

Morphological and Biochemical Comparative Studies on Control of Certain Ailments on Plants and Animals System of *Boerhaavia diffusa* and *Clerodendrum aculeatum*

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ABSTRACT: *Boerhaavia* and *Clerodendrum* are economically important plants because many of their species possess medicinal properties. Almost every part of these plants is credited with some medicinal properties and are employed in traditional Ayurvedic system of natural therapy.

KEYWORDS: Punarnava, Therapeutic property, Quinolizidine, Ayurvedic medicine, Alfalfa

I. INTRODUCTION

Boerhaavia is a genus of family Nyctaginaceae, order-thymilae, and phylum-angiosperm. In different parts of the world *Boerhaavia diffusa* is known by different names viz., in India it is known as, Punarnava, Biskhafra (Hindi), Thazhuthama (Malayalam), Mukaratte (Tamil), Gadhapurna (Bengali), Satodi (Gujarati), Itsit (Punjabi) etc., in America it is called as Hogweed, Pigweed while in Brazil it is known as Erva Tostao and in Unan as Ispast. *Boerhaavia diffusa* is a perennial herb and a long used drug in indigenous system of medicine. The name Punarnava ("Punah punarnava bhawati iti") is probably derived from its perennial habit, during summer it remains dormant, the aerial part of the plant dries out and only root stock represents the only surviving portion of the plants, which regenerates and forms aerial parts with the advent of rainy season. The presence of therapeutic property has been described as 'Karoti shariram punarnavam' (rejuvenates the body) (Wahi *et al.* 1997). *Clerodendrum* is a genus of family Verbenaceae, order-lamiales and Phylum-Angiosperm. *Clerodendrum spp.* is also a perennial shrub and has been used as drug plant in indigenous Ayurvedic medicine (Sharma 1998).

II. GEOGRAPHICAL DISTRIBUTION, ORIGIN AND HABITAT

Genus *Boerhaavia*, consisting of 40 species is distributed in tropical and sub-tropical regions (Heywood 1978) and warm climate. It is found in Ceylon, Australia, Sudan and Malay Peninsula, extending to China, Africa, America and Islands of the Pacific. Among 40 species of *Boerhaavia*, 6 species are found in India, namely *B. diffusa*, *B. erecta*, *B. rependa*, *B. chinensis*, *B. hirsuta* and *B. rubicunda* (Anonymous 1988). *Boerhaavia diffusa* in India, is found in warmer parts of the Country and throughout upto an altitude of 2,000 m in the Himalayan region. It is a perennial, spreading hog weed, commonly occurring abundantly in waste places, ditches and marshy places during rains. The plant is also cultivated to some extent in West Bengal (Anonymous 1988). The genus *Clerodendrum* (Verbenaceae) comprises about 560 species and varieties (Moldenke 1971).

The genus is chiefly native to the tropics and subtropics of the eastern hemisphere and consists of evergreen and deciduous herbs, shrubs and trees. Many have showy, handsome blooms and are cultivated as ornamental plants. Valuable medicinal, biocidal, anti-fungal and anti-viral properties in some species have been reported. Crude extracts from 8 species of *Clerodendrum* (*Clerodendrum aculeatum*, *C. indicum*, *C. infortunatum*, *C. phlomidis*, *C. serratum*,

C. inerme and *C. viscosum*), have been known to induce an Actinomycin-D sensitive systemic resistance to several viruses in different hosts (Verma *et al.* 1984).



Fig. 1: Field grown plants of *Boerhaavia diffusa* and *Clerodendrum aculeatum*
a. Field grown BD **b.** Uprooted BD plant
c. Hedge of CA **d.** A single plant of CA

III. CHEMICAL CONSTITUENTS OF *BOERHAAVIA DIFFUSA* AND *CLERODENDRUM ACULEATUM*

The *B. diffusa* plant is chemically very rich. It contains large number of compounds, which make it a very useful drug. Varieties of chemicals have been isolated from the punarnava and their structure has been elucidated. These chemicals belong to the groups such as flavonoids, alkaloids, steroids, triterpenoids, lipids and lignans. A typical alkaloid named punarnavine, isolated from this plant is $C_{17}H_{22}N_2O$, mp 236 – 237°C (Agarwal and Dutt 1936; Basu *et al.* 1947). The structure of this alkaloid is not known. It also contains quinolizidine alkaloids, besides two other unidentified alkaloids (Anonymous 1988). Quantitative assay of alkaloids showed its presence in the range 0.05% to 0.15% of total alkaloids in the root (Surange and Pendse 1972). Experimental work done on the screening of the root from garden-grown *in vivo* plants of *Boerhaavia diffusa* of different ages, showed that maximum alkaloid content 2.0% accumulated in the roots of three year old mature plants (Padhya 1993), whereas, only 0.02%-0.15% of the alkaloid punarnavine was extracted from the roots of micropropagated plants (Srivastava and Padhya 1995). Chemical analysis of roots and aerial parts have

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revealed quantitatively differential presence of other compounds also viz., boeraviinone A-F (Kadota *et al.* 1989 and Lami *et al.* 1990, 1992), hypoxanthine 9-L-arabinofuranoside (Ahmed and Hossain 1968), ursolic acid (Misra and Tiwari 1971; Sukhdev 2006) and punarnavoside (Jain and Khanna 1989). The roots yielded a glycoprotein having a molecular weight of 16,000 to 20,000 daltons (Verma *et al.* 1979). *C. aculeatum* (a constituent of dashamoola) plant is chemically very rich. Leaves contain a basic glycoprotein with molecular mass of 34 kDa (Verma *et al.* 1996). The c-DNA characterization for this 34 kDa basic anti-viral protein of *C. aculeatum* has also been done (Kumar *et al.* 1997).

Biological activity and economic importance of *B. diffusa* and *C. aculeatum* The whole plant of *Boerhaavia diffusa*, fresh or dried, is a very useful source of drug punarnava, which is official in Indian Pharmacopoeia as a diuretic. The active principle contained in the herb is an alkaloid, known as punarnavine. The roots and leaves with flowers have been found to be more potent than the stem (Anonymous 1988). In India it has a long history of use by indigenous and tribal people, and in Ayurvedic or natural/herbal medicine. The study by Anand (1995) revealed that this plant is harvested for medicinal purposes mainly for flushing out the renal system hence it is supposed to be dialytic in nature. It is also used for seminal weakness and blood pressure. *B. diffusa* finds its special cultural place during rainy season of sawan (July to August). Tribals with religious sentiments consume the plant in the form of vegetable curry. Pharmaceutical studies have demonstrated that punarnava possesses diuretic (Gaitonde *et al.* 1974) and anti-inflammatory (Bhalla *et al.* 1968) activities, maximum activity being present in samples collected in the rainy season (Mudgal 1975). A combination of these two activities makes punarnava a very useful drug for the treatment of inflammatory renal diseases and common clinical problems like nephrotic syndrome (Singh and Udupa 1972); it is particularly useful as a maintenance drug. The roots are employed for many purposes including treatments of liver, heart, gallbladder, kidney, renal and urinary disorders (Mudgal 1975). The plant roots have an important place in herbal medicine in Brazil also, where it is considered "a plant medicine of great importance, extraordinarily beneficial in the treatment of liver disorders" (Cruz 1995). The hepatoprotective (Rawat *et al.* 1997) and antiproliferative (anticancer) potential (Mehrotra *et al.* 2002) of *B. diffusa* are documented. In traditional system of medicine, the roots have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, abdominal pain (Kirtikar and Basu 1956) and as anti-stress agent (Dandiya 1991). The roots also have anticonvulsant and analgesic properties (Adesina 1979). Root powder when mixed with mamira is used in eye diseases. It cures corneal ulcers and night blindness and is also used for restoration of virility in man. It has also other multiple actions such as - stomachic, diaphoretic, anthelmintic, febrifuge, antileprosy, antiasthmatic, antiscabies and antiurethritis (Nadkarni 1976). It is also reported to be a laxative, expectorant and useful in anemia, cough and cold and as antidote for snake venom (Chopra *et al.* 1956). Punarnavoside present in plant exhibits significant antifibrinolytic activity (Jain and Khanna 1989). In vitro testing of root extract showed that it has antibacterial (Olukoya *et al.* 1993) and antinematodal (Vijayalakshimi *et al.* 1979) properties. The leaf juice serves as a lotion in ophthalmia. It is given internally as a blood purifier and to relieve muscular pain. It also helps to hasten delivery (Shah *et al.* 1983). The herb, including the roots, is eaten as vegetable in curries and soups. The root and seeds are added to cereal, pancakes and other food. They also serve as a bird feed. The herb is relished by sheep and goats and in West Bengal is given to milch cows to improve the yield of milk (Anonymous 1988). The derivatives from the plant appears to exert a more powerful effect on certain type of ascites and oedema (Anonymous 1988). Plant juice is used as an antidote to rat poisoning. Plant powder is used against abdominal tumour and cancer. In Ayurveda flowers and seeds are used as contraceptives and seeds are aphrodisiac in nature (Chopra *et al.* 1956). *C. aculeatum* is an important plant growing mainly for hedge purposes. It has been shown to contain a novel basic protein in leaves, which is capable of inducing resistance/immunity in several susceptible hosts against commonly occurring plant viruses (Verma *et al.* 1984, 1995, 1999). Induced resistance is manifested in terms of reduced/complete prevention of local lesions or systemic symptoms.

