EVALUATION OF PLANT EXTRACTS AGAINST *Ralstonia solanacearum* CAUSING BACTERIAL WILT

THESIS

By

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OF

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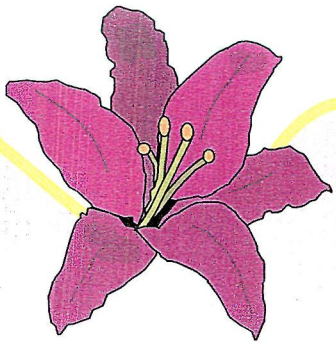

"What can be more clear and sound in explanation, than the love of a parent to his child?"

AFFECTIONATELY
DEDICATED
TO MY
INIMITABLE
PARENTS

Dr. A.K. Sood
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CERTIFICATE - I

This is to certify that the thesis entitled **"Evaluation of plant extracts against *Ralstonia solanacearum* causing bacterial wilt"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Plant Pathology** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Ms. Karishma Chauhan (A-2007-30-21)** daughter of **Sh. R.L. Chauhan** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

A.K. Sood 18.7.09
(A.K. Sood)
Chairman
Advisory committee

Place: Palampur
Dated: July 18, 2009

CERTIFICATE - II

This is to certify that the thesis entitled **"Evaluation of plant extracts against *Ralstonia solanacearum* causing bacterial wilt"** submitted by **Ms. Karishma Chauhan (A-2007-30-21)** daughter of **Sh. R.L. Chauhan** to the Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Plant Pathology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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'Needless to say, all errors and omissions are mine'

Place: Palampur
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(Karishma Chauhan)

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INTRODUCTION

Ralstonia solanacearum previously known as *Pseudomonas solanacearum* was originally described by E.F Smith (1896) as the causative agent of bacterial wilt of solanaceous plants (Hayward, 1991). It is a widely distributed and economically important plant pathogen. It invades the roots of diverse plant hosts from the soil and aggressively colonizes the xylem vessels, causing a lethal wilting. The bacterium affects over 200 species, representing over 50 botanical families and covering both monocots and dicots extending from annual plants to trees and shrubs, especially tropical and subtropical crop families, the most susceptible crops being potato, tomato, tobacco, eggplant, pepper, banana and groundnut. It is a serious disease and a major constraint in the production of solanaceous vegetables.

Bacterial wilt is well established in the mid hill sub-humid areas of Himachal Pradesh and appears endemically every year in Kangra, Mandi, Solan, Bilaspur, Hamirpur and Kullu Districts (Sood and Singh, 1993; Gupta et al., 1998). Besides Himachal Pradesh, it is widespread in other states of India like Assam, Bihar, Goa, Karnataka, Maharashtra, Orissa, West Bengal, etc causing yield losses up to 100 per cent (Kishun, 1987).

The control of bacterial wilt has proved to be very difficult because the (bacterium) has wide host range with exceptional ability to invade the roots of diverse plant hosts and colonize aggressively in the xylem vessels, all the

commercial cultivars are highly susceptible and chemical control is not feasible. Soil fumigants showed either slight or no effects (Murakoshi & Takahashi, 1984). Antibiotics such as streptomycin, ampicillin, tetracycline and penicillin also showed hardly any effect (Farag *et al.*, 1982). In fact, streptomycin application increased the incidence of bacterial wilt in Egypt (Farag *et al.*, 1986). Biological control has been investigated, but is still in its infancy. This pathogen has high genetic variation and new races or strains often occur (AVRDC, 1974).

Although integrated management of this disease utilizing various cultural (soil amendments, soil solarization, adjusting time of transplanting and biofumigation) and biological methods (antagonistic rhizobacteria and VAM) has been attempted at the CSK HPKV, Palampur with a limited success (Aggarwal, 2004 and Sood, 2004), but this approach is quite cumbersome and cost ineffective.

As an alternative strategy to prevent the spread of plant diseases, natural compounds of plants can be a source of new pesticides or serve a template for new, more effective compounds (Elkovich, 1988). During the last 10 years, herbal extracts have been used in human and animal disease therapy. Many reports on the use of herbal extract in plant disease control are also available but only a few of them are reported against phytopathogenic bacteria. Molina *et al.* (1999) reported that the amides (affinin) extracted from *Heliopsis longipes* at high concentration could inhibit the growth of *P. solanacearum* and capsaicin from chilli fruit could retard the bacterial growth. Wongkaew *et al.* (1997) reported that the crude extract of golden shower (*Cassia fistula*) leaf,

ringworm brush (*Cassia allota*) leaf and turmeric (*Curcuma longa*) rhizome could inhibit the growth of *P. solanacearum.* Tumeric rhizomes extract could inhibit at ED_{50} above 30,000 ppm while the extract of ringworm brush could inhibit at ED_{50} above 300,000 ppm. Leksomboon *et al.* (1998) reported that the extracts of guava (*Psidium guajava*) leaf and okra (*Hibiscus sabdariffa*) fruit could also inhibit the growth of *R. solanacearum.*

The herbal compounds are considered safer as they are biodegradable, non toxic and eco friendly and have been proved effective in controlling plant diseases. The natural plant products can be utilized in developing them as a part of integrated system for the control of soil borne pathogens. The roots of some medicinal plants are also known to possess antimicrobial activity against soil borne plant diseases (Ushiki *et al.*, 1996).

Further, many essential oils have been used in medicines as a solution for bacterial infections. The volatile nature of the essential oils allows them to penetrate soil, kill pests and then quickly evaporate leaving no residue.

Since the northwestern Himalayan region of India including the entire Himalayan region of Himachal Pradesh, particularly the higher reaches of Chamba, Kullu, Mandi, Kangra, Shimla, and Sirmour districts, is home to over 3,500 species of herbs and aromatic plants and these can be tried as alternative biodegradable components for the control of bacterial wilt. Moreover, Government's new National Mission on Medicinal Plants (NMMP) expects to bring some 80,000 -1, 00,000 hectares of land under medicinal plants through direct financial assistance for cultivation.

Many plant species also produce volatile essential oil compounds. These oils are considered to play a role in host defence mechanisms against plant pathogens. Essential oils and their components, usually derived from medicinal plants, are known to posses fungicidal effects. Not much information is available on the antibacterial activity of medicinal or other plants, commercially available organic formulations and essential oils against *R. solanacearum*. Once found effective *in vitro* and *in vivo* against *R. solanacearum*, they can be used as additional components for inclusion in the integrated management system of bacterial wilt. Further, it would be interesting to determine the efficacy of different fractions of the effective or potential botanicals against the wilt pathogen. Thus, keeping the foregoing in view, the present study "Evaluation of plant extracts against *Ralstonia solanacearum* causing bacterial wilt" is planned with the following objectives:

1. To determine the *in vitro* efficacy of aqueous and organic plant extracts against *Ralstonia solanacearum.*
2. To evaluate the potential of selected aqueous and organic plant extracts under *in vivo* conditions for the control of bacterial wilt.
3. Fractionation and evaluation of potential plant extracts against the wilt pathogen.

Review of Literature

Chapter-II

REVIEW OF LITERATURE

Ralstonia solanacearum poses a serious threat for successful cultivation of solanaceous vegetables in Himachal Pradesh. Due to many problems that can result from use of synthetic pesticides including environmental pollution, phytotoxicity and production of resistant pathogen strains, many scientists have conducted research on the integrated control of plant diseases with the aim of reducing the use of pesticides. However, not much information is available on the effectiveness of plant extracts, against the causal agent of bacterial wilt. The pertinent literature on this aspect is reviewed under the following heads:

- Use of plant extracts in plant disease management
- Use of essential oils in plant disease management
- Fractionation of plant extracts

2.1 Use of plant extracts in plant disease management

Hanudin and Djatnika (1986) used extracts from onion and garlic bulb roots and stem of *Crotolaria* spp., *Tagetus* spp. and Paw-Paw leaves for *in vitro* suppression of *R. solanacearum*. It was confirmed that the extracts from garlic bulbs inhibited the bacterial growth. Further, Hanudin (1987) observed that extracts of garlic, shallot and marigold suppressed the bacterial wilt disease.

Hutagalung (1988) conducted a study in west Java (Indonesia) and found that incidence of bacterial wilt of tomato decreased by adding 10ml suspension of 35g garlic bulb/77ml sterile water or 6g of ground garlic bulb to the rhizosphere. Additionaly, plant growth and fruit weight were increased.

Fukai *et al.* (1991) reported the antibacterial activity of tea polyphenols and crude theaflavins measured as MIC (minimum inhibitory concentration) against phytopathogenic bacteria which tend to infect commonly cultivated vegetables i.e., *Erwinia* (8 strains), *Pseudomonas* (10 strains) and a single strain of *Clavibacter, Xanthomonas* and *Agrobacterium*. They conducted lab tests to evaluate the effect of tea polyphenols, crude catechins and its four components (epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate(EGCg) against soft rot (*Erwinia* spp), blackleg, necrotic leaf spot, leaf spot (*Pseudomonaș spp*), bacterial wilt (*Ralstonia solanacearum*), leaf spot (*Xanthomonas* spp),canker (*Clavibacter michiganensis*) and crown gall (*Agrobacterium tumifaciens*) and observed that catechins, pyrogallol catechins *i.e.*, [(EGC) epigallocatechin and epigallocatechin gallate (EGCg)] were more effective than catechol catechins *i.e.* [(epicatechin) EC and epicatechin gallate (ECg)], and MICs being mostly effective below 100ppm. They suggested that tea polyphenols and crude theaflavins could be used to control bacterial diseases of vegetables.

Sobti *et al.* (1995) determined the effect of three plant extracts and two fungicides on three groundnut pathogens (*Aspergillus niger, Macrophomina phaseolina* and *Aspergillus flavus*) and observed that thiram, carbendazim and

extracts of the 3 plants (*Azadirachta indica, Polyalthia longifolia and Ocimum gratissimum*) significantly inhibited mycelial growth of the fungi, with the fungicides being most effective followed by *P. longifolia* extracts. Although carbendazim was the most effective but it had an adverse effect on crop production, soil and plant health. They concluded that as *P. longifolia* extracts were almost as effective as thiram but had no toxic hazards on crop production and are safer to use.

Antifungal and antibacterial properties of nine medicinal plant extracts were investigated by Thakur *et al.* (1995) against some cotton pathogens including *Myrothecium roridum, Alternaria tenuis [Alternaria alternata]* and *Xanthomonas campestris pv. malvacearum [X. axonopodis pv. malvacearum].* Two plant species, *Punica granatum* and *Datura metel* showed both antifungal and antibacterial activity.

Hassanien and Eldoksch (1997) studied antibacterial activity of five petroleum ether extracts from peppermint, thyme, caraway, chenopodium, eucalyptus and caravone both *in vivo* and *in vitro* against phytopathogenic bacteria. They found that caravone exhibited the strongest antibacterial activity against *R. solanacearum* with 100 percent growth inhibition. It had a broad spectrum of activity against all the tested bacterial strains with maximum inhibitory concentrations ranging between 125 to 500µg/ml.

Wood *et al.* (1997) tested antifungal and antibacterial activity of extracts from *Thymus vulgaris, Artemisia absinthium, Tanacetum vulgare* and *Rheum officinale* against two obligate (*Peronospora manshurica* and *Puccinia*

horiana) and 12 non-obligate parasites of plants (*Chondrostereum purpureum, Pythium irregulare, Stemphylium radicinum [Alternaria radicina], Botrytis cinerea, Phytophthora cactorum, Alternaria alternata, Fusarium avenaceum [Gibberella avenacea], Trametes versicolor [Coriolus versicolor], Aspergillus flavus, Penicillium expansum, Trichoderma viride* and *Mucor hiemalis)*. None of the 10 different extracts inhibited growth of the two obligate and 12 non-obligate pathogens at >1000 µg/ml. Among the bacteria, only *Erwinia carotovora* was found sensitive to *Thymus* and *Rheum* extracts.

Salahuddin *et al.* (1998) screened seed extracts and crude alkaloids of *Aegle marmelos (L) Corr.* for their antibacterial activity against pathogenic bacteria: *Bacillus cereus, B. subtilis, Escherichia coli* and *Pseudomonas sp.* Crude alkaloids were found to be active against almost all the test bacteria and showed a wide range of antibacterial properties. Aqueous seed extracts and crude alkaloids also exhibited antifungal activity with varied degrees of growth inhibition against seven phytopathogenic fungi (*Alternaria alternata, Botryodiplodia theobromae, Colletotrichum corchori, Curvularia lunata [Cochliobolus lunatus], Drechslera oryzae [Cochliobolus miyabeanus], Fusarium equiseti* and *Macrophomina phaseolina*).

Aqueous extracts of 36 plant species were screened by Lirio *et al.* (1998) for antibacterial activity against *Erwinia carotovora pv. carotovora, Xanthomonas campestris pv. campestris* and *Pseudomonas solanacearum [Ralstonia solanacearum*]. Twenty one plant species showed antibacterial activity, in which *Allium cepa, A. porrum, A. sativum, Euphorbia tirucalli* and *Piper betle* were most effective against all test pathogens.

Satish *et al.* (1999) screened aqueous extracts from leaves of 30 higher plants, collected from different localities for their *in vitro* for antibacterial activity against different pathovars of the phytopathogenic bacterium *Xanthomonas campestris*. Eight plant species (*Acacia arabica [A. nilotica], Achras zapota [Manilkara zapota], Enterolobium saman [Samanea saman, syn. Albizia saman], Lawsonia inermis, Oxalis corniculata, Prosopis juliflora, Punica granatum and Viscum orientale*) showed antibacterial activity, based on the zone of inhibition in a diffusion assay and rest of the 22 did not show any antibacterial activity. Significant antibacterial activity was however, observed in the aqueous extracts of *Prosopis juliflora, Oxalis corniculata and Lawsonia inermis.* The susceptibility of different pathovars of *(X. campestris pv. malvacearum, X. campestris pv. phaseoli* and *X. campestris pv. vesicatoria*) to these plant extracts varied. The antibacterial activity of extracts of a few plants was comparable with that of the synthetic antibiotics, bacterimycin and streptocycline. They also studied the potential of these plant extracts in the management of diseases caused by *X. campestris* in several important crop plants. Of the 30 plant extracts screened, eight showed significant antibacterial activity as evidenced by a zone of inhibition.

Kang-LiHua, (1999) reported that extracts from nodules of gray sheoak or long leaf ironwood (*Casuarina glauca),* a plant of Australian origin, inhibited the growth of *R. solanacearum.* Yu and Komada (1999) further reported that the volatile oils and non-volatile substances in Hinoki (*Chamaecyparis obtuse*) bark greatly reduced the growth of *R. solanacearum.*

Seoun Hur et al. (2009) screened methanol extracts of three cold-tolerant eucalyptus trees - *Eucalyptus darlympleana, E. gunnii* and *E. unigera*were for their antimicrobial activity against 22 phyto - pathogenic fungi. *E. unigera* showed the antagonistic activity against all the tested pathogens. Among the tested fungal pathogens, *Pythium* species were highly sensitive to the leaf extracts especially, *P. vanterpoolii*, a causal agent of leaf blight in creeping bentgrass (*Agrostis palustris*), was completely inhibited by the extracts. The eucalyptus extracts were also effective in inhibiting the fungal growth of *Botrytis cinerea* and *Phomopsis sp.* isolated from the lesions of kiwifruit soft rot during post-harvest storage. These findings suggested that the cold-tolerant eucalyptus species have antimicrobial properties that could serve the development of novel fungitoxic agents.

Porchezhian and Ansari (2001) investigated the *in vitro* antimicrobial properties of the petroleum ether, benzene, chloroform and methanol extracts (all at 30 and 60 mg) of *Alangium salvifolium* [*Alangium lamarckii*] roots on three bacteria (*Escherichia coli, Bacillus subtilis* and *B. cereus*) and four fungi (*Aspergillus niger, Aspergillus flavus, Candida albicans* and *Fusarium oxysporum*). They observed that methanol extract showed moderate to significant activity against all the bacteria at 30 and 60 mg. The chloroform extract at 60 mg showed marginal antibacterial activity and the chloroform and methanol extracts did not show any antifungal activities. The benzene and petroleum ether extracts did not show any antibacterial or antifungal activity.

