: 596.29)



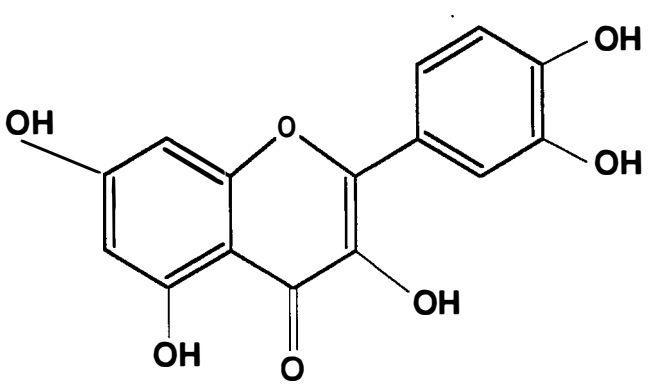
Salannin is a less polar triterpenoid compared to azadirachtin having properties similar to azadirachtin. This compound is chemically purified but is not yet marketed. Structure is simple when compared to azadirachtin group of limonoids (Govindachari *et al.*, 1996b). It has not been synthesized chemically so far. It was found to be more active when used in unison with azadirachtin. Found to be sensitive to pH variations, heat and light. Its biosynthesis still remains hypothetical and its bioactivity is not well studied as in the case of azadirachtin. Toxicity, feeding deterrence and mode of action has to be well studied. The compound is frequently used as an insectifuge (Schmutterer, 1995) and exhibits pronounced antimalarial property. It is found to be abundant in seed kernels and relatively low in leaves. The seeds of Thai neem, *A. siamensis* are known to have traces of salannin. Photooxidative products of salannin were found be equally active as azadirachtin (Morgan *et al.*, 2000; Gopalakrishnan *et al.*, 2001)

Nimbin ($C_{34}H_{36}O_9$, MW: 540.23)



Nimbin is another important tetranortriterpenoid having pronounced spermicidal action. Its melting point is 212^{O} C. This compound is chemically purified but is not marketed (Govindachari *et al.*, 1995) and is not well studied as in the case of azadirachtin (Schmutterer, 1995). The manufacturing of the formulated products based on the refined nimbin along with azadirachtin is forging ahead in many countries. It exists in highly oxidized state having many functional groups and is found abundant in seed kernels (Morgan *et al.*, 2000). It was the first compound isolated from neem bark but could not be chemically synthesized. Photooxidative products of nimbin were found be equally active as azadirachtin-A. The seeds of Thai neem, *A. siamensis* are known have nimbin which far less than the Indian Neem (*A. indica*) (Morgan *et al.*, 2000).

Quercetin ($C_{15}H_{10}O_7.2H_2O$, MW: 338.26)

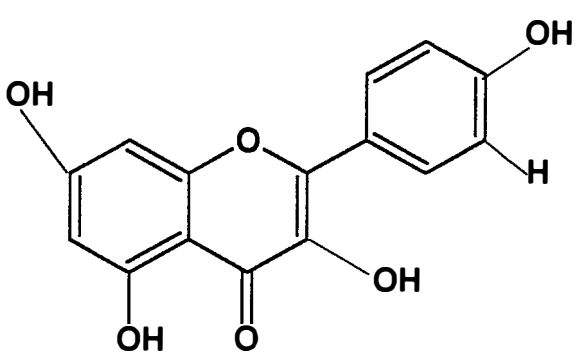


It is also found in other plants like *Ginkgo biloba*, *Hypericum perforatum*, *Sambacus canadensis* and also in apple, onion, tea,

berries and *Brassica* vegetables as well as in many seeds, nuts, flowers, bark and leaves of many plants (Anonymous, 1998). Chemically it is a flavonoid aglycone (meaning flavonoid without a sugar molecule) with similar activity compared to other compounds like rutin, isoquercetin and hyperoside having a specific sugar molecule in the place of one of the quercetin's hydroxyl groups on the C- ring (Miller, 1996; Anonymous, 1998). Quercetin appears to have many beneficial effects on human health including cardiovascular protection, anti cancer activity, antiulcer effects, antiallergic activity, cataract prevention, and antiviral and antiinflammatory effects (Miller, 1996) and also inhibits lipid peroxidation *in vitro* (Chen *et al.*, 1990). Paired with ascorbic acid, Quercetin reduced the incidence of oxidative damage to neurovasculature structures in skin and inhibited damage to neurons caused by experimental glutathione depletion (Skaper *et al.*, 1997). Quercetin's antiinflammatory activity appears to be due to its antioxidant and inhibitory effects on the inflammation producing enzymes like cyclooxygenase and lipooxygenase and their subsequent inhibition of inflammatory mediators including leukotrienes and prostaglandins (Kim *et al.*, 1998). Quercetin exerts antiviral activity against reverse transcriptase of HIV and the other retroviruses and was shown to reduce the infectivity and cellular replication of the viruses like Herpes simplex virus - type I, Polio virus - type I, Para