Virus inhibitory properties of *B. diffusa* and *C. aculeatum*

Virus inhibitors present in different parts of higher plants are known to inhibit several plant viruses. These inhibitors modify the susceptibility of plants towards virus infection and multiplication. The roots of *B. diffusa* are rich source of a basic protein, which is used for inducing systemic resistance in many susceptible crops against commonly occurring viruses (Verma and Awasthi 1979, 1980; Verma *et al.* 1979; Awasthi *et al.* 1984, 1985, 1989; Verma *et al.* 1995, 1999). This protein or antiviral agent was active against both spherical and tubular viruses, in hypersensitive hosts (such as *Datura metel*, *Nicotiana tabacum* var Ky-58, *N. glutinosa*/TMV, *Cyamopsis tetragonoloba*/SRV, *Vigna*

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sinensis/SRV etc.) and systemic hosts (like *Nicotiana tabacum* c.v NP-31/TMV, *Crotalaria juncea*/SRV, *N. glutinosa*/TRSV etc.), when applied a few hours (1-24 hours) before virus infection or when tested after mixing along with virus inoculum (Verma and Awasthi 1979; Awasthi *et al.* 1984). The virus inhibitor is a basic glycoprotein (70-80% protein +8-13% carbohydrate) with a molecular weight of 16-20 kDa as determined by gel-filtration chromatography (Verma *et al.* 1979). The protein has a PI greater than 9 and gives a molecular weight of 30 kDa on SDS-PAGE (Srivastava 1995). The resistance inducing protein (virus inhibitor) is found to be extremely thermostable (Verma and Awasthi 1979). Following treatment with the systemic resistance inducing protein, the host produces a circulatory virus inhibitory agent (VIA). The VIA shows characteristics of protein and reduces (neutralizes) infectivity of the viruses both *in vitro* and *in vivo* (Verma and Awasthi 1980). Upon gel filtration on Sephadex G-75, two active fractions exhibiting protein characteristics are recovered (Verma and Awasthi 1980). The protein occurring in *B. diffusa*, functions as a signal molecule and is of great interest as it has a role in stimulating the defense system of plants against viruses (Verma *et al.* 1984, 1995, 1998; Singh *et al.* 2004; Singh and Awasthi 2004; Awasthi *et al.* 2006). The VIA is produced both in treated and untreated leaves of plants (Verma and Awasthi 1980). Micropropagation of *B. diffusa* has also been done for large scale and uniform production of systemic resistance inducing protein (SRIP) for viral disease management (Gupta 1999; Gupta *et al.* 2004). Susceptible healthy hosts upon treatment with extract from leaves of *C. aculeatum* develop almost complete resistance against viruses within 4-6 hours. Treated plants when inoculated with viruses do not develop virus disease symptoms. The resistance induction following leaf extract treatment is reversed by simultaneous application of actinomycin-D (Verma *et al.* 1984). Treatment with systemic resistance inducer (SRI) occurring in *C. aculeatum* leaves triggers formation and accumulation of a new defensive virus- inhibitory agent (VIA) in treated and non-treated leaves of healthy host plants (Verma *et al.* 1984; Verma *et al.* 1996, 1999; Srivastava 1999). The SRI present in leaf sap is resistant to denaturation by organic solvents and is extremely thermostable (Verma *et al.* 1984). The resistance inducing activity of clarified CA-SRI is not affected by exogenous application of proteases (Verma *et al.* 1996) and upon storage *in vitro* for 30-40 days. The SRI containing leaf extract of *C. aculeatum* is very effective for controlling virus diseases in crop plants (Verma *et al.* 1995a; Srivastava 1999; Srivastava *et al.* 2004), as the extract when sprayed on susceptible host plants, prevented infection of mechanically and white fly transmitted viruses in several susceptible hosts viz. *Chenopodium amaranticolor*; *Datura stramonium*, *D. metel*, *Nicotiana glutinosa*, *N. tabacum* var. Ky-58; *Cyamopsis tetragonoloba*; *Nicotiana tabacum* cv. NP-31 etc. (Verma *et al.* 1984; Verma *et al.* 1995a). *C. aculeatum* is propagated by vegetative cuttings. Naturally propagated plants (by traditional methods) from different locations, as well in different seasons show variation in amount and potency of SRI (Verma *et al.* 1998). Development of tissue culture method for micro propagation of selected genotypes has been found to be useful to produce large number of highly potent, genetically uniform plants, in considerably less time. Therefore, a suitable and reproducible protocol for rapid micro propagation of *C. aculeatum* was developed. Relative effectiveness of isolated inhibitor among established cultures was determined. Significant variability generated through biotechnological approaches facilitated in the establishment and conservation of specific potent high SRI yielding lines of *C. aculeatum* plants (Srivastava *et al.* 2004). Table 1 lists the plants, which contain inhibitor(s) of virus infection and/or anticancer agents as reported by several workers.

Table 1: List of higher plants reported to contain virus-inhibitory agents

Scientific name	Common name	Family	Potential	Reference
<i>Amaranthus albus</i> <i>Amaranthus aureus</i>	Tumble pigweed	Amaranthac eae	Antiviral	Smookler 1971
<i>Alternanthera amoena</i> ; <i>Alternanthera brasiliiana</i>	Calico-plant Bouton blanc	Amaranthac eae	Antiviral	Nornha <i>et al.</i> 1983
<i>Acacia arabica</i>	Babul	Leguminaca e	Antiviral	Gupta and Raychaudhuri 1971a
<i>Acer insulera</i>		Aceraceae	Antiviral	Yoshi <i>et al.</i> 1954
<i>Aconitum napellus</i> L.	Monkshood	Rannuncula ceae	Heart and nerve sedative,	Duke <i>et al.</i> 1985; Shoyama <i>et al.</i> 1991;

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			anticarcinogenic, and reduce fever	Roers <i>et al.</i> 1997
<i>Acorus calamus</i> L. var. <i>Americanus</i> Wolff <i>A. tatarinowii</i> L.	Calamus, sweet flag Shi Change Pu	Araceae	Used as a panacea antiviral. Antibacterial, antifungal, antiseptic,.	Bown <i>et al.</i> 1987; Chaturvedi <i>et al.</i> 1996; Small <i>et al.</i> 1999
<i>Agave americana</i>	Century plant	Agavaceae	Antiviral	Simons 1963
<i>Ailanthus excelsa</i>	Maharuk	Simaroubac eae	Antiviral	Patel and Patel 1979
<i>Allium sativum</i> L.	Garlic	Liliaceae	Anticancer (intestinal tract), antimicrobial, antiviral Carcinogenesis	Joshi and Praksh 1978; Balandrin <i>et al.</i> 1988; Chevallier <i>et al.</i> 1996; Small 1997; Arivazhagan <i>et al.</i> 2004
<i>Alternanthera ficoidea</i>	Calico-plant	Amaranthac eae	Antiviral	Noronha <i>et al.</i> 1983
<i>Amaranthus caudatus</i>	Ram dana	Amaranthac eae	Antiviral	Smookler 1971
<i>Argemone mexicana</i>	Bharbhand	Papaveracce ae	Antiviral	Patel and Patel 1979
<i>Arnica latifolia</i> Bong. <i>A. montana</i> L. <i>A. chamissonis</i> L.	Arnica	Asteraceae	Antineoplastic, antibacterial, antifungal, antiviral anti-inflammatory, antiseptic.	Cavallini <i>et al.</i> 1991; Kaziro 1990; Levin <i>et al.</i> 1987; Small <i>et al.</i> 1999 Willuhn <i>et al.</i> 1988
<i>Azadirachta indica</i>	Margoss tree (Neem)	Meliaceae	Carcinogenesis	Arivzhagan <i>et al.</i> 2004
<i>Beta vulgaris</i>	Chukandar	Chinopodiac eae	Antiviral	Paliwal and Narinani 1965
<i>Boerhaavia diffusa</i>	Punarnava	Nyctaginace ae	Antiviral, anticancerous	Verma and Baranwal 1988 Mehrotra <i>et al.</i> 2002; Sukhdev 2006
<i>Borago officinalis</i> L.	Borage	Boraginacea e	Anti-inflammatory, demulcent, folk remedies for cancer, corns, sclerosis, tumor.	Duke <i>et al.</i> 1985; Mierendorff <i>et al.</i> 1995 Rogers <i>et al.</i> 1997
<i>Bougainvillea spectabilis</i>		Nyctaginace ae	Antiviral	Verma and Dwivedi 1983
<i>Brassica oleracea</i>	Cauliflower	Brassicaceae	Antiviral	Verma 1973
<i>Callistemon lanceolatus</i>	Bottlebrush	Myrtaceae	Antiviral	Gupta and Raychaudhuri 1971a, b;
<i>Cassia senna</i> L. <i>C. angustifolia</i> Vahl.	Tinnevely senna Alexandriasenn a	Caesalpinia eae	Laxative, stimulant, anticancer, cathartic.	Duke <i>et al.</i> 1985; Chevallier <i>et al.</i> 1996