. Amaresh and Nargund (2003) tested the aqueous plant extracts of 30 plant species *in vitro* against *Puccinia helianthi* causing sunflower rust. The plant extracts of *Allium cepa, Allium sativum, Pongamia glabra, Nerium olender* and *Melia azadirach* were found effective in inhibition of uredospore germination of *P.helianth*

Kaushik *et al.* (2003) screened 41 plant species for biocidal activity against bacteria (*Bacillus megaterium, Escherichia coli, Pseudomonas fluorescens* and *Xanthomonas spp.*). The aqueous and methanolic extracts of 24 plants showed antibacterial activity, while 17 plant species were not inhibitory to any of the test bacteria and aqueous extracts of 12 plant species exhibited bactericidal activity against one or more bacterial species. The aqueous extracts, fresh hot water (FHW) and dry hot water (DHW) extracts of *Berberis aristata* were more effective against *Bacillus megaterium* and *Xanthomonas spp.*, dry cold water (DCW) and dry hot water (DHW) extracts of *Mentha longifolia* against *Bacillus megaterium*, *Escherichia coli* and *Pseudomonas fluorescens*, DCW and DHW extracts of *Vitex negundo* against *Bacillus megaterium,* FCW and FHW extracts of *Aegle marmelos* against *Bacillus megaterium,* FCW and FHW extracts of *Cassia fistula* against *Bacillus megaterium* and FHW extract of *Cannabis sativa* against *Bacillus megaterium.* Aqueous leaf extracts of *Berberis aristata* and *Cassia fistula* were effective against all test bacteria. Aqueous extracts of *Lantana camara, Leonotis nepetaefolia* and *Polygonum hydropiper* inhibited only one or two test bacteria. *Berberis aristata* was the most promising for developing eco-friendly natural biocidal products. Dry methanol (DM) extracts of *A. marmelos*, *Berberis*

aristata, Cannabis sativa and *Cassia fistula* inhibited *Bacillus megaterium* and those of *Erigeron karvinskianus, Cleome viscosa, Mentha longifolia, Origanum vulgare* and *V. negundo* inhibited *Bacillus megaterium*, *Escherichia coli* and *Pseudomonas fluorescens*, and those of *Berberis aristata* and *Cleome viscosa* inhibited *Xanthomonas spp*.

Kumar *et al.* (2003) investigated water, ethyl acetate, ethanolic and chloroform extracts of leaves of aak (*Calotropis procera*), bhang *(Cannabis sativa*), datura (*Datura stramonium*), neem (*Azadirachta indica*), and garlic clove (*Allium sativum*) for their antibacterial activity against *X. axonopodis pv. cyamopsidis in vitro*. The water and ethyl acetate extract of garlic clove exhibited maximum antibacterial activity against the pathogen. The extracts were freeze dried and further compared for their bactericidal activity at four concentrations (1, 10, 100, 1000 and 10 000 µg/ml). The garlic clove preparation provided the maximum inhibition of the bacterium at all the concentrations used.

The efficacy of the extracts for the control of cluster bean blight was also evaluated under field conditions in Hisar (Haryana). The freeze dried extract preparations (at 10,000 µg/ml) were used as seed treatment for 1 h and foliar sprays. The first spray was given after 36 h of artificial inoculation of the bacterium, and the second and third sprays followed at 10 days interval. The disease could not be arrested by any of the preparations used as seed treatment alone. But, it protected the crop from the disease until 35 days of sowing. Three foliar sprays of garlic clove extract provided satisfactory control of the disease. The maximum reduction on overall severity of bacterial blight of cluster bean was obtained with seed treatment and three foliar sprays of garlic clove extract.

Pretorius *et al.* (2003) tested extracts from 26 plant species representing 16 families, collected in the Free State Province of South Africa, *in vitro* for their potential to inhibit the growth of seven plant pathogenic fungi (*Aspergillus niger, Botrytis cinerea, Colletotrichum acutatum, Fusarium oxysporum, Phoma sp.*, *Rhizoctonia solani* and *Phytophthora cinnamomi*) and five plant pathogenic bacteria (*Agrobacterium tumefaciens, Clavibacter michiganense pv. michiganense, Erwinia carotovora pv. carotovora, Ralstonia solanacearum* and *Xanthomonas campestris pv. phaseoli* (*X. axonopodis pv. phaseoli*). None of the crude extracts showed any mycelial growth inhibition of the seven test fungi. All the extracts inhibited the growth of one or more of the five plant pathogenic test bacteria, but to varying degrees. Crude extracts from *Acacia karroo* and *Elephantorrhiza elephantina* inhibited the growth of four bacteria, while that of *Euclea crispa, Acacia erioloba, Senna italica* and *Buddleja saligna* inhibited the growth of all five plant pathogenic bacteria. Of these, the crude extract of *Euclea crispa* was clearly superior to the rest as it compared more favourably to that of a commercial bactericide, Dimethyl Dodecyl Ammonium Chloride (DDAC). Subsequently, the *Euclea crispa* crude extract was fractionated by means of liquid-liquid extraction using four organic solvents, hexane, diethyl ether, chloroform and ethyl acetate, in order of increasing polarity. This was done in an attempt to assess the antimicrobial potential of the more concentrated fractions. Once again, none of the semi-purified fractions showed any antifungal activity. However, antibacterial activity was located in the more polar ethyl acetate fraction indicating that the substances involved were

very similar in polarity and/or structure. From this it seems justified to further purify the ethyl acetate fraction of the *Euclea crispa* extract and attempt to identify the active substance(s) involved.

Tiwari and Srivastava (2004) evaluated the efficacy of some plant extracts, i.e. neem (*Azadirachta indica*), eucalyptus (*Eucalyptus globutens* [*E. globulus* x *E. nitens*]), bougainvillea (*Bougainvillea spectabilis*), mint (*Mentha arvensis*), datura (*Datura alba* [D. metel]), lantana (*Lantana camara*), ramphal (*Annona reticulata*), sitaphal (*Annona squamosa*), mehndi (*Lawsonia inermis*), tulsi (*Ocimum sanctum* [*O. tenuiflorum*]) and ginger (*Zingiber officinale*), against the onion pathogens *Fusarium oxysporum* (damping off and basal rot), *Alternaria porri* (purple blotch), *Stemphylium vesicarium* (blight), *Aspergillus niger* (black mould) and *Sclerotium cepivorum* (white rot) in the laboratory. The antifungal activity of the extracts at 5, 10 and 20% was evaluated by measuring the pathogen mycelial growth in potato dextrose agar. All extracts exhibited significant antifungal activity. Mehndi, ginger, lantana and mint inhibited the growth of all pathogens, while bougainvillea, ramphal and sitaphal did not inhibit the growth of *Alternaria porri* and *F. oxysporum.*

Sasitorn (2003) studied the potential of some 11 kinds of Thai herbs, extracts by using 40, 60 and 95% ethyl alcohol for growth inhibition of *Ralstonia solanacearum*, the causal agent of bacterial wilt of tomato. They conducted efficacy test by the paper disc diffusion method. For 10 µl of each concentration of extract was dropped on a 0.6 cm diameter sterile paper disc by micropipette and placed the disc on the surface of double layer nutrient agar. The

results revealed that seven plant extracts from six kinds of Thai herbs could inhibit the growth of *R. solanacearum* on double layer NA and showed visible inhibition zones. Those included extracts from both fresh and dry fruit skin of pomegranate, fresh leaf of thong-phun-chang, fresh fruit skin of mangosteen, dry leaf of guava, fresh rhizome of tumeric, and dry root of nut sedge. The diameters of inhibition zone were different according to the kind and concentration of herbal extract. However, the inhibition zones were mostly visible when the concentrations were above 10,000 µg/ml.

Devi and Paul (2004) evaluated the fungitoxic activities of 10 plant extracts against wilt/root rot complex pathogens of pea. The plant extract of jaldhar (*Ranunculus muricatus*) completely inhibited the growth of all pathogens whereas that of devdar (*Cedrus deodara*) caused more than 70 per cent inhibition of *Fusarium solani* and *Sclerotinia sclerotiorum*.

Shimpi *et al.* (2005) studied the antibacterial activity of alcoholic extracts (petroleum ether, cyclohexane, DMF and acetone) each at 1, 3, 6 and 10% concentration of the locally available *Aristolochia bracteata* the Indian medicinal plant against *R. solanacearum* and found that the zones of inhibition for each solvent were increased with increasing concentration of extracts. Maximum antimicrobial activity was observed at 5% and 10% concentrations.

Aggarwal *et al.* (2005) conducted green house studies to ascertain the relationship between the wilt bacterium *R. solanacearum* and root knot nematode incidence and to manage bacterial wilt in tomato by using plant refuge or root extract of marigold (*Tagetes erecta*). The wilt incidence was adversely affected in

the presence of *T. erecta* refuge both in the presence or absence of nematode infestation. The nematode population was also affected adversely in soils treated with root extract of marigold, with or without the wilt pathogen.

Antibacterial activity of the aqueous and alcoholic extracts of the root, stem, leaf, flower and fruit of *Delphinium ajacis* (*Consolida ambigua*) was determined by Neelam and Bohra (2006) against *Agrobacterium tumefaciens* alone or in combination with antibiotics. All the extracts inhibited the growth of *A. tumefaciens*. Except for the alcoholic extract of the roots of *D. ajacis*. The growth of *A. tumefaciens* was inhibited by the aqueous flower and leaf extracts of *D. ajacis*, respectively.

Basim *et al.* (2006) investigated the *in vitro* antibacterial activities of Turkish pollen and propolis extracts against 13 different species of agricultural bacterial pathogens including *Agrobacterium tumefaciens, A. vitis, Clavibacter michiganensis subsp. michiganensis, Erwinia amylovora, E. carotovora pv. carotovora, Pseudomonas corrugata, P. savastanoi pv. savastanoi, P. syringae pv. phaseolicola, P. syringae pv. syringae, P. syringae pv.* tomato, *Ralstonia solanacearum, Xanthomonas campestris pv. campestris* and *X. axonopodis pv. vesicatoria*. Among the tested bacteria, *A. tumefaciens* was the most sensitive one to 1/5 concentration of pollen extract, and the sensitivity of the bacteria followed the sequence *A. tumefaciens* > *P. syringae pv. tomato, X. axonopodis pv. vesicatoria* > *E. amylovora, P. corrugata, R. solanacearum, X. campestris pv. campestris* > *A. vitis, C. michiganensis subsp. michiganensis* > *E. carotovora pv. carotovora, P. savastanoi pv. savastanoi, P. syringae pv. phaseolicola* > *P.*

syringae pv. syringae. P. syringae pv. phaseolicola was the most sensitive one to 1/10 concentration of propolis extract, and the sensitivity of the bacteria followed the sequence *P. syringae pv. phaseolicola* > *R. solanacearum* > *E. carotovora pv. Carotovora* > *P. syringae pv. Syringae* > *X. axonopodis pv. vesicatoria*. The least active concentrations towards the tested bacteria were 1/100 of the pollen extract and 1/1000 of the propolis extract. This study is the first report on the antibacterial activities of pollen and propolis against the plant pathogenic bacteria.

Pankaj (2007) evaluated 18 aqueous and organic plant extracts, 9 essential oils and 5 organic formulations against *R. solanacearum* under *in vitro* and *in vivo* conditions. The extracts of neem (*Azadirachta indica*), Jaldhar (*Ranunculus muricatus*) and nila phulnu (*Ageratum houstonianum*) at 100% concentration; neem oil at 20 µg ml^{-1}; and the organic formulations Wanis, Achook and Neemazal at 100 and 50% concentrations were found most effective against the bacterium.

Bhardwaj and Laura (2007) screened the aqueous extracts of twenty plants by agar diffusion methods for their antibacterial activity against *Rathyibacter tritici,* a causal organism of tundu disease of wheat. The bacterium was found most sensitive to the leaves extracts of *Camellia sinensis*. Some of the other plants showed the activity against the test bacteria in the following order *Aegle marmelos* > *Azadirachta indica* > *Callistemon lanceolatus* > *Calotropis procera* > *Acacia arabicae* > *Brassica campestris* > *Adhatoda vasica.*

Soon-OkOh *et al.* (2008) evaluated antifungal activities of natural substances from *Eucalyptus darlympleana, E. globules, E. gunnii* and *E.unigera* against postharvest pathogens of kiwifruits, (soft rot decay fungi) *Botrytis cinerea*, *Botryosphaeria dothidea* and *Diaporthe actinidiae*, to screen effective natural substances as an alternative to chemical fungicides. Methanol extract of the *Eucalyptus* trees showed strong antagonistic activity against the pathogenic fungi. Among them, E. *unigera* and *E. darlympleana* effectively inhibited mycelial growth of the pathogens. For chemical identification of the antifungal substances, the methanol extract of *E.darlympleana* leaves was successively partitioned with CH_2Cl_2, ethyl acetate EtOAc, *n*-BuOH and H_2O. Among the fractions, CH_2Cl_2 and *n*-BuOH showed strong inhibitory activity of mycelial growth of the fungi. Five compounds were further isolated from ethyl acetate EtOAc and *n*-BuOH fractions by SiO_2 column chromatograph. These compounds were identified by 1H-NMR and 13C-NMRspectroscopy. Among these, two were phenolic compounds (gallic acid and 3, 4-dihydroxybenzoicacid) and three flavonoid compounds (quercetin, quercetin-3-O-α-L-rhamnoside, quercetin-3-O-β-D-glucoside). Among them, only gallic acid was found to be effective in mycelial growth and spore germinationof *B. cinerea* at relatively high concentrations. The results suggested that gallic acid could be a safer and more acceptable alternative to current synthetic fungicides controlling soft rot decay of kiwifruit during post harvest storage.

2.2 Use of Essential oils for plant disease management

Many plant species produce volatile essential oil compounds. These oils are considered to play a role in host defense mechanisms against plant pathogens (Mihaliak *et al.,* 1991). Essential oils and their components, usually from medicinal plants, have been recognized as having fungicidal effects (Wilson

et al., 1997), but their efficacy as a biofumigant to manage bacterial wilt (*Ralstonia solanacearum*) in tomato was initiated in Florida (USA) in 1999. Preliminary *in vitro* and greenhouse experiments were conducted with several plant essential oils as soil fumigants. Potting mixture ("soil") infested with *R. solanacearum* was treated with the essential oils at 400 mg or µl and 700 mg or µl per litre of soil in greenhouse experiments. *R. solanacearum* population densities were determined just before and seven days after treatment. Populations declined to undetectable levels in thymol, palmarosa oil, and lemongrass oil treatments at both concentrations, and showed that thymol palmarosa and lemongrass oil were quite effective in reducing *R. solanacearum* populations and bacterial wilt incidence of tomato grown in infested soil (Momol *et al.*, 1999; Pradhanang *et al.*, 2003).

Ristic *et al.* (2000) evaluated the essential oils and an ethanol extract of *Phlomis fruticosa* for antibacterial and antifungal activities. The essential oils showed antibacterial activity against *Escherichia coli* and *Bacillus subtilis*. The essential oils extracted from the plants collected from two different localities in Yugoslavia showed similar antibacterial activities. The antifungal activity of the essential oils was positive against *Aspergillus niger, Cladosporium cladosporioides, Fusarium tricinctum* and *Phomopsis helianthi*. The ethanol extract showed antibacterial activity against *B. subtilis* and antifungal activity against *A. niger, C. cladosporioides, F. tricinctum* and *Phomopsis helianthi*.

Cantore *et al.* (2003) assayed essential oils extracted from fruits of cumin, caraway, fennel and coriander, *in vitro* for antibacterial activity toward strains of ten *Pseudomonas syringae* pathovars. Significant antibacterial activity

was shown by essential oils of cumin, caraway and coriander. Generally, the essential oil from fennel showed low activity. These preliminary results indicated the potential use of the above essential oils in the control of diseases caused by *P. syringae* pathovars on important crops.

Benkeblia (2004) investigated antimicrobial activity at different concentrations (50, 100, 200, 300 and 500 ml/l) of essential oil extracts of three type of onions (green, yellow and red) and garlic against three fungi, *Aspergillus niger, Penicillium cyclopium* and *Fusarium oxysporum*. The essential oil (EO) extracts of these Allium plants (garlic and onions) exhibited marked antibacterial activity, with garlic showing the highest inhibition and green onion the lowest. Comparatively, 50 and 100 ml/l concentrations of onions extracts were less inhibitory than 200, 300 and 500 ml/l concentrations. The fungus *F. oxysporum* showed the lowest sensitivity towards EO extracts, whereas *A. niger* and *P. cyclopium* were significantly inhibited particularly at low concentrations.