influenza virus type III and Respirtory syncytial virus (Kaul *et al.*, 1985). Much of the recent research on quercetin has been shown it to be an anticarcinogen to numerous cancer cell types including breast, colon, endometrial, gastric, leukemia, ovary and squamous cell (Larocca *et al.*, 1995; Pereira *et al.*, 1996; Caltagirone *et al.*, 1997). Biosynthesis and enzymes responsible for the synthesis of this compound is well documented (Anonymous, 2004). This compound is marketed as Quercetin dihydrate, a yellow crystalline powder, which is insoluble in water but soluble aqueous in alkaline solution.

Kaempferol ($C_{15}H_{10}O_6$, MW: 286.24)



This flavonol is abundant in fruits and edible parts of many tropical and sub-tropical plants. Rare in leaves and flowers. It has a stimulatory effect on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway (Prouillet *et al.*, 2004). It is also shown inhibit proliferation and increase mediator content in human leukemic mast cells (Alexandrakis *et al.*, 2003). Inhibition of estrogen receptor alpha expression and function

in MCF-7 cells by kaempferol was studied by Hung (2004). Kaempferol induced growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK (Nguyen *et al.*, 2003). Promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1 was reported by Miyake *et al.* (2003). In addition to this, kaempferol exhibits pronounced antihistaminic and antioxidant properties and is a reliable inhibitor of topoisomerase I. Chemically it is a flavonoid aglycone (Anonymous, 1998). Biosynthesis and enzymes responsible for the synthesis of this compound is well documented (Anonymous, 2004). No data were available showing teratogenicity or chromosomal effects of this compound in humans. No case report on epidemiological study of the carcinogenicity of kaempferol is available. Quercetin and kaempferol are found in neem flowers and leaves (Ramesh and Padhya, 1996).

OBJECTIVES

Tissue culture methods were devised to over produce five above-mentioned bioactive compounds considering their significance. The present investigation was aimed at the analysis of the accumulation of azadirachtin-A, salannin, nimbin, quercetin and kaempferol in callus (semi-organized cultures), cell suspension (unorganized cultures) and at various stages of somatic embryogenesis (organized cultures) of neem and to devise a method to convert low yielding cells to high yielding ones in terms of these five secondary compounds.

Callus cultures

I. Establishment of stock callus from 20 different explants collected from three different explant sources of neem viz., mature tree, seedlings (Field grown) and *in vitro* seedlings in a common growth regulator synergy in two different media MS and WPM

II. Identification and quantification of the five secondary compounds in callus

III. Selection of two stock calli one from each of the two media (MS and WPM) raised from similar explant based on growth and with production potential

Cell suspension cultures

I. Establishment of stock cell suspensions in MS and WPM with similar growth regulator in which stock callus were raised.

II. Identification of prospective production medium

III. Identification of best phase in cell growth that exhibited higher productivity for all the five compounds selected

IV. Extension of higher production phase by long-term maintenance

V. Selection of ideal carbon source and ideal growth regulator that favoured further enhancement of secondary compounds

VI. Elicitation of five secondary compounds by using precursors and permeabilisers in long-term cell cultures

VII. Histochemical localization of compounds in callus, cultivated cells and in plant tissues

Direct and Indirect Somatic Embryogensis

I. Establishment of somatic embryos from explants like root, internode, leaf, petal, cotyledon, cotyledonary node and from their respective stock calli raised in a common regulator MS medium

II. Quantification of metabolites selected during distinct transitory stages i.e. from non-embryogenic phase to embryogenic phase

Efficacy of pure samples and tissue culture crude extracts on colorectal cancer cell line

I. MTT viability assay for testing the effect of samples on HCT 116 colorectal cancer cell line.

II. Immunoflourescence examination of HCT 116 nuclear morphology using DAPI and Giemsa staining procedures