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<i>Carissa edulis</i>	Forssk	Apocynaceae	Antiviral	Tolo <i>et al.</i> 2006
<i>Celosia plumose</i>		Amaranthaceae	Antiviral	Patil 1973
<i>Chenopodium amaranticolor</i>		Chenopodiaceae	Antiviral	Smookler 1971 Alberghina 1976
<i>Chenopodium ambrosioides</i>	Mexican-tea	Chenopodiaceae	Antiviral	Verma and Barranwal 1983
<i>Catharanthus roseus</i>	Sadabahar	Apocynaceae	Anticancerous	Costa-Lotufo <i>et al.</i> 2005
<i>Cinchona ledgeriana</i>	Quinine	Rubiaceae	Antiviral	Gupta and Raychaudhuri 1971b
<i>Cinnamomum verum</i> J.Pres L.	Cinnamon	Lauraceae	A warming stimulant it is carminativeantiviral antispasmodic, antiseptic	Duke <i>et al.</i> 1985; Chevallier 1996
<i>Citrus limon</i> (L.) Burm. <i>C. bergamia</i> Risso & Poit, <i>C. reticulata</i> , <i>C. aurantifolia</i> <i>C. aurantium</i> L.	Lemon Bergamot orange Mandarin Lime,Bitter orange Grapefruit	Rutaceae	A contraceptive, treat cancer, heart and liver ailments, Antiseptic, Laxative.	Mansell <i>et al.</i> 1991 Chevallier 1996; Ajaiyeoba <i>et al.</i> 2003 Arias and Laca 2005 Kang <i>et al.</i> 2005
<i>Citrus medicatimonum</i>	Bara nimbu	Rutaceae	Antiviral	Ray <i>et al.</i> 1979
<i>Clerodendrum aculeatum</i>		Verbenaceae	Antiviral	Verma <i>et al.</i> 1984 Srivastava 1999; Srivastava <i>et al.</i> 2004
<i>Clerodendrum fragrans</i>	Glory-bower	Verbenaceae	Antiviral	Verma <i>et al.</i> 1984
<i>Cnicus benedictus</i> L.	Blessed thistle	Asteraceae	Antibacterial, carminative, Juice is used to treat tumors and warts	Chevallier 1996; Balandrin <i>et al.</i> 1988
<i>Cocos nucifera</i>	Coconut	Arecaceae	Antiviral	Gendron 1950
<i>Cucurbita maxima</i>	Red pumpkin	Cucurbitaceae	Antiviral	Weintraub and Willison 1983
<i>Cuscuta reflexa</i>	Amarbel	Cuscutaceae	Antiviral	Awasthi 1982
<i>Datura metel</i>	Sadahdlatura	Solanaceae	Antiviral	Verma and Awasthi 1975
<i>Datura stramonium</i>	Datura	Solanaceae	Antiviral	Paliwal and Narinani 1965
<i>Dianthus caryophyllus</i>	Carnation	Caryophyllaceae	Antiviral	Van Kammen <i>et al.</i> 1961
<i>Elaeagnus angustifolia</i> L.	Russian olive	Elaeagnaceae	Antimicrobial	Goncharva <i>et al.</i> 1990; Rogers 1997
<i>Eucalyptus tereticornis</i>	Blue gum	Myrtaceae	Antiviral	Ray <i>et al.</i> 1979

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<i>Embolia officinalis</i>	Avla	Euphorbiaceae	Carcinogenic/Anticancer	Rajeshkumar <i>et al.</i> 2003
<i>Eugenia jambolana</i>	Jambolan	Myrtaceae	Antiviral	Verma <i>et al.</i> 1969
<i>Euphorbia hirta</i>	Asthmaplant	Euphorbiaceae	Antiviral	Weeraratne 1961
<i>Gomphrena globosa</i>	Gloabamarauth	Amaranthaceae	Antiviral	Grasso and Shephard 1978
<i>Gyandropsis pentaphylla</i>	Hulul	Asteraceae	Antiviral	Paliwal and Nariani 1965
<i>Helianthus annuus</i>	Sun flower	Asteraceae	Antiviral	Johari <i>et al.</i> 1983
<i>Hordeum vulgare</i>	Barley	Gramineae	Antiviral	Leah <i>et al.</i> 1991
<i>Jasminum grandiflorum</i> L. <i>J. auriculatum</i> Vahl.	Royal jasmine	Oleaceae	Treatment of high fever, sunstroke, cancer (Hodgkin's disease)	Jonarad 1989; Brinda <i>et al.</i> 1998
<i>Lobelia pulmonaria</i> L.	Lungwort	Linaceae	Stimulate immune system, antitumor, cancer	Bunney 1992
<i>Mirabilis jalapa</i>	4'o; clock	Nyctaginaceae	Antiviral	Kataoka <i>et al.</i> 1991
<i>Narcissus pseudonarcissus</i> L.	Daffodil	Amayllidaceae	Treat coughs and colds, inhibits HIV-I and -2 infection of cells <i>in vitro</i>	Bracco 1973; Bastida <i>et al.</i> 1994; Kreh <i>et al.</i> 1995
<i>Oenothera biennis</i> L.	Evening primrose	Onagraceae	Antitumor, multiple sclerosis.	Bremness 1994; Mulherjee & Kiewitt 1987
<i>Opuntia robusta</i>	Wheel cactus	Cactaceae	Antiviral	Simonas <i>et al.</i> 19635
<i>Petunia hybrida</i>	Garden petunia	Solanaceae	Antiviral	Singh 1972
<i>Phyllanthus fraternus</i>	Jaramla	Euphorbiaceae	Antiviral	Saigopal <i>et al.</i> 1986
<i>Phytolacca dodecandra</i>		Phytolaccaceae	Antiviral	Reddy <i>et al.</i> 1984
<i>Podophyllum peltatum</i> L.	Mayapple	Berberidaceae	Skin cancer and leukemia, cholera	Fujii 1991; Stahein <i>et al.</i> 1991; Small <i>et al.</i> 1999
<i>Pseudoranthemum atropurpureum</i>		Acanthaceae	Antiviral	Verma <i>et al.</i> 1985
<i>Rabdosia rubescens</i>	Hara		Antitumor	Ren <i>et al.</i> 2006
<i>Rumex hastatus</i>	Amlora	Polygonaceae	Antiviral	Singh <i>et al.</i> 1977
<i>Solanum nigrum</i>	Makoi	Solanaceae	Antiviral	Vasudeva and Nariani 1952
<i>Salix caprea</i>	Goat willow	Salicaceae	Carcinogenesis	Sultana and Saleem 2004
<i>Sassafras albidum</i> (Nutt.) Nees	Sassafras	Lauraceae	Carcinogenic and mutagenic activities	Duke 1985; Heikes 1994
<i>Sedum rosea</i> (L.) Scop. Subsp. <i>Interifolium</i>	Roseroor	Crassulaceae	Improve learning and memory and reduce stress, anticancer	Zapesochaya <i>et al.</i> 1983; Small <i>et al.</i> 1999

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<i>Sesamum indicum</i> L. <i>S. orientale</i> L.	Sesame	Pedaliaceae	Antitumor,antiviral prevent breast cancer	Chung <i>et al.</i> 1995; Ogasawara <i>et al.</i> 1998
<i>Syringa vulgaris</i> L.	Lilac	Oleaceae	Antimicrobial, immunestimulating	Rogers 1997; Chapple 1991
<i>Syzygium aromaticum</i> (L.) Merr. & Perry	Clove	Myrtaceae	Therapeutic antiherpes simplex virus.	Duke <i>et al.</i> 1993; Zheng <i>et al.</i> 1992
<i>Taxus x media</i> Rehd <i>T. brevifolia</i> Nutt.	Yew	Taxaceae	Treat cancer, arthritis.	Wickremesinhe <i>et al.</i> 1998;Whiterup <i>et al.</i> 1990;Small <i>et al.</i> 1999
<i>Terminalia chebula</i>	Harra	Combretaceae	Antiviral	Gupta and Raychaudhuri 1971b
<i>Tetragonia expansa</i>	New Zealand (spinach)	Tetragoniaceae	Antiviral	Benda 1956
<i>Uncaria tomentosa</i> (Willd.) DC.U. <i>uianensis</i> (Aubl.) Gmel.	Cat's claw	Rubiaceae	Antiviral, Antitumor, Stimulating immunologic system	Wurm <i>et al.</i> 1998; Obregon 1995; Keplinger 1999
<i>Vinca minor</i> L.	Periwinkle	Apocynaceae	Leukemia, anticancer, hodgkin's disease	Duke 1985; Willuhn 1998
<i>Vitex negundo</i> L.	Chinese chaste tree	Verbenaceae	Breast cancer, malaria, poisonous bits.	Chevallier 1996
<i>Zingiber officinale</i>	Adrak	Zingiberaceae	Antiviral	Ray <i>et al.</i> 1979