Kzl *et al.* (2005) evaluated the antimicrobial and antifungal activities of essential oils of some medicinal plants against four plant pathogens at concentrations of 5, 10 and 15 µg and incubation times of 24, 48 and 72 h. The following seven medicinal plants traditionally used in medicines were subjected to preliminary screening against several pathogens: *Cuminum cyminum, Anethum graveolens, Coriandrum sativum, Pimpinella anisum, Mentha spicata, Hyssopus officinalis* and *Foeniculum vulgare*. Aqueous, hexane extracts of each plant were tested for their antimicrobial activity by using agar disk diffusion method. The pathogens used in the studies were: *Clavibacter michiganensis*

subsp. michiganensis, Pseudomonas syringae pv. tomato, Xanthomonas campestris [X. axonopodis] pv. malvacearum and Macrophomina phaseoli [Macrophomina phaseolina]. The results indicated that all plants exhibited antimicrobial activity against all pathogens, except *Xanthomonas campestris pv. malvacearum.* In addition, coriander and hyssop essential oils exhibited antibacterial activity against *Xanthomonas campestris pv. malvacearum.*

Vasinauskiene *et al.* (2006) studied essential oils from aromatic and medicinal plants for their antibacterial properties. In a preliminary evaluation, they investigated steam-distilled essential oils from oregano (*Origanum vulgare*), sweet flag (*Acorus calamus*), caraway (*Carum carvi*), peppermint (*Mentha piperita*), common (*Achillea millefolium*), fern leaf (*Achillea filipendulina*) and willow-leaved yarrow (*Achillea cartilaginea*) field accessions against growth of phytopathogenic bacteria. The disc-diffusion method was used for the assessment of *in vitro* inhibitory effects of the essential oils. Essential oil from oregano had the strongest inhibitory effect against the tested phytopathogenic bacteria. Less antibacterial effect was found with essential oils from caraway, peppermint, fern-leaf and willow-leaved yarrow, while the oils from common yarrow and sweet-flag manifested no inhibition. *Xanthomonas vesicatoria* 67 was the most sensitive to essential oils tested. A weak antibacterial activity was also found against some *Pseudomonas* spp. strains and *Erwinia carotovora subsp. carotovora.*

Kavidayal *et al.* (2006) studied the antibacterial activity of the essential oils of *Elsholtzia incisa, Elsholtzia pilosa* and *Elsholtzia densa* (against *Bacillus subtilis, Pseudomonas fluorescens* and *Escherichia coli*) as well as that of the

hexane, chloroform and methanol extracts of *Vernonia cinerea* (against *P. solanacearum* [*Ralstonia solanacearum*] and Escherichia coli) using the standard disc diffusion method. Essential oils of *Elsholtzia spp.* showed significant antibacterial activity at the tested concentrations (5, 15 and 25 micro l/disc). Maximum growth inhibition in all tested bacteria was recorded for *Elsholtzia incisa*. The essential oil was rich in thymol, 1, 8-cineole [eucalyptol], linalool, p-cymene, rosefuran and caryophyllene, which may have been responsible for the antibacterial activity. Among *V. cineraria* extracts, the methanol extract showed the greatest activity against all the tested bacteria. The activity of the chloroform extract was comparable to that of the methanol extract. In all cases, the activity increased with increasing concentration from 1000 to 3000 µg/ml.

Khan *et al.* (2007) tested essential oils of 14 aromatic/medicinal plants for their antibacterial properties against *Ralstonia solanacearum*. Inhibition zone technique (bacterial cells amended/seeded in medium) was used to screen the methanol extract/essential oils at 0.25, 0.50, 0.75 and 1.00 mg botanicals. All the seven essential oils from aromatic plants were effective at 1.00 mg with significantly more inhibition zone. Oils from *Mentha piperata [Mentha piperita]* restricted the bacterial growth to 9.42 mm and was statistically superior to others, i.e. palmarosa (8.42 mm), geranium (8.00 mm), M. arvensis (7.83 mm), citronella (7.75 mm), M. spicata (7.50 mm) and lemon grass (7.17 mm) at the highest concentration of botanicals.

Sunag *et al.* (2007) tested 39 essential oils for antifungal activities against five phytopathogenic fungi at a dose of 1 µl per plate. Five essential oils showed inhibitory activities against mycelial growth of at least one phytopathogenic

fungus. *Origanum vulgare* essential oil inhibited the mycelial growth of all of the five fungi tested. Both *Cuminum cyminum* and *Eucalyptus citriodora* oils displayed *in vitro* antifungal activities against four phytopathogenic fungi except for *Colletotrichum gloeosporioides*. The essential oil of *Thymus vulgaris* suppressed the mycelial growth of *C. gloeosporioides*, *Fusarium oxysporum* and *Rhizoctonia solani* and that of *Cymbopogon citratus* was active to only *F. oxysporum*. The chemical compositions of the five active essential oils were determined by gas chromatography-mass spectrometry. This study suggests that both *E. citriodora* and *C. cyminum* oils have apotential as antifungal preservatives for the control of storage diseases of various crops.

2.3 Fractionation of plant extracts

Coventry and Allan (2001) investigated the antimicrobial effects of the seed extracts of neem (*Azadirachta indica*) by using microbial growth inhibition assays. A laboratory-prepared neem seed extract and a commercially available formulated product (based on neemazal) were characterized using HPLC, and shown to be effective against a range of bacteria (*Bacillus mycoides, B. thuringiensis, B. subtilis, Nocardia sp. and Corynebacterium fascians [Rhodococcus fascians]*) in an agar diffusion assay. The active ingredient, i.e. the unformulated seed extract of the commercial product, also showed activity and this was further investigated in a biochromatogram, using the sensitive bacterium *B. mycoides*. The results showed antibacterial activity as three discrete inhibition zones that did not correspond to the Rf of the major neem metabolites, azadirachtin, nimbin and salannin. This suggested that these compounds were

not antibacterial. The colony radial growth rates of the fungal pathogens that cause 'take-all' (*Gaeumannomyces graminis* var. *tritici*) and 'snow mould' disease (Microdochium nivale [Monographella nivalis]) were both significantly affected when the commercial, unformulated, neem seed extract was incorporated into the growth medium. Experiments in liquid culture suggested that the effect was fungistatic. Conidial germination of the commercially important obligate pathogen *Sphaerotheca fuliginea* (powdery mildew) was reduced to 11%. The results showed that neem seed extracts possessed antimicrobial activity with notable effects on some fungal phytopathogens.

Becker *et al.* (2005) reported that *Lythrum salicaria* extracts showed activity against the phytopathogenic fungus *Cladosporium cucumerinum* and activity against the bacteria *Staphylococcus aureus*, *Proteus mirabilis* and *Micrococcus luteus*. Bioautography on thin-layer chromatograms was used to isolate the two antifungal triterpenoids oleanolic and ursolic acid. The hexahydroxydiphenoyl ester vescalagin was isolated as active principle of the antibacterial activity. Furthermore, the flavon-C-glucosides vitexin, isovitexin, orientin and isoorientin were also isolated.

Batra and Mehta (1985) reported that seed oil of *Argyreia speciosa* exhibited antifungal activity against four plant pathogens, especially *Geotrichum candidum*, and to a lesser extent *Alternaria solani, Helminthosporium* sp. and *Colletotrichum dematium*.

Anjana *et al.* (2008) analysed the chemical constituents of the plant extracts (bark of *Syzygium cumini*, leaves of *Lawsonia inermis*, fruits of *Terminalia bellerica)* by thin layer chromatography (TLC). The compounds were

determined by TLC-bioautography and were further confirmed by high performance liquid chromatography (HPLC). The TLC, TLC-bioautography and HPLC analysis showed that gallic acid and tannin present in ethanol extracts of *S. cumini,* tannin present in *L. inermis* and gallic acid present in *T. bellerica* might be responsible for the vibriocidal activity.

Boussaada *et al.* (2008) analysed the chemical composition of the volatile fractions obtained by steam distillation from the *capitula* (C) and the aerial parts of *Rhaponticum acaule* by GC-MS. From the 57 identified constituents, representing 95.5% and 96.3% of the two oils, respectively, methyl eugenol, epi-13 manool, beta -ionone, beta -bisabolol, 1-octadecanol, phytol and farnesyl acetate were found to be the main components. Furthermore, the oils were tested against six Gram-positive and Gram-negative bacteria and four phytopathogenic fungi. It was found that oils from both both parts of *R. acaule*, and especially that of *capitula* (C), exhibited interesting antibacterial activity, but no antifungal activity was observed.

Phytochemical evaluation of *Eucalyptus citridora* was done by Ahmed *et al.* (2002). TLC was used for separation and identification of constituents using ethyle acetate: petroleum ether: benzene solvent for resolution of maximum number of constituents in one single step. Further resolution of crude extracts was done by column chromatography which yielded several fractions among which fractions 1, 2, 3 exhibited the light brown colour, and fractions 4, 5, 6 and 7 exhibited red colour. This observation suggested that only two compounds have been extracted. Physico - chemical investigation showed that one of the isolated products was rutin (a major compound) whereas other compound remained unidentified.

Materials and Methods

Chapter-III

MATERIALS AND METHODS

The materials used and the methods employed in conducting the experiments are described in detail in this chapter.

3.1 Pure culture isolates of *Ralstonia solanacearum*

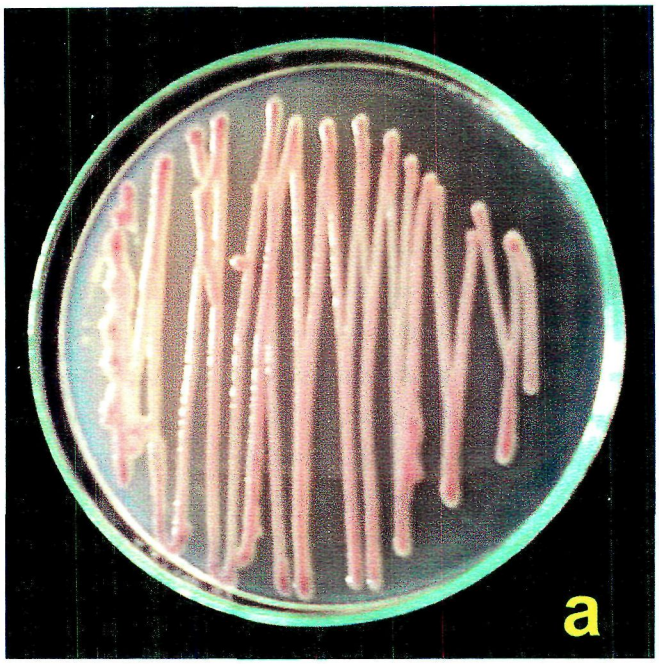
Fresh samples of bacterial wilt affected tomato, brinjal and capsicum plants were collected from farmers fields around Palampur or bacterial wilt sick plots of the Department of Vegetable Science and Floriculture, CSK HPKV, Palampur during the crop seasons of 2007 and 2008. The specimens were washed thoroughly in running tap water and presence of bacterium was confirmed by performing the ooze test. Sufficient bits of (2-3 mm) size were cut from the typical brown vascular tissue of wilted plants with the help of sterilized camel hair brush scalpel/blade, 2 to 3 cm above the crown region of the stem. The bits were surface sterilized by dipping in freshly prepared 0.1 per cent mercuric chloride or sodium hypochlorite solution for ½ to 1 minute followed by 3-4 washings in sterilized distilled water taken in sterilized petriplates. Two to three surface sterilized bits were then removed gently with the help of sterilized camel hair brush and were placed on sterilized clean slide containing a few drops of sterilized distilled water in the centre and crushed/macerated with the help of a sterilized blade and a dissecting needle.

After observing a waiting period of half to one minute for allowing the bacterial cells from the tissue to come out in water, a loopful of bacterial cell suspension sufficient for three plates was streaked on Kelman's (1953) 2,3,5

triphenyltetrazolium chloride (TZC medium - Appendix I) medium under aseptic conditions. In all, 20-30 petriplates per isolate were streaked. The inoculated petriplates were incubated at 28±1°C. After 48 hours of incubation, the isolated bacterial colonies were assessed for purity and selected for restreaking fresh TZC medium. The typical virulent colonies of *R. solanacearum*, on TZC medium were mucoid, irregular, shining and convex with production of lot of extracellular polysaccharide (EPS) around pinkish centers (Plate 3.1). Individual single colony was carefully picked up and streaked on two to three TZC plates to get a pure culture of the isolate. The pure culture of each isolate of *R. solanacearum* from all the three hosts' *viz.* tomato, eggplant and capsicum were established in a similar fashion using TZC slants.

3.1.1 Maintenance of pure culture isolates

Autoclaved screw capped glass vials (15 ml) each half filled with distilled water were sterilized twice by autoclaving at 1.09 kg/cm^2 for 20 minutes. One loopful of 36 -48 hour old bacterial growth of pure culture isolate was aseptically suspended in each of the three vials separately. The screw capped vials were properly labeled, wax sealed and stored at room temperature (25°C). A stock culture consisting of a set of at least three vials of each isolate was thus maintained. Whenever required, inoculum of the desired isolate was multiplied at 28±1°C by streaking a loopful of the stock suspension on freshly prepared TZC medium in petriplates and incubating for 36-48 hour. Typical virulent colonies so selected, was each streaked on TZC slants/petriplates for the multiplication of inoculum.



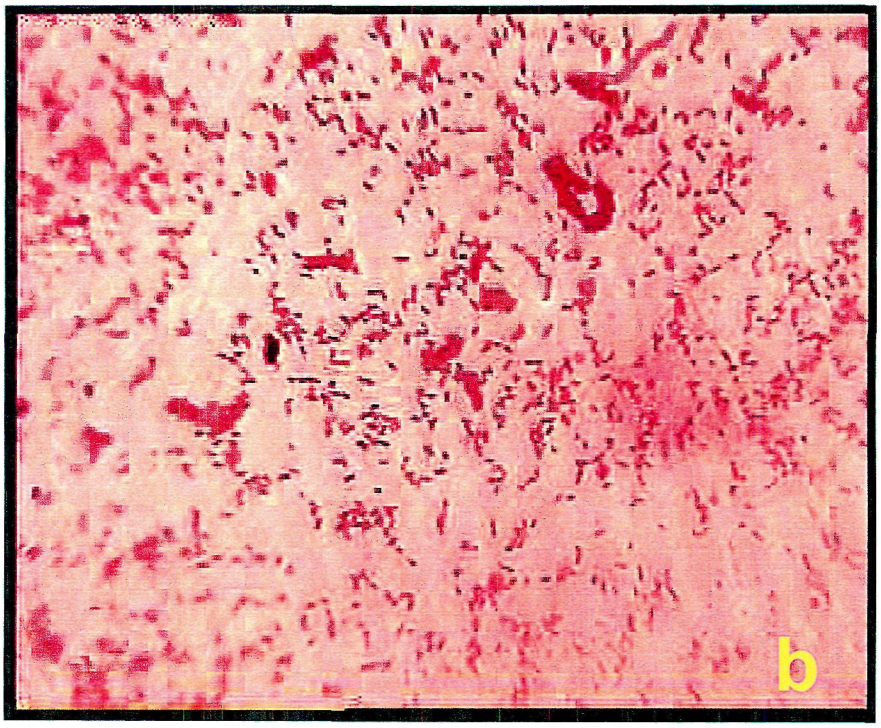


Plate 3.1 (a) Pure culture of *Ralstonia solanacearum* in TZC medium; (b) Gram negative bacilli of *R. Solanacearum.*

3.1.2 Inoculum preparation

As per requirement, sterilized distilled water (5 – 6 ml) was added aseptically in 36-48 hr growth of bacterium per slants. The bacterial suspension was prepared by gently rotating the slant between the palms and allowing to stand for some time. The bacterial suspension from different slants was pooled together in a sterlized flask/beaker (250-500 ml) and the cell suspension was adjusted with a spectrophotometer (Bausch and Lomb) to have 0.3 OD at 600 nm wavelength which corresponded to a cell concentration of 10^{10} cfu/ml by diluting the suspension with sterilized distilled water.

3.1.3 Testing cross pathogenicity

The pathogenicity of individual pure culture isolate was proved by inoculating it on the respective host as well as cross inoculating on all the three hosts *viz.* tomato (Solan Gola), brinjal (Pusa Purple Long), and capsicum (California Wonder) as per Kishun and Chand (1988) method.