Section I

Protection of crops from viral diseases by inducing immunity in plants

Resistance to plant viruses

A number of natural and synthetic products are known to alter the ability of viruses to infect susceptible plants and animals (reviewed by Bawden 1954), Matthews (1970), Verma *et al.* (1974), Verma *et al.* (1979), Lakhmatova (1991), Verma and Prasad (1992), Kowalewski and Schmitt (1993), Day (1993), Metraux *et al.* (1993), and Verma *et al.* (1995). Such products are obtained from different parts of the plants mostly leaves or roots from bacteria, fungi, insects, enzymes, milk, blood, egg white, organic and inorganic chemicals and inactivated viruses (Stanley 1935; Bawden 1954; Bartels 1955; Ramakrishna 1963; Gibbs and Harrison 1975; Horsfall and Cowling 1977). Resistance is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor. Disease resistance in plants is manifested by mold/or no symptoms, reflecting the inability of pathogen to grow or multiply and spread (Agrios 1988). Gianinazzi (1982) identified two classes of substances which affect the course of disease viz. the antiviral agents and the inducers of resistance. The antiviral agents were further split into those agencies which possess the ability to inhibit virus infection *in vitro* and *in vivo*. Some of these inducers are pathogenic in origin, others synthetic, or obtained from extracts of a few higher plants. Induced resistance is the phenomenon that a plant, once appropriately stimulated, exhibits resistance upon challenge inoculation with a pathogen (Hammerschmidt and Kuc 1995). A number of inducers incite antiviral state only at site of application, which is termed as local induced resistance (Ross 1961a; Lobenstein 1972), while that induced by others, extends beyond the point of treatment, and is called as systemic induced resistance (Ross 1961b). Gilpatric and Weintraub (1952) were the first to report development of systemic resistance in the upper non-infected leaves of *Dianthus barbatus* whose lower leaves had been inoculated with Carnation mosaic virus (CarMV). Systemic resistance inducers from pathogens, chemicals and

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extracts are known to alter the susceptibility of the host to subsequent challenge infection by triggering an induced-resistance state in the plant tissue.

Mechanism of inhibition

Mechanism of induction of systemic resistance by the systemic resistance inducer (SRI) occurring in a few plants was first detailed by Verma and Mukherjee (1975). SRIs induce an anti-viral state in susceptible hosts through mechanisms which are actinomycin-D (AMD) sensitive and presumably involve synthesis of some new virus-inhibitory agent (VIA) by the host (Verma 1980). AMD is an inhibitor of protein synthesis at transcription level giving an indication of SRI-induced resistance being a host-mediated response. The VIA formation was also observed in several hosts upon treatment with *Pseuderanthemum bicolor* leaf extract (Khan and Verma 1990), *B. diffusa* root extract (Verma and Awasthi 1980), *Bougainvillea spectabilis* leaf extract (Verma and Dwivedi 1984). The VIA(s) synthesized is neither virus-specific nor host-specific. Extract containing VIA, when incubated with viruses reduced their infectivity. The time for maximum production of VIA varies in hosts treated with different plant extracts (Srivastava 1995). VIA is presumably present in very low amounts in non-treated plants but following treatment with a SRI, its accumulation increases dramatically. Thus it appears that certain specific plant constituents can act as signal molecules activating pre-existing plant defense responses.

Nature of virus inhibitors from plants

Kuntz and Walker (1947) made an attempt, for the first time to investigate the nature of the inhibitory property of spinach extract. Kassanis and Kleczkowski (1948) were the first to purify the virus inhibitor from pokeweed (*Phytolacca americana*). They suggested that the inhibitor was probably a glycoprotein. First inhibitor was characterized from *Dianthus caryophyllus* and shown to be proteinaceous (Ragetli and Weintraub 1962). Wyatt and Shepherd (1969) later on found that active moiety in the inhibitor was only protein. Mostly virus inhibitory substances showed characteristics of proteins and glycoprotein's (Ragetli and Weintraub 1962; Irvin 1975) but some may be carbohydrates (El-Kandelgy and Wilcoxson 1966), phenolics (Thresh 1956) or alkaloids (Leven *et al.* 1982). There are two well characterized classes of basic virus inhibitory proteins from higher plants. One class of basic proteins catalytically damage eukaryotic ribosomes and have been called ribosome inactivating proteins (RIPS) (Barbieri and Stirpe 1982). Yet another class of basic proteins induce an antiviral state in plants through formation of *de novo* synthesis of defensive protein(s) and perhaps are active in signaling the activation of defense mechanism in susceptible host and hence have been called as systemic resistance inducer (SRI) (Verma *et al.* 1996; Prasad *et al.* 1995; Srivastava 1999). Inhibitors from different plant extracts get inactivated upon heating at different time intervals. Inhibitor from French bean was inactivated at 50°C (Nart 1972), *Bougainvillea spectabilis* at 80°C, of *Mirabilis jalapa* at 90°C (Verma 1985), of *Phytolacca Americana* at 100°C (Kassanis and Kleczkowski 1948) and of *Rumex hastatus* at 120°C (Singh *et al.* 1977). There occurs a closeness in molecular weights of anti-viral proteins obtained from different plant extracts. The molecular weight of antiviral proteins from some plants were- 30 kDa from *Boerhaavia diffusa* (Srivastava 1995), 29 and 34 kDa from *Clerodendrum inerme* (Prasad *et al.* 1995), 34 kDa from *C. aculeatum* (Verma *et al.* 1996), 29 kDa (Irvin 1975), 30 kDa (Irvin *et al.* 1980) and 31 kDa (Barbieri *et al.* 1982) from *Phytolacca americana*, 30 and 32 kDa from *Dianthus caryophyllus* (Stirpe *et al.* 1981) and 24 kDa from *Mirabilis jalapa* (Kubo *et al.* 1990).

IV. CHARACTERISTICS OF ANTIVIRAL PROTEINS MEDIATING VIRAL DEFENCE IN PLANTS

Ribosome inactivating proteins

Pokeweed Antiviral Proteins (PAPs)

The antiviral protein present in the leaves of *Phytolacca americana* (Duggar *et al.* 1925) was purified to homogeneity and its molecular weight determined as 29 kDa (Irvin 1975). This protein, called PAP, had a pI of 8.1 (Irvin 1983). The antiviral effect of PAP was most pronounced when it was co inoculated with the virus. PAP also inhibited virus infection when applied prior to virus challenge. Local lesion formation by TMV on *N. tabacum* cv. Xanthi-nc was inhibited by nearly 70% even after 48 hours of treatment. The virus inhibitory effect of PAP increased with the decrease in time lapse between treatment and challenge inoculation. PAP was less effective in preventing virus infection when applied a short time after virus inoculation. No inhibition was observed when PAP was applied 50 minutes after virus infection (Chen *et al.* 1991). PAP reduced infectivity of several mechanically transmitted RNA and

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DNA viruses when the purified virus or sap from virus infected plants was mixed with an equal volume of PAP solution and the mixture rubbed on the leaves of the local-lesion hosts (*N. glutinosa* /TMV; *Chenopodium quinoa* /CMV; *C. amaranticolor*/TMV, CMV, alfalfa mosaic virus, PVY; *Gomphrena globosa* /PVX) or systemic hosts (*Brassica campestris*/cauliflower mosaic virus; *N. benthamiana*/ African cassava mosaic virus). PAP, thus, appears to be a general inhibitor of virus infection (Tomlinson *et al.* 1974; Stevens *et al.* 1981; Chen *et al.* 1991). PAP also shows antiviral activity against several animal viruses. It is toxic to cells infected with poliovirus (Ussery *et al.* 1977), influenza virus (Tomlinson *et al.* 1974) and inhibits multiplication of herpes simplex virus type 1 (Arnon and Irvin 1980) & human immunodeficiency virus (Zarling *et al.* 1990). PAP did not show any detectable activity against two bacterial and six fungal pathogens (Chen *et al.* 1991). *P. americana* is now known to contain three other proteins (PAP I, II and III) with similar biological properties.

Carnation Antiviral Proteins (DIANTHINS)

Sap from carnation leaves shows virus inhibitory activity (Van Kammen *et al.* 1961; Ragetli *et al.* 1962). Dianthin 30 and Dianthin 32 were isolated from the leaves of *Dianthus caryophyllus* (Ragetli *et al.* 1962). Local lesion production by TMV on *N. glutinosa* was inhibited by 100% when the inhibitor was co inoculated with the virus (Stevens *et al.* 1981). These are probably the same proteins purified by Ragetli and Weintraub (Ragetli *et al.* 1962). The MW as determined by SDS-PAGE is 29.5 and 31.7 kDa, respectively (Stripe *et al.* 1981). Immunoelectrophoretic methods revealed that Dianthin 32 is distributed in the growing shoots and in the young and old leaves of *D. caryophyllus* and Dianthin 30 is distributed throughout the plant (Reisbig and Bruland 1983). The two are glycoproteins containing mannose and show a weak cross reaction. The nucleotide sequence of cDNA coding for Dianthin 30 has been determined (Legname *et al.* 1991). The carnation proteins are also inducers of systemic resistance (Plobner and Leiser 1990).