3.2 Plants used as botanicals

Fourteen botanicals were use in the present study and the information regarding the botanicals and specific part/parts used for evaluation against *R. solanacearum* is presented in Table 3.1.

3.2.1 Collection and preservation of botanicals

The leaves of peppermint and geranium were collected from the herbal Garden of the Agroforestry and Organic Agriculture Department of C.S.K.H.P.K.V; Palampur. Leaves and flowers of artemisia were collected from field bunds and wastelands around Palampur. Green needles of deodar were

Table 3.1 Plants and their part/parts used

Sr. No	English name	Botanical name	Local name	Part/parts used
1	Peppermint	*Mentha piperita*	Peppermint	Leaves
2	Geranium	*Geranium stramonium*	Geranium	Leaves
3	Deodar	*Cedrus deodara*	Devdar	Needles (Green)
4	Toothache tree	*Zanthoxylum alatum*	Tirmir	Bark
5	Chaste tree	*Vitex negundo*	Banah	Leaves, Bark
6	Eucalyptus	*Eucalyptus citridora*	Safeda	Leaves, Seeds
7	Prickly pear	*Opuntia ficus-indica*	Nagphana	Leaves
8	Guava	*Psidium guajava*	Amrood	Leaves
9	Agave	*Agave americana*	Bara Kanwar	Leaves
10	Jatropha	*Jatropa curcus*	Ratanjot	Leaves, Bark
11	Indian oleander	*Nerium indicum*	Kaner	Leaves
12	Ageratum	*Ageratum houstonianum*	Nila phulnu	Leaves
13	Yellow oleander	*Thevetia nerifolia*	Pile kaner	Leaves
14	Artemisia	*Artimisia maritima*	Kirmala	Leaves, Flowers

collected from the university campus. Bark and leaves of chaste tree, agave and toothache tree were collected from wastelands near Bilaspur and Palampur. Leaves of eucalyptus, jatropha and guava were collected from the campus of C.S.K.H.P.K.V., Palampur. Leaves of Indian oleander were collected from sides of water streams near Palampur. Leaves and flowers of ageratum were collected from waste land near Palampur.

The leaves and bark of eucalyptus, prickly pear, agave, deodar and toothache tree were oven dried by spreading them on the shelves of a hot air oven over two to three layered blotting sheets at 50°C for 5 to 6 hours for two to three days. The leaves of peppermint, geranium, artemisia, chaste tree, jatropha, guava, Indian oleander, nila phulnu were shade dried at room temperature for five to six days. After drying, the respective plant material was ground in a wearing blender to obtain fine dry powder. Sufficient powdery biomass of all the botanicals was stored in paper bags (Tassel bags) at room temperature for further use.

3.3 Preparation of extracts

Aqueous and organic plant extracts of botanicals used in the present study were prepared as given below:

3.3.1 Preparation of aqueous plant extracts

Fifty gram fine powder of each botanical was soaked overnight in 100 ml of sterlized distilled water (1:2 w/v) in a conical flask (500 ml). Next day, the extract obtained was strained/filtered through a double layer of muslin cloth and twice through Whatmann No.1 filter paper to get the clear filtrate.

3.3.2 Preparation of organic plant extracts

Fifty gram fine powder of each botanical was soaked over night in 100 ml of 95% methanol (methyl alcohol) in a conical flask (500 ml). The flask was covered with aluminium foil to avoid evaporation of the solvent. The extract obtained was strained/filtered through double layer of muslin cloth and twice through Whatmann No.1 filter paper to get the clear filtrate.

3.3.3 Sterilization of aqueous and organic plant extracts

All organic and aqueous plant extracts obtained after filtration were sterlized with the help of a JSGW seitz glass filter (300 ml). Before filtration, the seitz filter assembly (flask, membrane filter and cup) was sterlized by autoclaving at 1.09 kg/cm^2 for 20 minutes. Different parts were assembled and connected to a vacuum pump and plant extract was sterilized. The sterilized plant extract was stored in another sterilized flask (250 or 500 ml) and marked as mother extract. The mother organic and aqueous extracts with 100 per cent concentration was further diluted to the 50 per cent by adding Dimethyle Sulphoxide (DMSO) in organic extract and sterilized distilled water proportionately in aqueous extracts and stored in the refrigerator for further use.

3.3.4 Removal of solvent from organic extracts

The organic solvent (methanol) was removed from all the organic extracts with the help of Flash Evaporator (LABORATA 4000) (Plate 3.2) at 50°C and 60 rpm for one hour. Dried extracts were reconstituted in 25 ml Dimethyle Sulphoxide (DMSO) and stored in screw capped vials (30ml) in deep freezer at 5°C.

3.3.5 Quantification of aqueous and organic plant extracts

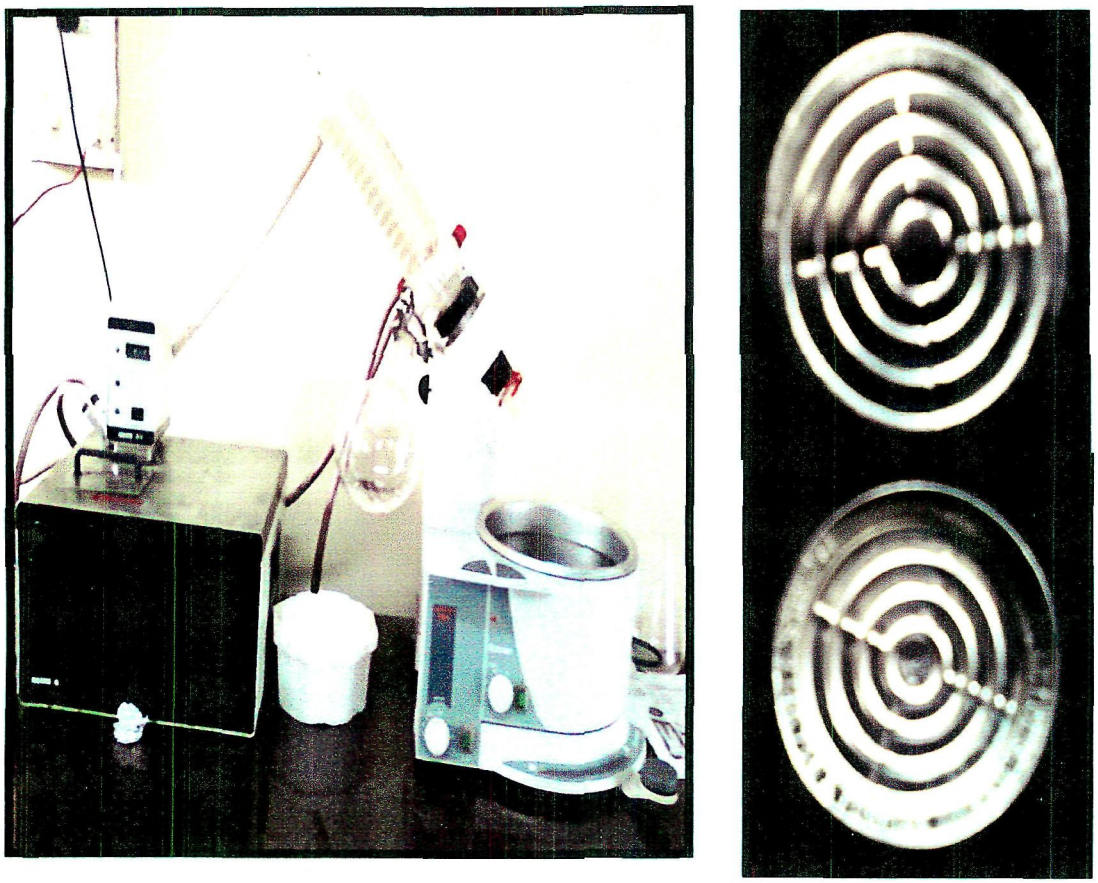
Quantification of each aqueous and organic plant extract was done by using steel chips (3 cm diameter) (Plate 3.2). Weight of each chip was recorded individually and then 0.5 ml of each extract was poured separately on steel chip in duplicate and recorded the wet weight of chip lined with extract. The chip were then dried over night in the hot air oven at 50°C and the dry weight of the chip lined with extract was taken and accordingly amount of material (crude compound + active ingredient) was worked out in 0.5 ml of each extract. Further amount of material (crude compound + active ingredient) was computed for 25 ml, 5ml and 0.01ml.

3.4 Essential oils

Besides 14 plant extracts, five essential oils viz. terpinol oil, citronella oil, palmarosa oil, neem oil, lemon grass oil procured from M/S Kanta chemical company, Tilak Bazar, Khari Baoli, Delhi, were also evaluated against *R. solanacearum* in the present study.

3.5 *In vitro* evaluation of plant extracts against *Ralstonia solanacearum*

Aqueous and organic plant extracts were evaluated *in vitro* at 100 and 50 per cent concentrations by paper disc, Spectrophotometeric and plate count methods. Streptocycline and copper oxychloride at 100 and 50 µg/ml and 0.25 and 0.125 per cent concentration, respectively were included as check treatments.



a b

Plate 3.2 **(a)** Flash Evaporator (LABOROTA 4000)
(b) Steel chip used for quantification

3.6 *In vitro* evaluation of essential oils against *Ralstonia solanacearum*

Essential oils were evaluated *in vitro* at 100, 50 and 25 percent by paper disc, agar well diffusion and plate count methods. Streptocycline and copper oxychloride at 100, 50 and 25 µg/ml and 0.25, 0.125 and 0.625 per cent concentration were included as check treatments.

3.7 Seeding of TZC medium with bacterial suspension

TZC medium was prepared and sterilized by autoclaving at 1.09 kg/cm^2 corresponding to 121°C for 15-20 minutes. At the time of pouring, 2 ml of bacterial suspension (10^{10} cfu/ml) of *R. solanacearum* was added per 100 ml of the medium and mixed thoroughly by gentle shaking. The seeded medium was then poured into sterilized petriplates under aseptic conditions.

3.8 Paper disc method

Circular paper discs (6 mm diameter) of Whatman No.1 filter paper were cut with the help of paper puncture and collected in a petriplate. The paper discs were sterilized by autoclaving. Three discs for each treatment were picked up with the help of a sterilized needle and placed equidistantly over the seeded TZC medium in a petriplate. With the help of a micropipette, each paper disc was impregnated with 10 µl extract of each concentration along with the test chemicals streptocycline and copper oxychloride. Likewise, paper discs impregnated with 10 µl DMSO served as control for organic extracts and with sterlized distilled water served as control for aqueous extracts. The three discs placed in a single petriplate served as three replications of each treatment, two such petriplates comprised the six replications of each treatment. The petriplates were then placed in lower most shelf of a refrigerator for half an hour at 5°C,

thereby allowing the plant extracts and test chemicals to diffuse into medium. The plates were then shifted to the incubator at 28± 1°C. The inhibition zone (mm) formed around each of the three discs in a plate of different treatments was measured with the help of a 15 cm scale after 48 hours of incubation. The mean inhibition zone was worked out for each treatment and compared with the control.

3.9 Spectrophotometeric method

Plant extracts, along with the test chemicals streptocycline and copper oxychloride were evaluated *in vitro* for antimicrobial activity against *R. solanacearum* by Spectrophotometeric method given by Manav and Thind (2001). 2.5 ml of the stock solution of each plant extract and test chemical was added into two test tubes (15 x 150 mm), each containing 2.5 ml of sterlized double strength TZC broth. The two test tubes comprised the two replications of each treatment. The mixture in each test tube was inoculated with 1 ml of bacterial cell suspension (10^{10} cfu/ml) of *R. solanacearum* and incubated at 28 ± 1°C for 48 hours. Likewise, two test tubes containing inoculated and uninoculated TZC broth and DMSO served as control for organic extract and inoculated and uninoculated TZC broth and distilled water served as control for aqueous extract. After incubation, the turbidity of bacterial growth in each treatment was measured by recording optical density (OD) with the help of a spectrophotometer (Bosch and Lomb) at 600 nm wavelength. Each time, the spectrophotometer scale was set at 'zero' optical density with blank which consisted of double strength TZC broth mixed with equal quantity of sterlized water.

3.10 Plate count method

Plant extracts and test chemicals along with inoculated and uninoculated control were also evaluated by pour plate technique. In each flask containing 50 ml of seeded TZC medium, 2.5 ml of each concentration of a plant

extract and essential oil was added, separately before pouring. The ingredients were mixed thoroughly by shaking the contents gently. Twenty to twenty five ml each of the mixture was aseptically poured in two sterlized petriplates, representing two replications of each treatment. The plates were incubated at 28±1°C for 24 hours. The inoculated and uninoculated plates without plant extracts/essential oil served as control. After incubation, the number of *R. solanacearum* colonies formed in each plate were counted with the help of a 'STUART' colony counter. Mean colony count per treatment was then worked out.

3.11 Evaluation of essential oils by agar well diffusion method

Purified essential oils namely terpinol, lemongrass, citronella, neem and palmarosa oils were evaluated for their antimicrobial activity against *R. solanacearum*, by agar well diffusion method at 100, 50 and 25 percent concentrations. The seeded TZC medium (20-25 ml) was poured in each sterlized petriplate and allowed to solidify. Three wells separated equidistantly, representing three replications, were cut with the help of a sterilized cork borer. Each of the three wells in petriplate was filled with the respective concentration of each oil, separately. Likewise, wells in the petriplate filled with sterlized distilled water served as control treatment. The plates were incubated at 28±1°C for 48 hours and inhibition zone (mm) around each well was measured with the help of 15 cm scale. The mean inhibition zone per treatment was then worked out.

3.12 *In vivo* evaluation of plant extracts against *Ralstonia solanacearum*

Based on *in vitro* evaluation, six organic plant extracts (peppermint, eucalyptus, ageratum, guava, devdar and tirmir) were selected for determining their *in vivo* efficacy. Apparently healthy tomato (Solan Gola) nursery was raised

in sterlized soil taken in iron trays (30 x 21cm) from 1st March to 25th March 2009. Twenty five days old seedlings were uprooted gently and washed in running tap water to remove the adhering soil. These seedlings were then washed with sterilized distilled water. The seedlings were dipped in 100 per cent concentration of each aqueous and organic plant extract for 30 and 60 minute duration, respectively. Likewise, tomato seedlings dipped in sterlized distilled water and DMSO for the same duration served as control treatments for aqueous and organic extracts, respectively. The treated seedlings were then transplanted in sterilized soil in pots separately on 26th March, 2009. Three seedlings each transplanted in a separate pot comprised three replications of different treatments. The seedlings were first kept in the cage house and allowed to stabilize in the pots. After five days seedlings were inoculated with the test bacterium by pouring 20 ml of inoculum (10^{10} cfu ml^{-1}) per plant. The inoculated plants were kept in polyhouse at 35-45°C. The inoculated plants were observed daily for the development of symptoms regularly for 30 days.

3.13 Fractionation of effective plant extracts

Based on *in vitro* and *in vivo* studies, five effective organic plant extracts *i.e.* peppermint, eucalyptus, ageratum, guava and devdar were selected for fractionation. With the help of thin layer chromatography (TLC), fractionation of these five extracts was done.

3.13.1 Thin layer chromatography (TLC) analysis of effective plant extracts

Thin layer chromatography (TLC) analysis of the effective organic plant extract of peppermint, eucalyptus, ageratum, guava and deodar was done on glass plates using silica gel G (Sharma and Dawra, 1991; Stahl, 1969). The

plates were washed using chromic acid. The dried plates were wiped with acetone so as to remove any traces of grease. Slurry was prepared by mixing 50 g of silica gel G in 90-100 ml of water. The slurry was immediately transferred to the spreader (adjusted to 0.2 mm thickness) and spread over the plates. The plates were allowed to dry at room temperature followed by activation at 110°C for 1 hour. The solvent system used was made up of methanol (B.P 64.7 °C) chloroform (B.P 61.2°C) (10:90) v/v. The developed plates were dried at room temperature. The detection was done as per Sharma and Dawra (1991) and Stahl (1969) methods.

3.13.1.1 Iodine vapours

The plates were exposed to Iodine vapours in an iodine saturated chamber for about 15 minutes. Such an exposure yielded dark yellow spots on the TLC plates.

3.13.1.2 Ethanol-sulphuric acid reagent

Ethanol-sulphuric acid reagent was prepared by mixing ethanol and H_2SO_4 (90:10) in ice cold conditions. After spraying, the plates were heated at 110°C for 10 to 15 minutes.

3.14 Testing efficacy of crude fractions of effective extracts against *R. solanacearum*

Efficacy of plant extracts was evaluated by Contact Bioautography (Fisher and Lautner, 1961; Nicolaus *et al.*, 1961). Spots of plant extracts (10 µl) were applied from the base on duplicate precoated silica TLC plates and were developed with chloroform : methanol (90:10) as the developing solvent. One set was used as the reference chromatogram whereas the other set was used for

bioautography (comparison of organic compounds separated by chromatography by means of their effect on test bacterium) The chromatogram was placed face down onto the inoculated TZC agar layer and left for 30 minutes in the lower most shelf of refrigerator at 5 °C to enable diffusion. Then the chromatogram was removed under sterilized conditions and the TZC agar plate was incubated at 28±1 °C for 48 hours. The inhibition zones were observed on the TZC agar surface in the places where the spots of antibacterial fractions of plant extracts were allowed to diffuse to the agar. The spots on reference TLC plates were developed in iodine chamber. Formation of Inhibition zones indicated the presence of active compounds in the extracts. Inhibition zones were compared with the *R*eference of the related spots on reference TLC plates.

3.15 Statistical analysis

The statistical analysis was done as per Gomez and Gomez (1984).The data recorded were subjected to factorial RBD analysis. The significance of difference was tested at 5 per cent level.

Results

Chapter-IV

RESULTS

The results obtained during present investigation are presented under following heads:

4.1 Quantification of plant extracts

4.1.1 Quantification of aqueous extracts

4.1.2 Quantification of organic extracts

4.2 Evaluation of aqueous plant extracts against *R. solanacearum*

4.2.1 *In vitro* evaluation

4.2.1.1 Paper disc method

4.2.1.2 Spectrophotometeric method

4.2.1.3 Plate count method

4.2.2 *In vivo* evaluation

4.3 Evaluation of organic plant extracts against *R. solanacearum*

4.3.1 *In vitro* evaluation

4.3.1.1 Paper disc method

4.3.1.2 Spectrophotometeric method

4.3.1.3 Plate count method

4.3.2 *In vivo* evaluation

4.4 Evaluation of essential oils against *R. solanacearum*

4.4.1 *In vitro* evaluation

4.4.1.1 Paper disc method

4.4.1.2 Agar well diffusion method

4.4.1.3 Plate count method

4.5 Fractionation of effective plant extracts

4.6 Evaluation of crude fractions from effective plant extracts against *R. solanacearum*

4.1 Quantification of plant extracts

4.1.1 Quantification of aqueous extracts

The results of quantification of aqueous plant extracts are presented in Table 4.1. Quantity of crude material + active material was worked out for 25 ml, 2.5 ml and 0.01 ml of extracts. The extract of chaste tree and deodar contained the maximum quantity of active ingredient + crude material at all the three concentrations and extract of agave, Indian oleander and ageratum were at par with deodar and chaste tree where as extract of jatropha contained minimum quantity of active ingredient + crude material at all the three concentrations.

4.1.2 Quantification of organic extracts

The results of quantification of organic plant extracts are presented in Table 4.2. Like aqueous extracts quantity of crude material + active material was also worked out for 25 ml, 2.5 ml and 0.01 ml of organic extracts. The extract of Indian oleander contained the maximum quantity of active ingredient + crude material at all the three concentrations and extracts of peppermint, artemisia, chaste tree and prickly pear were at par with Indian oleander where as extract of geranium and toothache tree contained minimum quantity of active ingredient + crude material at all the three concentrations.

4.2 Evaluation of aqueous plant extracts against *R. solanacearum*

4.2.1 *In vitro* evaluation

4.2.1.1 Paper disc method

The results of *in vitro* efficacy of aqueous plant extracts against *R. solanacearum* are presented in Table 4.3. Eucalyptus at 100 percent concentration showed maximum inhibition of 6.20 mm against *R. solanacearum*

Table 4.1 Quantification of active ingredient + crude material in aqueous extracts of plants

	Dry weight of active ingredient + crude material (µg)*		
Plant extract	**25 ml**	**2.5 ml**	**0.01 ml**
Peppermint	1983.0	198.3	0.793
Toothache tree	1984.0	198.4	0.793
Guava	1985.0	198.5	0.794
Artemisia	1985.0	198.5	0.794
Yellow oleander	1989.0	198.9	0.795
Agave	1990.5	199.0	0.796
Chaste tree	1995.0	199.5	0.798
Prickly pear	1985.5	198.5	0.794
Eucalyptus	1983.0	198.3	0.793
Geranium	1989.0	198.9	0.795
Jatropha	1978.5	197.8	0.791
Deodar	1995.0	199.5	0.798
Indian oleander	1991.0	199.1	0.796
Ageratum	1994.0	199.4	0.797
CD	**6.6**	**4.2**	**0.007**
CV %	**0.2**	**0.5**	**0.430**

*Average of two replications

Table 4.2 Quantification of active ingredient + crude material in organic extracts of plants

	Dry weight of active ingredient + crude material (µg)*		
Plant extract	**25 ml**	**2.5 ml**	**0.01 ml**
Peppermint	1995.0	199.5	0.798
Toothache tree	1975.5	197.5	0.790
Guava	1985.5	198.5	0.794
Artemisia	1993.5	199.3	0.797
Yellow oleander	1978.5	197.8	0.791
Agave	1985.0	198.5	0.794
Chaste tree	1995.0	199.5	0.798
Prickly pear	1990.5	199.0	0.796
Eucalyptus	1984.0	198.4	0.793
Geranium	1975.0	197.5	0.790
Jatropha	1979.5	197.9	0.791
Deodar	1994.0	199.4	0.797
Indian oleander	1997.0	199.7	0.799
Ageratum	1985.5	198.5	0.794
CD	**10.9**	**6.7**	**0.005**
CV (%)	**0.3**	**0.8**	**0.310**

*Average of two replications

Table 4.3 ***In vitro*** **efficacy of aqueous plant extracts against** ***R. solanacearum*** **by paper disc method**

Treatment	Concentration (%)/µgml^{-1}	Inhibition zone (mm)*
Peppermint	100	5.40
	50	2.87
Toothache tree	100	2.10
	50	0.17
Guava	100	4.60
	50	1.80
Artemisia	100	1.10
	50	0.40
Yellow oleander	100	0.13
	50	0.00
Agave	100	0.57
	50	0.10
Chaste tree	100	0.17
	50	0.00
Prickly pear	100	0.00
	50	0.00
Eucalyptus	100	6.20
	50	3.10
Geranium	100	0.00
	50	0.00
Jatropha	100	0.13
	50	0.00
Deodar	100	2.90
	50	0.50
Indian oleander	100	0.00
	50	0.00
Ageratum	100	4.10
	50	1.50
Streptocycline**	100	8.70
	50	6.00
Copper oxychloride	0.25	5.83
	0.125	3.67
Control		0.00
CD (P=0.05)		**0.11**
CV (%)		**3.65**

***Average of six replications**
****concentration in µgml^{-1}**

followed by peppermint, guava and ageratum with inhibition zones of 5.40, 4.60 and 4.10 mm, respectively; however eucalyptus was statistically superior to rest of three extracts. At 50 percent concentration also, the aqueous extract of eucalyptus (3.10 mm) was found maximum inhibitory followed by peppermint (2.87 mm), guava (1.80 mm) and ageratum (1.50 mm), respectively. In case of the test chemicals streptocycline was more inhibitory than all the extracts and eucalyptus at 100 percent concentration was more inhibitory than copper oxychloride. The extracts of prickly pear, geranium and Indian oleander were not at all inhibitory at any of the concentrations. The extracts of jatropha and yellow oleander were least inhibitory among all the aqueous extracts. Inoculated control without any plant extract or test chemical did not form any inhibition zone.

4.2.1.2 Spectrophotometeric method

In vitro evaluation of aqueous plant extracts by spectrophotometeric method revealed that aqueous extract of eucalyptus at both the concentrations (100 and 50 percent) was most inhibitory against *R. solanacearum* with O.D values of 0.18 and 0.24 followed by peppermint, ageratum and guava, respectively. Other plant extracts were comparatively less inhibitory. In case of test chemicals streptocycline was more inhibitory (0.11 and 0.15) than all the extracts at both the concentrations. The extract of eucalyptus at both the concentrations was more inhibitory (0.18 and 0.22) than copper oxychloride (0.19 and 0.24). The inhibitory effect of different organic extracts decreased correspondingly with decrease in the concentration of extracts (Table 4.4).

Table 4.4 ***In vitro*** **efficacy of aqueous plant extracts against *R. solanacearum* by spectrophotometeric and plate count methods**

Treatment	Concentration (%)/µgml^{-1}	Spectrophotometeric method (OD*600nm)	Plate count method (cfu/ml*)
Peppermint	100	0.21	221.0
	50	0.27	276.0
Toothache tree	100	0.66	464.3
	50	0.70	515.0
Guava	100	0.43	367.0
	50	0.51	468.0
Artemisia	100	0.81	566.3
	50	0.85	608.0
Yellow oleander	100	0.87	665.0
	50	0.94	751.0
Agave	100	0.89	623.0
	50	0.92	712.7
Chaste tree	100	0.87	567.0
	50	0.95	591.7
Prickly pear	100	0.91	877.7
	50	0.96	904.0
Eucalyptus	100	0.18	217.3
	50	0.22	266.3
Geranium	100	1.08	885.0
	50	1.13	892.0
Jatropha	100	0.98	932.0
	50	1.18	977.0
Deodar	100	0.63	445.0
	50	0.68	486.7
Indian oleander	100	1.11	707.7
	50	1.15	793.0
Ageratum	100	0.32	316.0
	50	0.46	355.7
Streptocycline**	100	0.11	208.3
	50	0.15	225.0
Copper oxychloride	0.25	0.19	321.0
	0.125	0.24	365.7
Inoculated control		0.98	995.0
Uninoculated		0.0	0.0
CD (P=0.05)		**0.15**	**1.9**
CV (%)		**1.38**	**0.2**

***Average of two replications**
****concentration in µgml^{-1}**

4.2.1.3 Plate count method

The aqueous extract of eucalyptus at both the concentrations was found to be most inhibitory against the tomato isolate of *R. solanacearum* by plate count method with 217.3 and 266.3 cfu/ml values followed by peppermint, nila phulnu and guava. In case of test chemicals, streptocycline was more inhibitory than all the extracts at both the concentrations, where as extracts of eucalyptus and peppermint at both the concentrations were more inhibitory than copper oxychloride (Table 4.4).

4.2.2 *In vivo* evaluation

The results of *in vivo* evaluation of aqueous plant extracts against *R. solanacearum* have been presented in Table 4.5. Eucalyptus and peppermint extracts were at par with each other and resulted maximum survivability days of 17.7 and 16.7 after 60 minutes dip at 100 percent concentration, followed by ageratum and guava. However, tirmir was least inhibitory. After 30 minutes dip at 100 percent concentration, eucalyptus was most inhibitory followed by peppermint and ageratum with survivability days of 15.7, 14.3 and 12.3, respectively. Inoculated plants survived up to 8 days after inoculation but the uninoculated plants could survive up to 30 days after transplantation. The survivability days of tomato plants after 60 minute dip and 30 minute dip in aqueous extracts have been graphically illustrated in Fig. 4.1.

Table 4.5 ***In vivo*** **evaluation of aqueous plant extracts against** ***R. solanacearum***

Treatment	Concentration (%)	Survivability (DAI)** Duration of dipping	
		30 min*	60 min*
Peppermint	100	14.3	16.7
Eucalyptus	100	15.7	17.7
Guava	100	10.7	12.8
Ageratum	100	12.3	15.0
Deodar	100	8.6	10.0
Toothache tree	100	8.0	9.3
Inoculated control		8.3	8.0
Uninoculated control		30.0	30.0
CD (P=0.05)		**0.9**	**1.2**
CV (%)		**6.8**	**7.8**

***Average of three replications**
****Days after inoculation**

4.3 Evaluation of organic plant extracts against *R. solanacearum*

4.3.1 *In vitro* evaluation

4.3.1.1 Paper disc method

The results of *in vitro* efficacy of organic plant extracts against *R. solanacearum* by paper disc method are presented in Table 4.6. Peppermint at 100 percent concentration produced maximum inhibition zone of 8.10 mm against tomato isolate of *R. solanacearum.* Its efficacy was followed by eucalyptus, ageratum and guava with inhibition zones of 7.37, 7.10 and 6.53 mm respectively (Plate 4.1) which were significantly lower than that of peppermint.

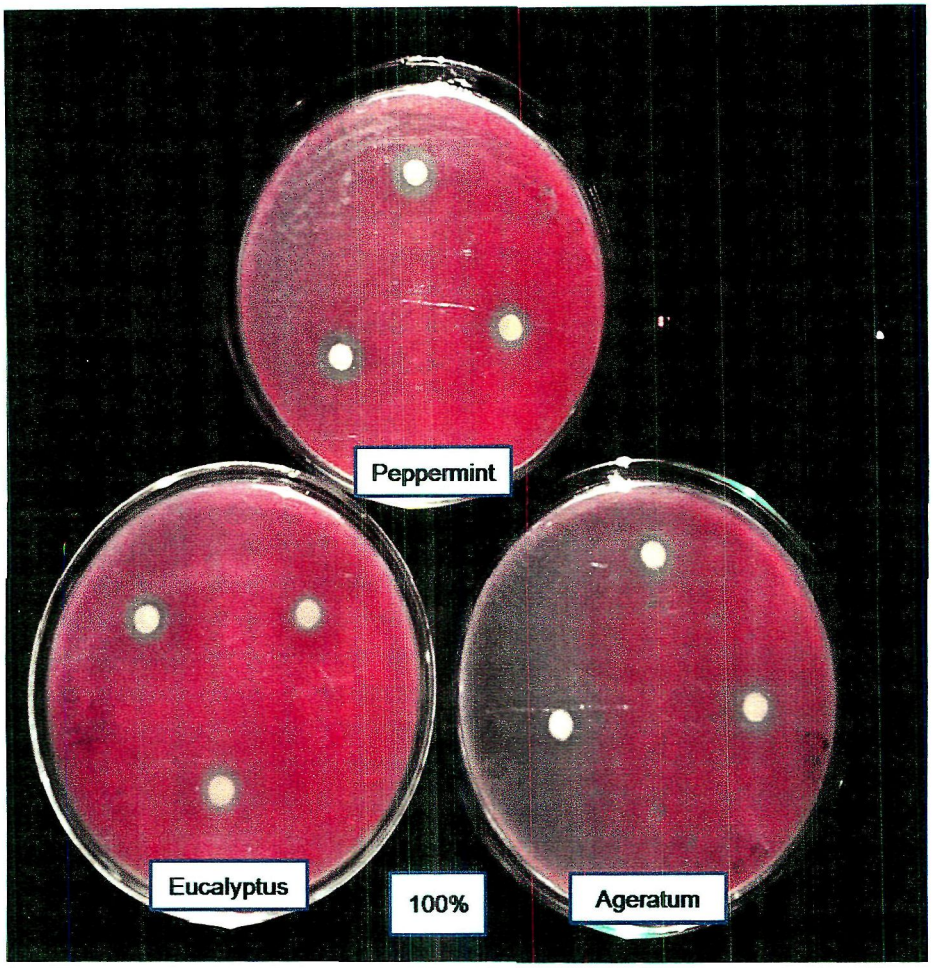
At 50 percent concentration also peppermint showed maximum inhibition zone of 4.87 mm followed by eucalyptus and ageratum with inhibition zones of 4.07 and 3.80 mm, which was lower than peppermint.

Among all the plant extracts, the extracts of geranium and Indian oleander didn't show any inhibition irrespective of concentration. The extracts of yellow oleander, prickly pear and jatropha were effective at 100 percent concentration only. With the decreasing concentration of organic extracts and test chemicals, correspondingly lower inhibition zones were produced against *R. solanacearum.* In case of the test chemicals, streptocycline was more inhibitory than all the extracts. The extracts of peppermint at both the concentrations and that of eucalyptus, guava and ageratum at 100 percent concentration were more inhibitory than copper oxychloride. The inoculated control without any plant extract or test chemical did not form any inhibition zone.