Mirabilis Antiviral Protein (MAP)

The roots, leaves and stem of *Mirabilis jalapa* show strong inhibitory activity against plant viruses. The *Mirabilis jalapa* leaf extract, when used as a foliar spray 24 hours prior to virus inoculation, suppressed the disease symptoms on a few systemic hosts (tomato/tomato yellow mottle virus; *Cucumis melo* var. *momordica*/CMV; *Cucumis sativus* /cucumber green mottle mosaic virus; tomato/tomato yellow mosaic virus; urd/yellow mosaic of urd) (Verma and Kumar 1980). A 50-60% reduction of the virus content in the treated plants was observed in the infectivity assays. *Mirabilis jalapa* extract was able to check the population of aphids and whiteflies and, thereby, control the natural spread of a few viruses on the systemic hosts (Verma and Kumar 1980). The Mirabilis antiviral protein (MAP) roots inhibits mechanical transmission of TMV, PVY, cucumber green mottle mosaic, virus and turnip mosaic virus on local-lesion and systemic hosts and induced systemic resistance of low order, when applied to basal leaves of Xanthi-nc tobacco, 24 hours prior to TMV inoculation of upper leaves (Kubo *et al.* 1990). The purified protein consists of a single polypeptide without a sugar moiety and has a MW of 24.2 kDa. It is a basic protein rich in lysine content, with a pI of 9.8 (Kubo *et al.* 1990). The complete amino acid sequence of MAP has been determined. It consists of 250 amino acids and its MW as determined from the amino acid sequence is 27,833 Da. The native MAP was resistant to protease digestion (Habuka *et al.* 1989). MAP produced by *Mirabilis jalapa* cells in suspension culture showed comparable biological activity with that of the roots and leaves and also reacted positively with anti-MAP serum (Ikeda *et al.* 1987). Several nutritional and hormonal factors also affect the formation of MAP by *M. jalapa* cells in suspension culture (Ikeda *et al.* 1987).

Mechanism of Systemic Induced Resistance by Botanicals

Systemic induced resistance against virus infection by botanicals has not been fully understood. The botanical resistance inducers themselves do not act on the virus directly. Verma and Awasthi (1979), Verma *et al.* (1980) demonstrated the *de novo* synthesis of an antiviral substance(s) in untreated leaves of *N. glutinosa*, whose basal leaves were treated with root extract of *B. diffusa*. These induced substances upon incubation with viruses inhibited almost completely tobacco mosaic virus infection in *N. glutinosa*, *Datura stramonium* and *D. metel* but inhibition of tobacco ring spot virus or gomphrena mosaic virus in *Chenopodium amaranticolor* was less pronounced. In another study Verma and Dwivedi (1984) found that virus inhibiting agent (VIA) induced in *C. tetragonoloba* inhibited completely the infection of tobamoviruses in all the 7 hypersensitive hosts tested. Yet in another study by Khan and Verma (1990), it was observed that VIA produced in *C. tetragonoloba* following treatment with extract of *Pseuderanthemum bicolor*

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inhibited completely SRV, TMV, cucumber green mottle mosaic virus and PVX in their respective hypersensitive hosts viz., *C. tetragonoloba*, *D. stramonium*, *C. amaranticolor* and *G. globosa*. No attempt has been made about comparative studies for VIA production in different hosts by different resistance inducers. However, the VIA produced by *B. spectabilis* in *N. tabacum* cv. samsun NN, *N. glutinosa*, *D. stramonium* was less effective against TMV (Verma and Dwivedi 1984). Thus it seems induction of systemic resistance by botanicals is non-specific and is effective against a broad spectrum of viruses. It has been demonstrated that the production of VIA is maximum after 24 hours of application of *B. diffusa* root extract in *N. glutinosa*. However, *B. spectabilis* induced maximum VIA activity after 48 hours of its application in *N. glutinosa*. Thus VIA production in a host is time specific but a general phenomenon. Properties of VIA produced in *C. tetragonoloba* after application of *B. spectabilis* leaf extract has been studied by Verma and Dwivedi (1984). The VIA could be precipitated by ammonium sulphate and hydrolyzed by trypsin but not by ribonuclease indicating that it was proteinaceous rather than a nucleic acid. The VIA induced by *Pseuderanthemum bicolor* in *C. tetragonoloba* was also proteinaceous with a MW of 15 kDa (Khan and Verma *et al.* 1990). The production of VIA was sensitive to actinomycin D (Verma and Dwivedi 1984). Some mobile signal produced VIA in the entire plant system. It appears that the putative messenger becomes active soon after induction and starts producing VIA, reaches a maximal concentration and then starts to decline. The role of salicylic acid (SA) as endogenous signal in systemic acquired resistance has been studied (Gaffney *et al.* 1993) but role of SA in systemic resistance induced by botanical substances has not yet been studied. Commonly associated with systemic acquired resistance, induced by pathogens is the systemic synthesis of several families of serologically distinct low molecular weight pathogenesis related (PR) proteins. The localization and timing of some PR proteins suggested their possible involvement in acquired resistance against viruses. However, definite proof that the induction of PR proteins causes the acquired resistance against viruses has not been given. Plobner and Leiser (1990) did not find the production of PR proteins during systemic resistance induced by carnation extract in Xanthi-nc tobacco plants. Absence of PR proteins during induction of SAR by botanicals suggests that another biochemical chain of reaction, other than one operating in pathogen/chemical induced SAR, might be operating during botanical induced systemic resistance against virus infection in plants. The agriculture role of endogenous antiviral substances of plant origin has been reviewed by Verma *et al.* (1995). Hansen (1989), while reviewing antiviral chemicals for plant disease control, gave the following characteristics of ideal antiviral compounds that will serve all purposes for virus disease management in crops.

1. Soluble in water or non phytotoxic solvents.
2. Effective against at least some agriculturally or horticultural important viruses at non phytotoxic concentration.
3. Easily taken up by plants and distributed throughout the system.
4. Non toxic by itself and its catabolic forms to humans, plants and wildlife.

Botanical resistance inducers can be classified as ideal virus suppressing agents as they qualify all the characteristics of ideal antiviral compound. The resistance inducing proteins from *Boerhaavia diffusa* and *Clerodendrum aculeatum* can be applied directly by spraying on systemic host for management of some commonly occurring virus diseases under greenhouse conditions or field conditions in micro plots. However, their effectiveness on larger scale in fields needs to be evaluated. The induced antiviral state in *N. glutinosa* by SRI from *C. aculeatum* decreased considerably after 3 days (Verma and Varsha 1994). However, the resistance inducing ability of *C. aculeatum* SRI could be enhanced up to 6 days by priming it with certain proteinaceous additives (Verma and Varsha 1994). The activity is probably enhanced due to modification or enhanced stability of proteinaceous inducers by these additives. The expression of resistance inducers in transgenic plants may overcome the constraint of their large scale production. Thus, one unexploited approach to engineering virus resistance is the manipulation of inducible defence in plants. The production of systemic resistance by use of botanical genes will be effective against a broad spectrum of viruses and will not break down when plants will be exposed to high temperatures.

Management of virus diseases

Plant viruses are a major cause of concern among agriculturists because of their economic impact on crop they infect. To reduce losses, scientists have explored several strategies to control virus infection. However, it is the exploitation of inherent resistance phenomenon and manipulation of inducible defense in plants which is receiving much attention by researchers. The common approach for introducing resistance against virus in crops has been through conventional plant breeding. Limited success has been achieved through this method. Recent advances in molecular biology of resistance to virus infection have opened new approaches of making susceptible crops resistant against virus infection.

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These approaches include pathogen derived resistance to viruses (coat protein mediated resistance, movement protein mediated resistance, replicas and protease mediated resistance) and virus resistance through transgenic expression of antiviral proteins of non-viral origin (Baulcombe 1994).

Section II

***In vitro* stimulation of growth of plants and virus resistance using phytoproteins**

Micro propagation

The goal of micro propagation is to mass propagate genetically identical, physiologically uniform, and developmentally normal plants that can be acclimatized quickly and inexpensively (Kozai and Jeong 1993). Clonal multiplication of plants via tissue culture can be carried out by four methods, namely:

- a. enhanced axillary bud breaking
- b. production of adventitious buds
- c. callus cultures and regeneration
- d. somatic embryogenesis

Plant cell and tissue culture research is a "growth industry" today. The term plant tissue culture denotes generally all cell tissue and organ cultures. The most significant advantage of clonal propagation (Micro propagation) over the conventional methods is that in a relatively short time and space a large number of plants can be produced starting from a single individual (Murashige 1974). Its impact on agriculture began with Morel's (1960) suggestion to explore the applicability to clonal multiplication of flower and ornamental crops. During the last 25 years, *in vitro* culture of higher plants has become an important tool for the following possible reasons:

1. Available methods for vegetative propagation may be too slow and/or non profitable,
2. Conventional vegetative propagation may not be possible,
3. Need for a large number of plants of selected genotype
4. The need of plants which are free from pathogen (viruses, bacteria, molds and nematodes).
5. Production of pharmaceutical and other natural products,
6. Genetic improvement of crops,
7. Recovery of disease-free clones and preservation of valuable germplasm and
8. Rapid clonal multiplication (micro propagation) of selected varieties and elites.