Table 4.6 ***In vitro*** **efficacy of organic plant extracts against** ***R. solanacearum*** **by paper disc method**

Treatment	Concentration (%)/µgml^{-1}	Inhibition zone* (mm)
Peppermint	100	8.10
	50	4.87
Toothache tree	100	3.60
	50	1.10
Guava	100	6.53
	50	3.67
Artemisia	100	1.50
	50	0.67
Yellow oleander	100	0.30
	50	0.00
Agave	100	1.20
	50	0.27
Chaste tree	100	0.57
	50	0.17
Prickly pear	100	0.17
	50	0.00
Eucalyptus	100	7.37
	50	4.07
Geranium	100	0.00
	50	0.00
Jatropha	100	0.17
	50	0.00
Deodar	100	4.10
	50	1.43
Indian oleander	100	0.00
	50	0.00
Ageratum	100	7.10
	50	3.80
Streptocycline**	100	8.33
	50	6.03
Copper oxychloride	0.25	5.87
	0.125	4.23
control		0.00
CD (P=0.05)		**0.14**
CV (%)		**3.19**

***Average of six replications**
****concentration in µgml^{-1}**



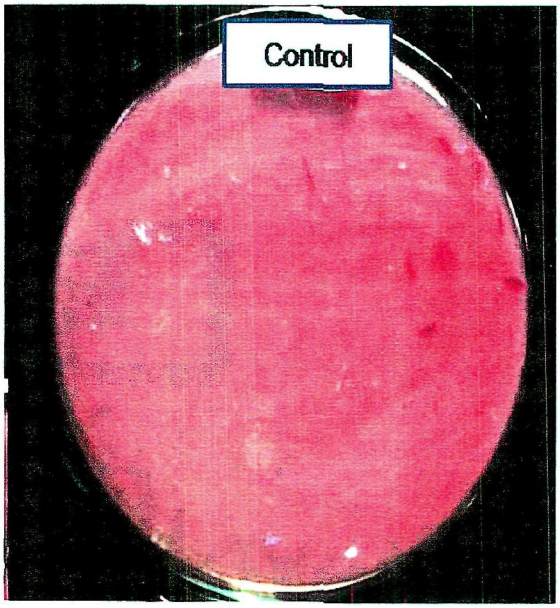


Plate 4.1 Inhibition zone (mm) produced by organic extract of peppermint, eucalyptus and ageratum

4.3.1.2 Spectrophotometeric method

In vitro evaluation of organic plant extracts by spectrophotometeric method revealed that the organic extract of peppermint at both the concentrations (100 and 50 percent) was most inhibitory against *R. solanacearum* with O.D values of 0.11 and 0.16, respectively followed by eucalyptus, ageratum and guava. Other plant extracts were comparatively less inhibitory (Table 4.7). In case of test chemicals, inhibitory effect of streptocycline was more as compared to all the plant extracts and copper oxychloride at both the concentrations, whereas peppermint extract at both the concentrations was more inhibitory than copper oxychloride. The inhibitory effect of different organic extracts decreased correspondingly with decrease in the concentration of extracts.

4.3.1.3 Plate count method

The organic extract of peppermint at both the concentrations was found to be most inhibitory against the tomato isolate of *R. solanacearum* by plate count method with 211.7 and 246.7 cfu/ml followed by eucalyptus and ageratum. In case of test chemicals, streptocycline was more inhibitory than all the extracts and copper oxychloride at both the concentrations. However, the extracts of peppermint and eucalyptus at both the concentrations were more inhibitory than copper oxychloride.

Table 4.7 ***In vitro*** **efficacy of organic plant extracts against** ***R. solanacearum*** **by spectrophotometeric and plate count methods**

Treatment	Concentration (%)/µgml^{-1}	Spectrophotometeric method	Plate count method
		OD* (600nm)	cfu/ml*
Peppermint	100	0.11	211.7
	50	0.16	246.7
Toothache tree	100	0.37	426.7
	50	0.43	454.0
Guava	100	0.28	326.7
	50	0.31	448.3
Artemisia	100	0.44	528.0
	50	0.67	607.3
Yellow oleander	100	0.61	540.0
	50	0.88	566.7
Agave	100	0.57	443.3
	50	0.68	534.3
Chaste tree	100	0.81	466.7
	50	0.92	480.3
Prickly pear	100	0.90	720.0
	50	0.95	838.7
Eucalyptus	100	0.17	215.0
	50	0.19	248.0
Geranium	100	0.93	599.0
	50	0.96	684.7
Jatropha	100	0.91	731.3
	50	0.96	843.0
Deodar	100	0.33	325.3
	50	0.35	348.0
Indian oleander	100	0.90	584.3
	50	0.94	634.3
Ageratum	100	0.24	316.3
	50	0.28	439.0
Streptocycline**	100	0.09	192.7
	50	0.12	209.0
Copper oxychloride	0.25	0.15	310.0
	0.125	0.17	337.0
Inoculated control		0.98	992.3
Uninoculated		0.00	0.0
CD (P=0.05)		**0.015**	**2.1**
CV (%)		**1.77**	**0.3**

****concentration in µgml^{-1}**

***Average of two replications**

4.3.2 *In vivo* evaluation

The results of *in vivo* evaluation of organic extracts against tomato isolate of *R. solanacearum* are presented in Table 4.8. Peppermint extracts resulted in maximum survivability of 22.7 days after 60 minutes dip and 18.0 days after 30 minutes dip at 100 percent concentration (Plate 4.2) followed by eucalyptus (Plate 4.3) and guava (Plate 4.4) with survivability days of 19.7 and 18.3 days after 60 minutes dip. After 30 minutes dip, eucalyptus and guava were at par with each other with survivability days of 15.3 and 15. Inoculated control plants survived up to 8 days after inoculation however uninoculated control plants survived up to 30 days after inoculation (Plate 4.5). Wilted plants were confirmed by conducting ooze test (Plate4.6). The survivability days of tomato plants after 60 minute dip and 30 minute dip in organic extracts has been presented graphically in Fig. 4.2.

4.4 Evaluation of essential oils against *R. solanacearum*

4.4.1 *In vitro* evaluation

4.4.1.1 Paper disc method

The results of *in vitro* efficacy of essential oils by paper disc method against *R. solanacearum* are presented in Table 4.9. Terpinol oil at all the three concentrations (100, 50, and 25 per cent) showed maximum inhibitory effect with inhibition zones of 13.33, 9.37 and 3.60 mm, respectively followed by citronella oil, lemon grass (Plate 4.7) and palmarosa oil. However, neem oil was inhibitory only at 100 per cent concentration. As the concentration of oils decreased, there was corresponding decrease in size of inhibition zones also. In case of check

treatments streptocycline produced smaller inhibition zones than essential oils terpinol, citronella at all the three concentrations and lemon grass oil at 100 per cent concentration. Whereas copper oxychloride produced inhibition zones smaller than terpinol, citronella, lemon grass and palmarosa oil at all the three concentrations. The inoculated control however did not produce any inhibition zone.

Table 4.8 ***In vivo*** **evaluation of organic plant extracts against** ***R. solanacearum***

Treatment	Concentration (%)	Survivability (DAI)** Duration of dipping	
		30 min*	60 min*
Peppermint	100	18.0	22.7
Eucalyptus	100	15.3	19.7
Guava	100	15.0	18.3
Ageratum	100	12.0	15.8
Deodar	100	10.7	12.8
Toothache tree	100	8.0	12.0
Inoculated control		7.7	8.3
Uninoculated control		30.0	30.0
CD (P=0.05)		**1.0**	**0.9**
CV (%)		**6.6**	**5.3**

***Average of three replications**
****Days after inoculation**

Plate 4.2 Root dip in 100% concentration of organic extract of peppermint (*Mentha piperita*)

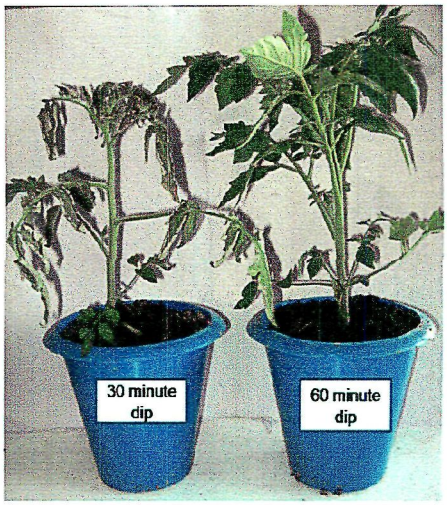


Plate 4.3 Root dip in organic extract eucalyptus (*Eucalyptus citridora)*

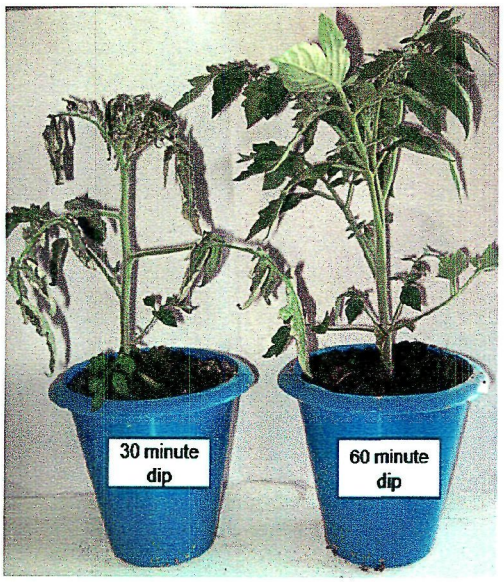


Plate 4.4 Root dip in 100% concentration of organic extract of guava (*Psidium guajava)*

Plate 4.5 Uninoculated and inoculated control, 8 days after inoculation

Plate 4.6 White milky bacterial ooze streaming from cross section of wilted tomato plant

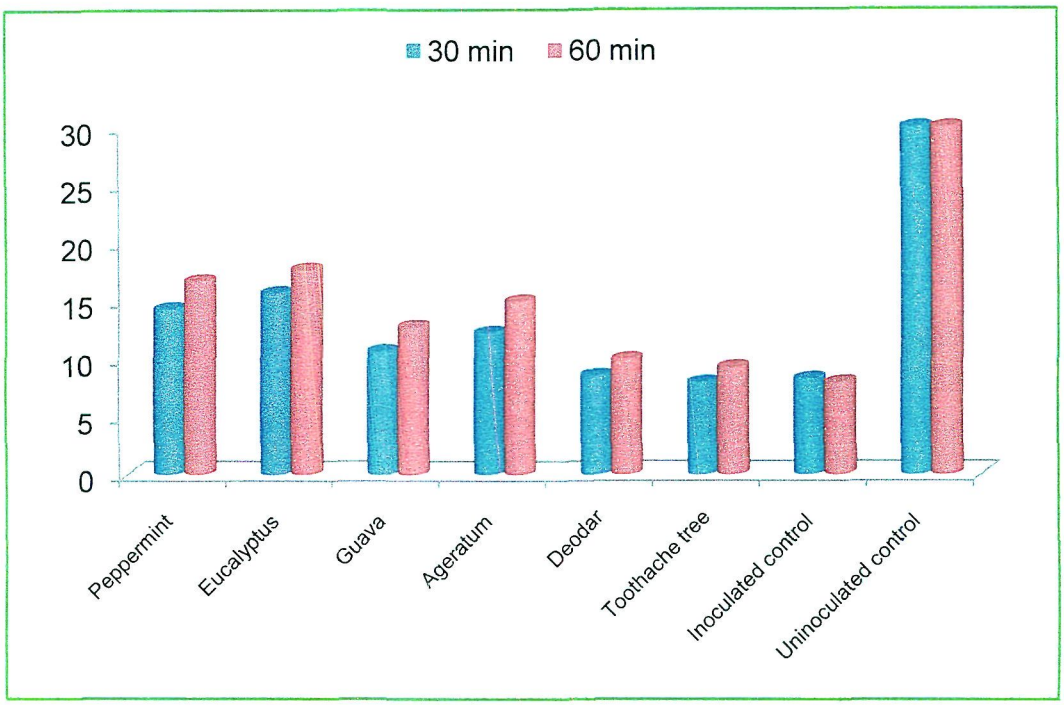


Fig. 4.1 *In vivo* evaluation of aqueous plant extracts against *R. solanacearum*

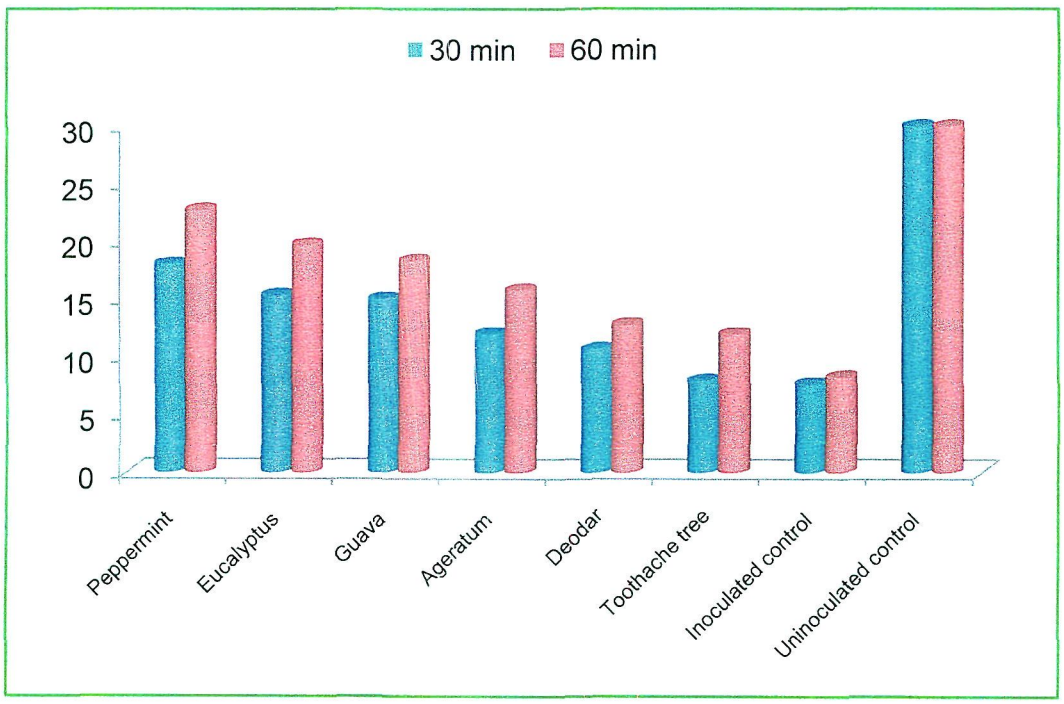


Fig. 4.2 *In vivo* evaluation of organic plant extracts against *R. solanacearum*

Table 4.9 ***In vitro*** **efficacy of Essential oils against** ***R. solanacearum*** **by Paper disc method**

Treatment	Concentration(%)/µgml^{-1}	Inhibition zone (mm)*
Terpinol	100	13.33
	50	9.37
	25	3.60
Citronella	100	10.10
	50	7.40
	25	3.10
Lemon grass oil	100	8.93
	50	5.83
	25	2.53
Palmarosa oil	100	5.60
	50	3.83
	25	1.20
Neem oil	100	0.80
	50	0.00
	25	0.00
Streptocycline**	100	7.73
	50	5.60
	25	2.43
Copper oxychloride	0.25	4.97
	0.125	2.63
	0.062	0.77
Control		0.00
CD (P=0.05)		**0.15**
CV (%)		**1.87**