V. *IN VITRO* ESTABLISHMENT

The concept of developmental stages proposed by Murashige (1974) is:

Stage I: Establishment of aseptic cultures,

Stage II: Multiplication of propagules and

Stage III: Establishment of plants in soil

This concept suggests that a single medium usually is not sufficient for *in vitro* plant multiplication and regeneration. The age and physiological state of the parent plant may contribute to the success of organogenesis in cultures (Thorpe and Biondi 1984; Das *et al.* 1996; Agrawal *et al.* 1997; Singh 2001). The season can affect callus initiation from explants, especially when the donor plant is field grown (Tisserat 1985; Chaturvedi *et al.* 1993; De Clerck and Korban 1994; Komalavalli and Rao 1997; Chaturvedi and Agnithotri 2002). Seasonal variation in the concentration of endogenous auxins has been observed by Wodzicki (1978). Expanding shoots soon after bud break help in successful establishment of culture *in vitro* (Poessel *et al.* 1980; De Clerck and Korban 1994). Seasonal change also influenced the explant viability and contamination of cultures in scot pine (Hohtola 1988). Culture establishment with mature plant material was more difficult due to problems like microbial contamination (Debergh and Maene 1981), phenolic exudations (Marks and Simpson 1990), reduced growth and rooting ability (Pierik 1993). Size (Das *et al.* 1996; Komalavalli and Rao 1997) and position (Komalavalli and Rao 1997; Puddephat *et al.* 1997) of the explants also determined the survival of the cultures. Plant growth regulators (Agrawal *et al.* 1997) and additives like ascorbic acid and gibberelic acid (Komalavalli and Rao 1997; Puddephat *et al.* 1997) were shown to affect the process.

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VI. CULTURE MEDIA

The success of plant propagation is greatly influenced by the constituents of the culture medium (George and Sherrington 1984; Komalavalli and Rao 1997; Puddephat *et al.* 1997; Das *et al.* 1996). Low concentration of nitrogen and potassium were found suitable for axillary bud induction (Garton *et al.* 1981; Goh *et al.* 1988). Use of original and modified MS formulation for culture establishment was reported by Kuberski *et al.* (1984), Chaturvedi and Jain (1994). The specific protocols for the different media ingredients have been developed starting with the original experiments of White (1963). Most of the basic formulations are of Murashige and Skoog (MS) (1962), White (1963), Linsmeir and Skoog (1965), Gamborg *et al.* (1968). MS medium contains a high concentration of nitrogen as ammonium (20 mM), while White's medium (White 1963) is ammonium free. White's medium is a low salt medium, while the other media are classified as high salt media. For the growth of a new variety of plant cells, the inorganic ingredients are generally used as specified. Only the plant growth hormones are varied both in kind and concentration. The source of energy in the form of exogenously supplied carbohydrate is very important for growth and proliferation of cultures (Leifert *et al.* 1995). The organic supplements required in plant culture media include a carbon source like sucrose at a concentration of 2-3% in most media (van Uden *et al.* 1991; Woerdenbag *et al.* 1993; Berlin 1988; Singh 2001). Other sugars are used less often. Some special cultures may require glucose or another carbohydrate. Although some of the media formulations call for a number of vitamins, most plant cells grow very well with thiamine only. However, additional vitamins may have to be added to obtain optimum growth. Vitamins most often added to culture medium include inositol, nicotinic acid, pyridoxine, thiamine, calcium pantothenate, and biotin. Thiamine is required for plant growth. Vitamin-D may enhance root formation (Buchala and Schmid 1979). Most media contain at least 100 mg/l of myo-inositol. Its function is not known, however, addition to the medium increases growth rate. The medium is adjusted to pH 5.8 before autoclaving. Media ingredients are stable to autoclaving except 6-benzyl adenine and indole acetic acid which require filter sterilization.

Hormones

Exogenous auxins are required for shoot growth (Dale 1975), because in some cases resting bud may not contain endogenous auxin for shoot growth (Hu and Wang 1983). Normal proliferation pattern change in presence of exogenous auxin (Murashige 1974). Low level of auxin had synergetic effect on shoot bud induction while higher concentration results in callus formation at the base of the explants (Kaur *et al.* 1998). GA helped in shoot bud induction (Komalavalli and Rao 1997) and shoot elongation (Schnabdrauch and Sink 1979; Singh 2001).

Culture of shoot tips and nodal stem segments

There are very few reports of shoot proliferation from culture of its good-sized shoot tips or from nodal stem segments and perhaps none by using explants of field-grown mature trees despite several attempts made in this direction. Rangaswamy (1975) cultured shoot tips or apices of seedling of *C. microcarpa* and could grow them only as single shoots. Kitto and Young (1981) attempted shoot tip culture of seedlings of sour orange (*C. aurantium*), Carrizo citrange (*C. sinensis* X *P. trifoliata*) and Cleopatra mandarin (*C. reshni*), but only Carrizo citrange showed appreciable shoot proliferation. Also, Barlass and Skene (1982) reported that shoot tip culture has been successful only in Carrizo citrange, though they experimented with a number of *Citrus* species, including *C. reshni*, *C. limonia* and *C. sinensis*. However, by using nodal stem segments of seedlings of Carrizo citrange, *P. trifoliata*, *C. limonia*, *C. reshni* and *C. sinensis*, proliferation of shoots could be obtained (Barlass and Skene 1982). Chaturvedi and Sharma (1987, 1988) also reported shoot proliferation from single-node stem segments of *C. aurantifolia*, *C. sinensis*, *C. karna* and *C. jambhiri* of aseptically-grown seedling plants except in case of *C. aurantifolia*, where a limited success could be achieved with explants of field-grown trees. Subsequently, proliferation of shoots and recovery of complete plantlets from nodal stem segments of several *Citrus* species and cultivars have been reported again by using seedling explants (Barlass and Skene 1986; DuranVila *et al.* 1989, 1992). However, regeneration of nodal explants and shoot tips of one-and-a-half-year-old grafted plants of *C. mitis* (*C. microcarpa*), which otherwise has been reported to be highly regenerative and is highly polyembryonic, has been obtained (Sim *et al.* 1989). In general, a nodal stem segment has been more responsive than a shoot tip for shoot culture. Medium formulations of Murashige and Skoog (1962) and Murashige and Tucker (1969) have been generally employed for culturing shoot tips and nodal stem segments. Generally, BAP has been a commonly used cytokinin to stimulate shoot proliferation in citrus, while other cytokinins have been less effective (Chaturvedi and Mitra 1974; Bhansali and Arya 1978; Kitto and Young 1981; Barlass and Skene 1982). Addition of

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auxin to a BAP containing medium has been found beneficial for shoot proliferation (Chaturvedi and Mitra 1974; Bhansali and Arya 1978), albeit not in all cases (Barlass and Skene 1982). Of the three nitrogenous complex substances, viz., malt extract, casein hydrolysate and yeast extract, the first one has been found to be beneficial for improving growth of shoots regenerated under the influence of BAP or BAP and auxin (Chaturvedi and Mitra 1974; Chaturvedi and Sharma 1988; Singh 2001).

Shoot multiplication

The rate determining factors include culture conditions and medium composition (Chu 1992), nature of propagule (Dhawan 1993) and age of the plants (Chalupa 1987). The phenomenon of apical dominance is controlled by the interaction of growth regulators. The detipping (Gumbley and Dodd 1990) or application of cytokinin overcome the apical dominance effect and stimulate lateral buds (Pierik 1993). Induction of multiple shoots was best in presence of BA (Bhojwani 1980; Siril and Dhar 1997; Sujatha and Dhingra 1993). Various concentrations of BA showed differential response (Jackson and Hobbs 1990). Chance of somaclonal variation was low when multiplication was conducted by growing shoots in BA-free medium (Hosoki and Katahira 1994) and by means of axillary branching induced by cytokinin (George and Sherrington 1984). Increased concentration of BA inhibited shoot elongation (Santos *et al.* 1990; Brassard *et al.* 1996). Cytokinin in combination with auxin promoted shoot growth and elongation (Santos *et al.* 1990; Agarwal *et al.* 1992).