***Average of six replications**
**** Concentration in µg/ml**

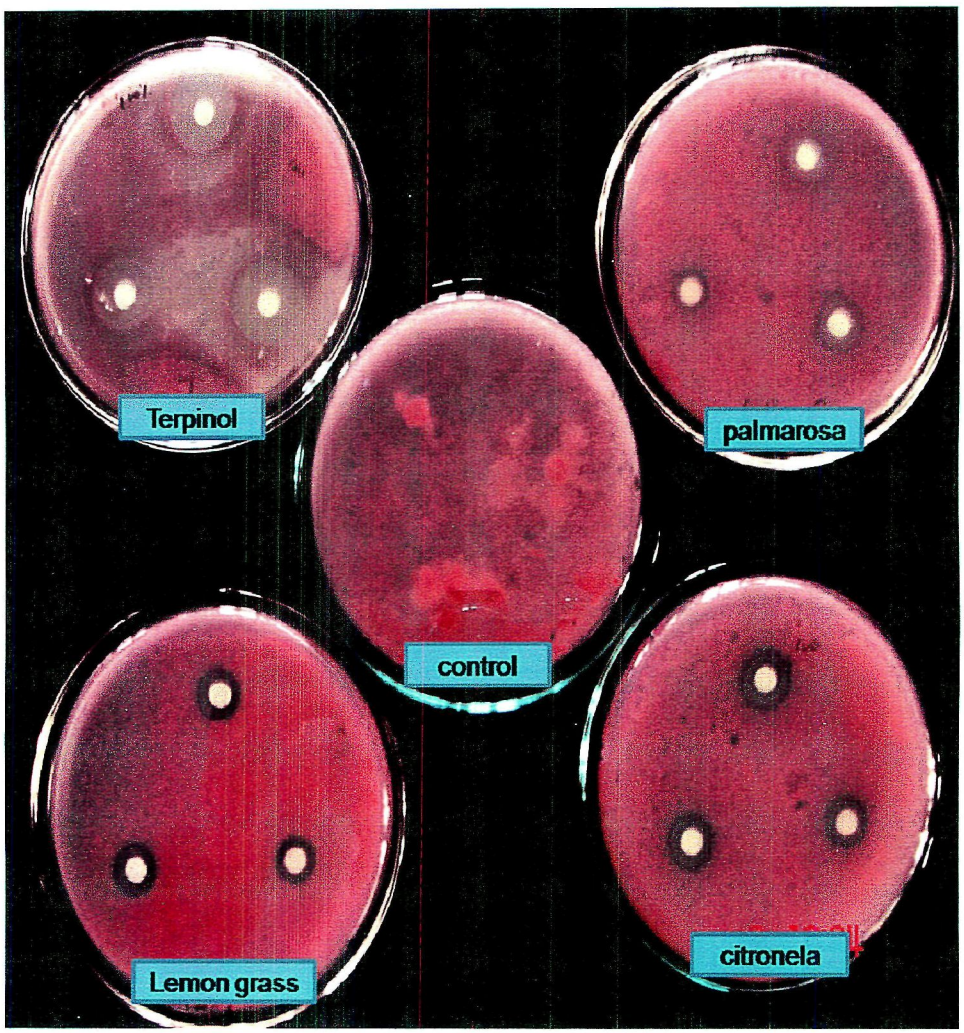


Plate 4.7 Inhibition zones (mm) produced by essential oils at 100 percent concentration

4.4.1.2 Plate count method

The results of *in vitro* efficacy of essential oils by plate count method are presented in Table 4.10. All the five oils were found inhibitory against tomato isolate of *R. solanacearum.* Terpinol oil at all the concentrations was found to be most inhibitory against the tomato isolate of *R. solanacearum* with 166.0, 186.7 and 266.0 cfu/ml followed by citronella, lemon grass oil and palmarosa oil. In case of check treatments, streptocycline was less inhibitory than the essential oils terpinol and citronella at all the three concentrations. However, copper oxychloride was less inhibitory than the essential oils terpinol, citronella, lemon grass and palmarosa oil at all the three concentrations. Inoculated and uninoculated controls without any essential oil or test chemical did not show any inhibition.

4.4.1.3 Agar well diffusion method

The results of *in vitro* efficacy of essential oils by agar well diffusion method are presented in Table 4.11. Terpinol oil at all the three concentrations (100, 50, and 25 percent) produced maximum inhibition zones of 13.10, 9.07, and 3.40 mm followed by citronella oil (Plate 4.8), lemon grass and palmarosa oil (plate 4.6). However, neem oil was inhibitory only at 100 percent concentration. As the concentration of oils decreased, there was corresponding decrease in size of inhibition zones also. In case of check treatments streptocycline formed smaller inhibition zones than the essential oils terpinol, citronella and lemon grass oil (Plate 4.9) at all the three concentrations and copper oxychloride produced inhibition zones smaller than terpinol, citronella, lemon grass oil and palmarosa oil. The inoculated control however, did not produce any inhibition zone.

Table 4.10 ***In vitro*** **efficacy of essential oils against** ***R. solanacearum*** **by plate count method**

Treatment	Concentration (%)/µgml^{-1}	CFU/ml*
Terpinol	100	166.0
	50	186.7
	25	266.0
Citronella	100	176.7
	50	265.0
	25	308.7
Lemon grass oil	100	227.3
	50	337.0
	25	467.0
Palmarosa oil	100	345.7
	50	434.7
	25	591.7
Neem oil	100	502.7
	50	563.3
	25	702.7
Streptocycline**	100	209.0
	50	292.7
	25	376.7
Copper oxychloride	0.25	323.0
	0.125	366.0
	0.062	515.0
Inoculated control		975.0
Uninoculated		0.0
CD (P=0.05)		**2.1**
CV (%)		**0.4**

***Average of two replications**
**** Concentration in µg/ml**

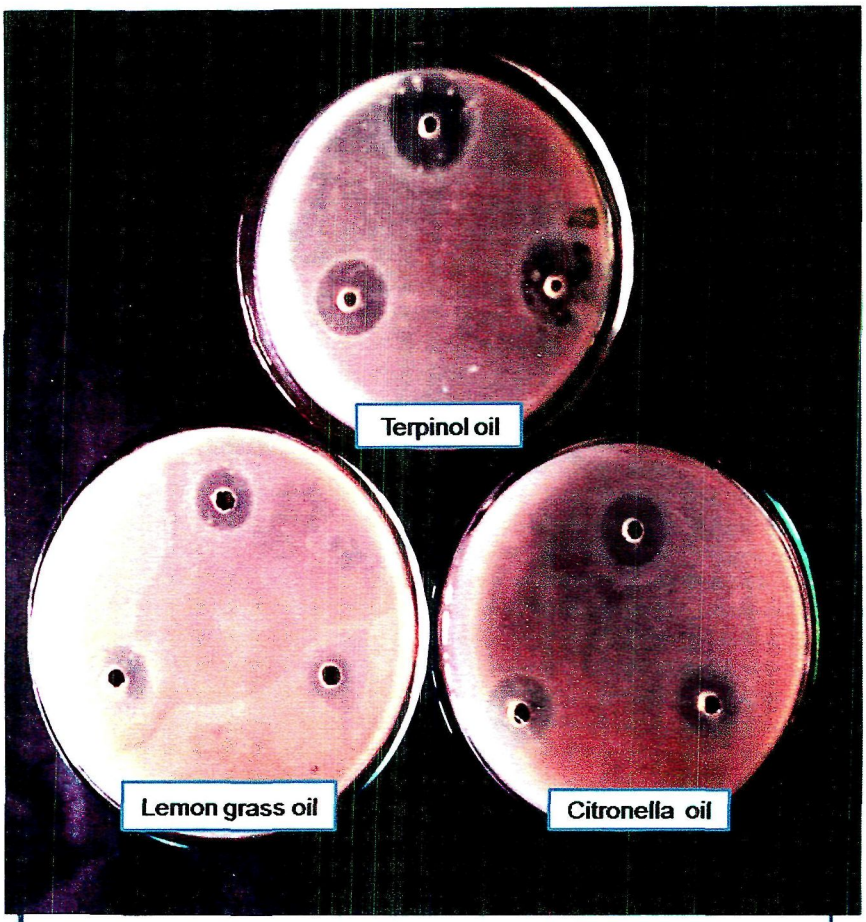


Plate 4.8 Inhibition zone (mm) by essential oils at 100 percent concentration (Agar well diffusion method)

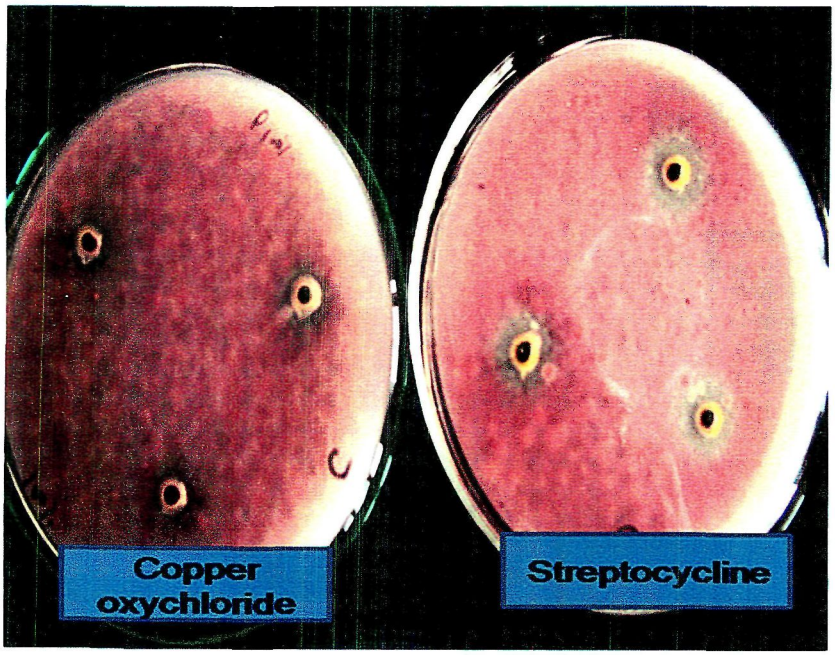


Plate 4.9 Inhibition zones by test chemicals at 100 percent concentration

Table 4.11 ***In vitro*** **efficacy of essential oils against** ***R. solanacearum*** **by Agar well diffusion method**

Treatment	Concentration(%)/µgml^{-1}	Inhibition zone (mm)*
Terpinol	100	13.10
	50	9.07
	25	3.40
Citronella	100	9.73
	50	7.27
	25	2.93
Lemon grass oil	100	8.63
	50	5.60
	25	2.57
Palmarosa oil	100	5.20
	50	3.53
	25	1.03
Neem oil	100	0.83
	50	0.00
	25	0.00
Streptocycline*	100	7.70
	50	5.10
	25	2.40
Copper oxychloride	0.25	4.93
	0.125	2.60
	0.062	0.70
Control		0.00
CD (P=0.05)		**0.12**
CV (%)		**1.56**

***Average of six replications**
**** Concentration in µg/ml**

4.5 Fractionation of effective plant extracts

On the basis of efficacy of plant extracts under *in vitro* and *in vivo* conditions, five effective plant extracts *viz.* peppermint, eucalyptus, ageratum, guava and deodar (Plate 4.10) were selected for fractionation by thin layer chromatography. Different bands were formed by these plant extracts depending upon the fractions present in the components of respective extracts.

4.6 Evaluation of crude fractions from effective plant extracts against *R. solanacearum*

Fractions of effective plant extracts were evaluated through contact bioautography following Fisher and Lautner (1961) and Nicolaus *et al.* (1961). Peppermint (Plate 4.11), eucalyptus (Plate 4.12) and ageratum (Plate 4.13), produced larger inhibition zones thereby showing that the fractions present in these extracts were more inhibitory against *R. solanacearum*. However, fractions of guava (Plate 4.14) and deodar (Plate 4.15) correspondingly proved less inhibitory.

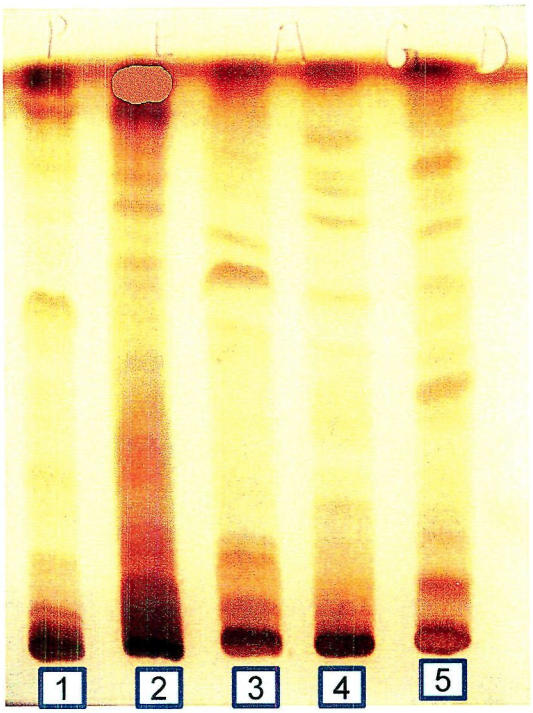


Plate 4.10 Thin Layer Chromatography of Peppermint (*Mentha piperita*) lane 1, Eucalyptus (*Eucalyptus citridora*) lane 2, Ageratum (*Ageratum houstonianum*) lane 3, Guava (*Psidium guajava*) lane 4 and Deodar (*Cedrus deodara*) lane 5 (10µl Extract applied at bottom of each lane)

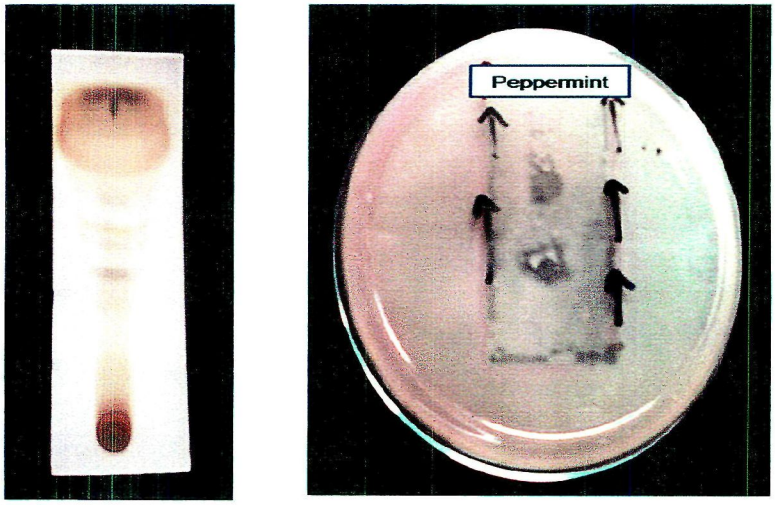


Plate 4.11 (a) TLC of peppermint (*Mentha piperita)* (b) Inhibition by components of peppermint organic extract (10µl)

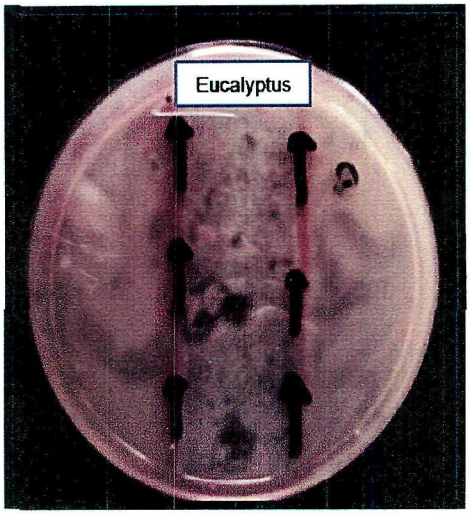
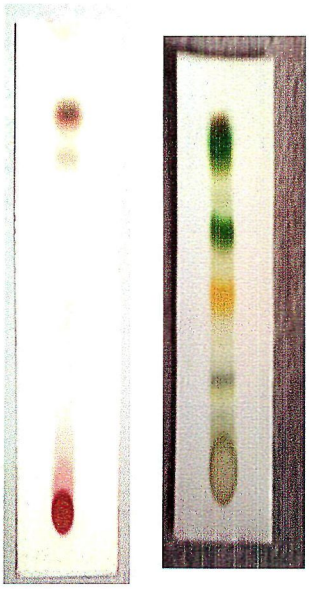


Plate 4.12 (a) TLC of eucalyptus (*Eucalyptus citridora*) organic extract (10µl) (b) Inhibition by components of Eucalyptus



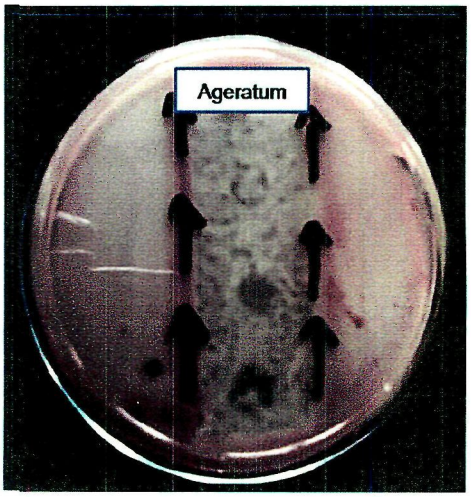
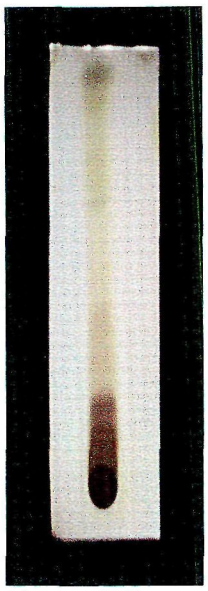