Induction of rooting in micro shoots

All the methods of citrus micro propagation apart from embryogenesis result in the production of shoots, which must be rooted to yield plantlets. In citrus, differences have been reported in the rooting responses of the *in vitro* – regenerated shoots of different *Citrus* species and cultivars (Duran-Vila *et al.* 1989, 1992). The majority of rooting experiments have been conducted with Murashige and Skoog's (1962) or Murashige and Tucker's (1969) medium and with low salt media, such as, White's (1943) medium (Barlass and Skene 1982), half-strength Murashige and Skoog's (1962) medium (Sim *et al.* 1989) as also with that of Chaturvedi's (1979); Singh (2001) medium having reduced concentrations of NH_4NO_3 , KNO_3 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The most commonly used auxins for root induction are NAA or IBA or IAA. Once root has been initiated, shoots are transferred to nurturing (elongation) medium (auxin-free medium), where root growth continues (Kito and Young 1981; Barlass and Skene 1982; Duran-Vila *et al.* 1989; Ghorbel *et al.* 1998). In most of the rooting experiments, 2-3% sucrose has been used. Some *Citrus* species have successfully been rooted even at its higher concentration of 5% (Chaturvedi and Mitra 1974; Murashige and Tucker 1969). Besides, the size of regenerated shoots also affected the rooting of citrus (Duran-Vila *et al.* 1989). Therefore, Ghorbel *et al.* (1998) selected shoots of Alemow (*C. macrophylla*) measuring not less than 0.5 cm in length for rooting and got 84% success and upon transplanting the rooted plantlets to soil, their 16% survival has been reported. The intervening callusing between root and shoot, which prevents proper vascular connections, may explain this low rate of transplant success. As an alternative to rooting, *in vitro* grafting of regenerated shoots onto rootstock seedling has been successfully performed by many workers to recover whole plants of many Citrus species (Pena *et al.* 1995a, b; Ghorbel *et al.* 1998; Singh 2001). Chaturvedi (1979), Chaturvedi *et al.* (1982), Chaturvedi and Sharma (1988) have reported high percentage of rooting in the isolated callus-regenerated shoots of several *Citrus* species, viz., *C. grandis*, *C. sinensis*, *C. aurantifolia*, *C. karna* and *C. jambhiri* with sole supplement of 0.5 or 0.75 mg/l NAA. However, such rooted shoots have not survived after transplantation to soil; until tailoring of root from many roots per shoot proceeded the callusing to tap root-like system, without intervening callusing has been achieved. The tailoring of root system has been effected by reducing the concentrations of mainly the NH_4HO_3 , KNO_3 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as also of the vitamins and of sucrose. By tailoring the root system, the *in vitro*-rooted shoots have survived even 100% (Chaturvedi 1979; Singh 2001; Chaturvedi and Agnihotri 2002).

Rooting

Effectiveness of different auxins on adventitious rooting has been reported by De Klerk *et al.* 1997; Hussain *et al.* 1998; Singh 2001. The presence of auxin nullifies the suppressive effect of high cytokinin and restores normal size and growth (Lundergan and Janick 1980). Callusing at basal end due to action of accumulated auxins in presence of cytokinin has been reported by Marks and Simpson (1994); Toa and Verbelen (1996). Inhibition of root development was noticed at high concentration of auxin due to enhancement of ethylene biosynthesis (Wareing and Phillips 1981).

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Hardening of in vitro raised plantlets Transplantation of *in vitro* raised plants into soil is a crucial step (Singh 2001). Poor transplantation is caused by lesser development of cuticle (Donnelly and Vidaver 1984), impaired stomatal mechanism (Brainerd and Fuchigami 1982), poor photosynthetic ability (Grout and Aston 1988), intervening callusing and unsterilized soil (Dwivedi *et al.* 1990).

Disease resistance studies in tissue cultures

Tissue culture systems have been utilized in past many years to investigate numerous aspects of host-pathogen interactions (Ingram 1977; Ingram and Helgeson 1980). Spoor and Lennard (1992) described the methods for management of diseases in tissue culture. Tissue cultures offer many advantages over intact plants for these studies, including (a) exclusion of contaminating microorganisms, (b) control of environmental parameters, e.g., temperature, light, and nutrients (c) ability to inoculate host cells without wounding (d) control of pathogen inoculation levels and number of host cells (e) ability to alter the nature of the host-pathogen interaction by altering components of the growth medium (f) the presence of only one or a few major host cell types and (g) the ease of application or removal of materials, e.g., labeled precursors, from cultured cells.

There are many examples of tissue culture propagated plants showing inhibition to diseases including fungal, bacterial and viral. Resistance to PLRV, PVX and PVY from *Solanum brevidens* to potato was achieved by somatic hybridization and transfer to TMV resistance was achieved by asymmetric protoplast fusion. Callus tissue culture systems have been used widely for studies of the expression of race-specific and non-host resistance (Ingram and Robertson 1965; Maronek and Hendrix 1978; Helgeson *et al.* 1972; Keen and Horsch 1972; Warren and Routley 1970; Huang and Van Dyke 1978; Holliday and Klarman 1979; Campbell 1979) and offer several advantages over suspension cultures, isolated cells or protoplasts.

The tissue culture system that has been most carefully defined and presents the best evidence for the applicability of *in vitro* systems to the study of host-parasite interactions is the tobacco (*Nicotiana tabacum* L.) / *Phytophthora parasitica* var. *nicotianae* (Ppn) system (Helgeson *et al.* 1972; Helgeson and Haberlach 1980). Callus tissues derived from all the resistant plants were resistant, and those derived from susceptible plants, were susceptible. Since there were no deviations from the correlation of callus tissue reactions of Ppn with the reaction of the plants from which they were derived. These results indicate that the resistance gene that is expressed in the intact tobacco plants is also expressed in tissue culture. Quantitatively inherited resistance to Ppn is also expressed in tissue culture. Deaton *et al.* (1982) found that the response of callus tissue from field resistant barley cultivars to Ppn was highly correlated with the whole plant response to the pathogen. The expression of resistance in tobacco callus cultures is affected by several factors, including temperature, inoculum concentration, callus morphology, and particularly, the balance of phytohormones in the tissue culture medium (Helgeson *et al.* 1972).

The expression of race-specific resistance in tissue culture has also been demonstrated from potato (*Solanum tuberosum* L.) in response to *Phytophthora infestans* (Ingram and Robertson 1965). Zhuk *et al.* (1990) have shown the resistance in regenerated plants of sugar beet to the beet mosaic virus. Other systems in which the expression of disease resistance in tissue cultures has been investigated include:

- a. tomato (*Lycopersicon esculentum*) / *P. infestans* (Warren and Routley 1970)
- b. soybean (*Glycine max*) / *Phytophthora megasperma* (Holliday and Klarman 1979)
- c. tobacco / *Pseudomonas* spp. (Huang and Van Dyke 1978), and
- d. tobacco / tobacco mosaic virus (Beachy and Murakishy 1971)

Phytoalexins in culture

Phytoalexins are low molecular weight, antimicrobial compounds formed by plants in response to infection and exposure to various toxic and non-toxic compounds and are thought to play a role in disease resistance (Kuc 1976; Stoessel 1980; Keen 1981; Heath 1981a). Plant cells grown in culture retain the capacity to produce phytoalexins in response to biotic or abiotic phytoalexin-inducing agents (elicitors). In some cases phytoalexins are produced in the absence of added elicitors, but in general, the level of constitutive, or non-induced, phytoalexin production in culture is low (Dixon 1980) and decreases with continual subculture (Bailey 1970; Hargreaves and Selby 1978; Dixon 1980). Both the expression of disease resistance (Helgeson *et al.* 1972; Haberlach *et al.* 1978; Holliday and Klarman

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1979; Campbell 1979) and production of phytoalexins (Dixon and Fuller 1976, 1978) are influenced by the concentration of auxins and cytokinins in the medium.

Antiviral in tissue culture

Anti-plant viral activity has been demonstrated in the cell cultures of *Phytolacca americana*. The cultured cells produced very active anti-plant virus substances. Ribosome inactivating protein was produced from *in vitro* culture of *Phytolacca dodecandra* by Thomson *et al.* (1991), Bonnes and Mabry (1992). Attempts have been made to produce antiviral proteins from plant cells *in vitro* using different culture systems. Using cell culture technique, antiviral substances were obtained from *Phytolacca americana* (Misawa *et al.* 1975), *Agrostemma githago* (Takayama *et al.* 1987) and *Mirabilis jalapa* (Ikeda *et al.* 1987a, b, 1988a, b), *Phytolacca dodecandra* (Thomsen *et al.* 1991, Bonnes and Mabry 1992), *Boerhaavia diffusa* (Gupta 1999; Gupta *et al.* 2004), *Clerodendrum aculeatum* (Srivastava 1999; Srivastava *et al.* 2004). In *Luffa cylindrica* L., antiviral proteins were obtained through hairy root culture (di Toppi *et al.* 1996). Testing systems for antiviral production *in vitro* are of different types. Antiviral contents were evaluated by measuring inhibition of protein synthesis using animal system. These plant proteins strictly come in category of ribosome inactivating proteins viz. in *Phytolacca dodecandra*, inhibition of protein synthesis was measured in a cell free rat liver extract; antiribosomal activity of the culture extracts in same plant was tested by *in vitro* translation (Bonnes and Mabry 1992). In *Luffa cylindrica* crude extract cloned lines of hairy roots inhibit protein synthesis in a reaction mixture based on rabbit reticulocyte lysate (di Tipo *et al.* 1996). Production of antiviral substances was determined by local lesion assay method on susceptible host plants viz., in *Mirabilis jalapa* degree of inhibition was tested on tobacco against TMV (Ikeda *et al.* 1987a). Application of culture extract of *Phytolacca americana* and *Agrostemma githago* on the leaves of tobacco and tomato inhibited plant virus infection.

Section III

Studies on human breast cancer cell line and semliki forest virus (sfv) in mice

The medical importance of viruses is in their ability to cause a very large number of human diseases. Viral diseases range from minor ailments such as the common cold to highly fatal diseases, such as rabies or yellow fever. They may be sporadic such as mumps, endemic such as rabies, epidemic such as measles or pandemic such as influenza. Some dangerous human viral diseases are: Encephalitis, Se Aente Respiratory Syndrome (SARS), Dengue Haemorrhage Fever (DHF) or Dengue Shock Syndrome (DSS), the emergence and pandemic spread of the acquired immunodeficiency syndrome (AIDS). The animal viral disease, Foot and mouth disease, Africa horse sickness, viral pneumonia of pigs, chicken embryo virus, simliki forest virus (SFV). Simliki Forest Virus (SFV) causes encephalitis, paralysis and death of mice. SFV can cause encephalitis in horses, mice rat, hamsters, rabbits and guineapigs (Bradish *et al.* 1971; Robin *et al.* 1974; Atkins *et al.* 1990). Severity and type of diseases are dependent on the age of the animal at the time of infection, on the route of inoculation and on the virulence of the strain of SFV used for infection (Grimley *et al.* 1970, Bradish *et al.* 1971). Mice have been most extensively studied and the strains of inbred mouse infected also can influence the outcome (Suckling *et al.* 1980). Viruses can cause cancer in animals and in human beings as well. The association of virus with malignancy dates from the discovery by Ellerman and Bong (1908), of the viral etiology of fowl Leukemia. Rous (1911) showed that a solid malignant tumor, fowl sarcoma, was caused by virus. Viruses causing tumours in animals were first demonstrated by Shope, who isolated the rabbit fibroma virus in 1932 and Papilloma virus in 1933 (Ananthanarayan and Paniker 1995). Bittner (1936) proposed that breast cancer in mice could be caused by a virus transmitted from mother to offspring through breast milk.

Viruses that produce tumors in their natural hosts or in experimental animals, or induce malignant transformation of cells in culture, are known as oncogenic viruses. These include Retroviruses, Papovavirus, Poxvirus, Herpes virus and Hepatitis B Virus (Ananthanarayan and Paniker 1995). Transformation represents the various changes that accompany the conversion of a normal cell into the malignant cell. The herbal medicines are the mainstay of about 75-80% of the world population, in both developing and developed countries, for primary health care because of their efficacy, safety, compatibility with the animal and human bodies and fewer side effects. They also offer therapeutics for age related disorders like memory loss, osteoporosis, immune disorders etc. for which no modern medicine is available. India and Brazil despite their rich traditional knowledge heritage of herbal medicines and large biodiversity have a dismal share of the world market due to export of crude extracts and drugs. Therefore, to start with, there is a need to understand the mechanism of action and scientific basis of the herbal drugs already in the market, followed by identification and

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analysis of new medicinal plants and their bioactive molecules in detail. The herbal drugs which can act magically and cure even incurable diseases have not been properly/systematically investigated for their antiviral effects against human and animal viral infections. Antiviral activity of some of these herbal drugs against plant viruses has been reported by Abidi *et al.* (1977), Khurana (1971) and Verma *et al.* (1969), In addition to the antiviral effect of herbal medicines against plant viruses, Verma and Verma (1963), Verma (1971) Verma and Awasthi (1980) and Prasad *et al.* (1995), reported antiviral activity of some bio chemical drugs against plant viruses as well. In the traditional system of medicine, roots have been widely used for the treatment of liver disorders (including jaundice and hepatitis), Gall bladder pain and stones, urinary tract disorder, renal disorders and calculi, cystitis nephritis, stomachic, laxative and emmenagogue to treat gonorrhea, internal inflammation of all kinds, Dropsy, menstrual problems, anemia and liver, gallbladder, and kidney disorder. The roots of the plant are normally softened in boiling water and then mashed up and applied as a paste or poultice to the affected areas to kill the worms and express them from the skin and as an antistress agent (Dandiya 1991). Some well known Ayurvedic preparations are also made from punaravana, viz. "Punarava vastaaka Kvatha", "Punaravana Kishar" and "Punaravana Taila". Pharmacological studies have demonstrated that punaravana possess diuretic (Gaitonde *et al.* 1974), anti-inflammatory (Bhalla *et al.* 1968) anti fibronolytic (Jain and Khanna 1989), anticonvulsant (Adesina 1979) and antibacterial activities (Olukoya *et al.* 1993) which make it a very useful drug. Premanathan *et al.* (1996) carried out *in vitro* screening of mangrove plant extracts for anti-immunodeficiency virus activity. HIV infected MT-4 cells were incubated with the extract and anti-viral activity was detected using tetrazolium based colorimetric assay. The potential role of various plants in cancer therapy has been reported. The antitumor effect of the crude extract of *Centella asiatica*, *Withania somnifera*, *Janakia aryalpathra*, *Podophyllum hexamdrum*, *Ocimum sanctum*, *Hygrophilic spinosa* etc. have been reported (Dahanukar *et al.* 2000). Modulation of the immune response by *Withania somnifera*, *Allium sativum*, *Azadirachta indica* and *Piper longa* etc. through stimulation or suppression may help in maintaining a disease free stage (Dahanukar *et al.* 2000). The alkaloid fraction of *Boerhaavia diffusa* significantly restored the suppressed humoral response in stressed rats as observed by Mungantiwar *et al.* (1997), where in *B. diffusa* increased the suppressed antibody titres.

Alcoholic extract of *B. diffusa* inhibited the growth of several cancer cell lines of mouse and human origin, and may have antiproliferating (anticancer) potential (Mehrotra *et al.* 2002). It is quite conceivable that this activity may be due to urosolic acid (Sukhdev 2006) which has been isolated from *B. diffusa* and which is known to have anticancer activity. The Vinca alkaloids, Vinblastine and Vincristine, isolated from *Catharanthus roseus*; Etoposide and Teniposide (epipodophyllotoxin) isolated from species of the genus *Podophyllum*; Taxanes isolated from species of the genus *Taxus*; Camptothecin, Irinotecan and Topotecan isolated from *Camptotheca acuminata* (Costa-Lotufo *et al.* 2005) also have been reported to possess anti-cancer activity. Estrogen (E2) initiates and regulates a cascade of events following interaction with the estrogen receptors (ERs), which are copiously expressed in human breast cancer-derived cell lines such as MCF-7, T47D and ZR75 (Westley and Rochefort 1980). The MCF-7 cell line, a human breast adenocarcinoma consisting of epithelial cells isolated from the pleural effusion of a post-menopausal female, has thus far provided an ideal tool for evaluating E2-dependent phenomena *in vitro* (Saxena *et al.* 1995; Levinson and Jordan 1997; Seo and Leclercq 2002). This has significantly led to the identification of tamoxifen (TAM) and other antiestrogens (AEs) for breast cancer therapy (Lerner and Jordan 1990; Muss 1992; Jordan 1994). Besides, other estrogen-related phenomena such as the expression of proteases and antiproteases, which *prima facie* regulate the invasion and metastasis, have also been investigated using MCF-7 and other ER-positive cell lines (Seo and Leclercq 2002). One such enzyme cathepsin-D (Cath-D), an acidic lysosomal aspartyl endoprotease ubiquitously present in all the cells, constitutively expressed in ER-negative cells, and over-expressed in ER-positive breast cancer cell lines (Rochefort *et al.* 2000), has attracted enormous research attention. The roots of *B. diffusa* are the oldest and most frequently used for traditional medicine in many Asian countries including India. It is a drug of choice to cure the chemical induced liver damage in Ayurveda, an Indian system of medicine, because of the presence of novel phytochemicals, including flavonoids, steroids and alkaloids and a basic protein BD- SRIP (Chopra *et al.* 1956; Rawat *et al.* 1997; Verma and Baranwal 1999). It is also known to be protective against several plant viruses by stimulating the inbuilt resistance of plants when treatments were done prior to virus infection. The roots of *B. diffusa* have also been found to have diuretic, anti-inflammatory, fibrinolytic, nephrotoxic syndrome and anti convulsant activities (Bhalla *et al.* 1971, Singh and Udupa 1972a, b, c, Mudgal 1975; Satyavati *et al.* 1976; Chakraborti and Handa 1989). The worldwide use of *B. diffusa* roots for various liver complaints and disorders was validated when researches demonstrated that its root extracts provided antihepatotoxic properties (Misra *et al.* 1980, Chandan *et al.* 1991, Rawat *et al.* 1997). The leaves of *C. aculeatum*

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contain a potent endogenous virus inhibitor which confers strong systemic resistance in several susceptible plants against a number of plant viruses (Verma *et al.* 1984, 1996). In the present study, we have investigated the morphological and numerical changes in MCF-7 cells *vis-à-vis* E2 and isolated proteins from the roots of *B. diffusa* and leaves of *C. aculeatum* and to see their antiestrogenic and antineoplastic potential, and then antiviral activity against semliki forest virus (SFV) on albino mice model.

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