Plate 4.13 (a) TLC of ageratum (*Ageratum houstonianum)* organic extract (10µl) (b) Inhibition by components of ageratum



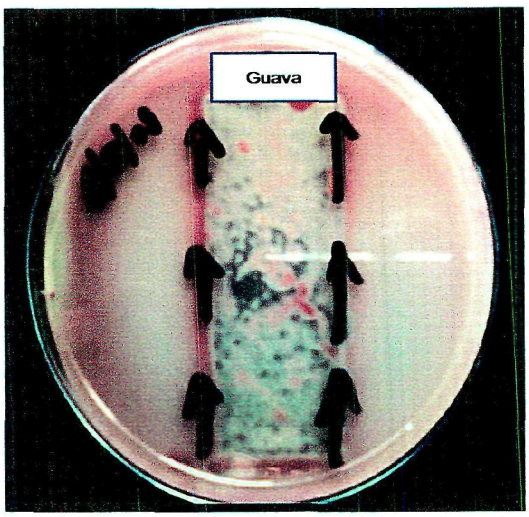
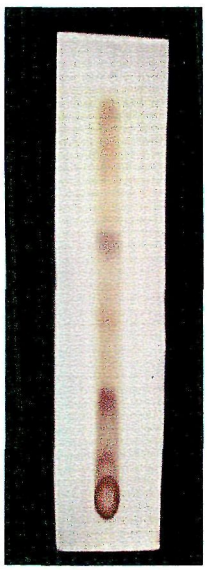


Plate 4.14 (a) TLC of guava (*Psidium guajava*) organic extract (10µl)

(b) Inhibition by components of guava



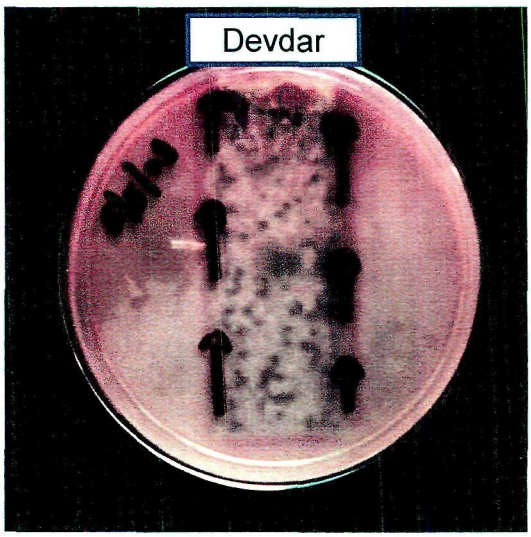


Plate 4.15 (a) TLC of deodar *(Cedrus deodara)* organic extract (10µl)

(b) Inhibition by components of deodar

Discussion

Chapter-V

DISCUSSION

Pathogenic microorganisms affecting plant health are a major threat to food production and ecosystem stability world wide. As agricultural production intensified over past few decades, producers became more and more dependent on agrochemicals. However, increasing use of chemical inputs has caused several negative effects. Ecofriendly management of plant diseases with the help of biopesticides and plant based pesticides is thus being considered as an alternative or supplement of reducing the use of chemicals in agriculture.

Bacterial wilt caused by *Ralstonia solanacearum* is very difficult to manage because the pathogen has a wide host range with exceptional ability to survive in the roots of non host plants and in soil. Resistance against the disease is rarely available and is linked to small fruit size and also sensitive to high temperature and rainfall. All the commercial cultivars including hybrid varieties are highly susceptible and chemical control is not feasible. The ability of the bacterium to survive in the rhizosphere and weed hosts without causing symptoms (Kishun, 1987) further aggravates the situation. Despite decades of efforts by many national and international organizations, this disease continues to be a considerable problem throughout the world. The plant pathologists and plant breeders in the tropics are frustrated in their attempts to control the disease. Efforts to intregrate host resistance with various location specific, cultural and biological measures seem to be the cheapest strategy for its

effective management and satisfactory approach from an ecological point of view. Efforts in this direction have been made in India (Miah *et al.*, 1995; Michel *et al.,* 1997; Yao *et al.* 1994; Aggarwal, 2004; Sood, 2004) and abroad (Akiew *et al.*, 1996; Terblanche and de Viliers, 1996).

Microbial diseases are responsible for widespread mortality in the world. For centuries plants have been used throughout the world as drugs and remedies for various diseases. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as soil microorganisms, animals and plants (Bhavani *et al.*, 2000). Decreased efficacy of antibiotics and resistance of pathogens to antibiotics has necessitated the development of new alternatives (Smith *et al.*, 1994). Making antimicrobial drug therapy effective, safe and affordable has been the focus of interest during recent years (Sharma *et al.*, 2002). There are several reports on antimicrobial activity of different herbal extracts (Adelkun *et al.*, 2001).

The problems caused by synthetic pesticides and their residues have increased the need for effective biodegradable pesticides with greater selectivity. Alternative strategies have included the search for new types of pesticides which are often effective against a limited number of specific target species, are biodegradable into nontoxic products and are suitable for use in integrated pest management programme.

The natural plant products derived from plants effectively meet this criterion and have enormous potential to influence modern agrochemical research. When extracted from plants, these chemicals are referred to as

botanicals. The use of botanical pesticides is now emerging as one of the prime means to protect crops and their products and the environment from pesticide pollution. Botanicals degrade more rapidly than most chemical pesticides, and are, therefore, considered relatively environment friendly and less likely to kill beneficial pests than synthetic pesticides with longer environmental retention. Most of the botanical pesticides generally degrade within a few days and some times within a few hours.

Identification of additional cost effective components of management for inclusion in the integrated management strategy is needed for further refinement of technology. Therefore, 14 such botanicals along with five essential oils were evaluated in the present study against *R. solanacearum* with the objective of identifying additional components for inclusion in the integrated management system for bacterial wilt. The present study indicated the potential of aqueous and organic plant extracts and essential oils in the management of bacterial wilt. The results obtained are discussed under the following heads:

5.1 Evaluation of aqueous and organic plant extracts against *R. solanacearum*

5.2 Evaluation of essential oils *R. solanacearum*

5.3 Fractionation of effective plant extracts and testing the efficacy of against *R. solanacearum*

5.1 Evaluation of aqueous and organic plant extracts against *R. solanacearum*

Evaluation of 14 plant species against tomato isolate of *R. solanacearum* was done by paper disc, spectrophotometeric and plate count methods. Irrespective of the method of evaluation, the aqueous and organic

extracts of peppermint and eucalyptus were found most inhibitory against the bacterium at both the concentrations (100 and 50 percent), followed by ageratum and guava. These results corroborate the findings of Yepola and Adeniyi (2008) who reported that the methanol leaf extracts of *Eucalyptus camaldulensis* were inhibitory against *Pseudomonas aeruginosa and Bacillus subtilis.* Likewise, the methanol leaf extracts of three cold-tolerant eucalyptus trees - *Eucalyptus darlympleana, E. gunnii* and *E. unigera* have been reported to possess antimicrobial activity against *Pythium, Botrytis cinerea and Phomopsis sp.* (Jae-Seoun Hur, *2000*). Sasitorn Vudhivanich (2003) also found that organic extract of guava has high potential for inhibiting growth of *R. solanacearum* and might be applicable for bacterial wilt control. Rest of the plant extracts were found less inhibitory. The efficacy of all the extracts decreased with the decrease in concentration. The test chemical streptocycline was more inhibitory than all the extracts at both 100 and 50 per cent concentrations. The organic extracts of peppermint at both the concentrations, and that of eucalyptus, ageratum and guava at 100 per cent concentration and aqueous extracts of eucalyptus at 100 per cent concentration were more inhibitory than copper oxychloride. A fairly large number of plants are known to possess antibacterial properties against *R. solanacearum* (Hanudin and Djatnik, 1986; Hanudin, 1987; Hutalung, 1988) and other bacterial phytopathogens (Kodama *et al.* 1991; Kelanibangoda, 1997; Terblanche and De Villiers, 1997; Ushiki *et al.*, 1998; Satih *et al.*, 1999).

Six of 14 aqueous and organic extracts found inhibitory under *in vitro* conditions and were also evaluated under *in vivo* conditions and the survivability of seedlings after 60 and 30 minutes dipping in the respective extract was determined. Irrespective of the type of extracts, extracts of eucalyptus and

peppermint were found most inhibitory against the *R. solanacearum*, however organic extracts were comparatively more inhibitory under *in vitro* as well as *in vivo* conditions. The probable reason may be better extraction of active ingredients in organic solvent (methanol) than in water. This observation is supported by Zhang and Lewis (1997) who found that the aqueous extracts possessed low antimicrobial activity.

In general, greater the duration of dipping, more inhibitory was a particular extract. This differential variation may be explained because of differences in the relative content and composition of active inhibitory compounds present in the respective plants. Contrarily, the consistency in the performance of peppermint and eucalyptus extracts at both the dipping durations against the test bacterium *in vitro* and *in vivo* may have occurred due to the presence of qualitative antibacterial substances in relatively higher concentrations in these plants. For example, peppermint and eucalyptus are known to contain 3-p-Menthanol, two phenolic compounds (gallic acid and 3, 4-dihydroxybenzoic acid) and three flavonoid compounds (quercetin, quercetin-3-O-α-L-rhamnoside, quercetin-3-O-β-D-glucoside), respectively (Ahmed *et al*; 2002). The organic and aqueous extracts of geranium and ghanira were not at all inhibitory against *R. solanacearum* at any of the concentration.

Laboratory screening of plant extracts in the present study has given encouraging results, indicating their potential use in the management of bacterial wilt. Since this disease is soil borne in nature, the pot and field experiments involving treatments like soil amendments or drenching applications need to be

conducted in future. The increased awareness of the environmental problems associated with antibiotics has led to the search for non-conventional chemicals of biological origin for the management of plant diseases because of their eco-friendly nature (Bolkan and Reinert, 1994).

Mostly, leaves alone of different plants were used for preparing the aqueous or organic extracts in the present study and fairly good inhibitory effects against the test bacterium were obtained. However, the possibility of their enhanced efficacy could further be investigated by testing different parts of these plants since antimicrobial activity of plant extracts has been reported to vary with test organism and the part of plant used for preparing the plant extracts (Gourinath and Manoharachary, 1990).

The relative inhibitory effect of a plant extract against a particular pathogen depends upon the content and type/s of phenols, alkaloids, flavanoides, tannins, costic acid, etc. present in that particular plant material. For example, the leaf extract of *Geranium pretense* had an inhibitory effect on *Fusarium oxysporum* and *Rhizoctonia solani* while its root extract did not exert any significant effect on these fungal pathogens (EL-Gammel and Mansour, 1986). Similarly, the antimicrobial activity of some plant species was higher against *Streptomyces scabies* than four different fungi (Ushiki *et al.*, 1996; Ushiki *et al.,* 1998). The differential efficacy of different plant extracts against *R. solanacearum* in the present study could be explained on this basis also. The antimicrobial activity of aqueous and acetone extracts of *G. thumbergii* was reported due to the presence of 1.6 per cent 'geraniin' per fresh weight which constituted much of tannin in this plant (Okuda *et al.,* 1997). Similarly, the

antimicrobial activity of *Sanguisorba officinalis* is due to the presence of tannin and some ellagitannins (Nonaka *et al.*, 1982) and of root exudates from *Eupatorium fortune* against *Bacillus subtili* is attributed to the presence of costic acid (Rao and Alvarez, 1981). The antibacterial activity of ethanol extract (1:1) of the leaves and seeds of *Datura metel* and *Datura stramonium* against *Xanthomonas campestris* pv. *malvacearum* was reported earlier (Bambawale *et al., 1995*). Bacterial leaf blight of rice (*X. oryzae* pv. *Oryzae*) has also been reported to be controlled by spraying of aqueous extract of basuti (*Adhatoda vasica*) leaves (Madhiazhagen *et al.*, 2002). Amides affinin extracted from *Heliopsis longipes* has been reported to inhibit the growth of *P. solanacearum* at high concentration and capsaicin from chilli fruit has been reported to retard the bacterial growth (Molina *et al.,* 1999). The crude extracts of golden shower (*Cassia fistula*) leaf, ringworm brush (*Cassia allota*) leaf and tumeric (*Curcuma longa*) rhizome could inhibit the growth of *P. solanacearum.* Turmeric rhizomes extract could inhibit *P. solanacearum* above 30,000 ppm concentration while the extract of ringworm brush could inhibit it above 300,000 ppm concentration (Wongkaew *et al, .*1997). The extracts of guava (*Psidium guajava*) leaf and okra (*Hibiscus sabdariffa*) fruit could also inhibit the growth of *R. solanacearum* (Leksomboon *et al.* 1998). *Eucalyptus citridora* is known to posess insect repellent effect (Menendez, 1992; Collen, 1993). Yasinashita (1989) isolated rhaponticin, an antifouling substance from the leaves of *E. rubida*. Chopped leaves of *E. Citridora* resulted in reduced nematode population (Akhtar and Alam, 1989).

5.2 Evaluation of essential oils against *R. solanacearum*

Among the essential oils tested, terpinol was found most inhibitory against *R. solanacearum* at all three concentrations (100, 50 and 25 per cent) followed by citronella, lemon grass and palmarosa oils. On the other hand neem oil formulation (Max neem oil) made from neem kernels was inhibitory to the test bacterium at 100 per cent concentration only. The leaf extracts of neem have been reported effective against the fungi *Alternaria solani* and *Fusarium oxysporum* pathogenic to tomato (Hassanein et al., 2008). Interestingly, the essential oils, terpinol and citronella were even more inhibitory than test chemical streptocycline at all the three concentrations, whereas oils of terpinol, citronella, lemon grass and palmarosa were found more inhibitory than copper oxychloride at all the three concentrations, though they were not evaluated under *in vivo* conditions in the present study. These results corroborate the findings of Pradhanang *et al.*, (2003) who reported thymol, palmarosa oil, and lemon grass oil effective for managing bacterial wilt in tomato under *in vivo* conditions in the greenhouse experiments.

Essential oils are the volatile oils, odorous which occur in certain plants or specified parts of plants recovered by accepted procedures such that the nature and composition of the product is, as nearly as practicable, uncharged by such procedures. Essential oils have been extracted from over 3,000 plants, of which about 200-300 are commonly traded on world markets. Himachal pradesh's unique climate and flora provide opportunities for new crop development and agricultural diversification. Changing consumer preferences in

favour of natural over synthetic substances has a strong impact on pharmaceutical, cosmetic and 'green pesticides' industries which has transplanted into growing demand for essential oils and plant extracts.

Recent investigations in several countries confirm that certain plant extracts and essential oils have contact fungicidal action against some important plant pathogens. As part of an effort aimed at the development of reduced risk pesticides based on plant essential oils, toxic and sub lethal effects of some essential oil terpenes and phenols have been investigated (Isman, 2000). Further, investigations in several countries confirm that some plant essential oils not only repel insects but have contact and fumigant insecticidal action against specific pests and fungicidal action against some important plant pathogens. Their potential as one of the components for integrated management of bacterial wilt with exact mode of action and environmental impact as crop protectants needs further investigation.

Himachal Pradesh is a rich repository having a large variety of medicinal aromatic plant species. Out of total 45,000 plants species in India, as many as 3,245 species are reported to exist in Himachal Pradesh. Of these, about 150 species are recognized for their medicinal and aromatic value (Choudhary *et al.*, 2005). But many of these species are at the verge of extinction due to their unscientific extraction and over exploitation in the past. No systematic efforts have been made to domesticate these herbal plants for commercial purpose, although the state offers diversified agro climatic conditions ranging from sub tropical to cold deserts.

Although crude extracts of various plant species have exhibited antibacterial activity against *R. solanacearum* in the present study, modern ecofriendly products can be developed after extensive investigation of their bioactivity, mechanism of action, therapeutic action and toxicity by conducting extensive experiments. As the global scenario is now changing towards the use of non toxic plant products having traditional medicinal use, development of modern drugs from different plants (peppermint, eucalyptus, ageratum, guava, deodar, etc.) found effective in the present study should be emphasized for the control of various diseases.

5.3 Fractionation and evaluation of potential plant extracts *R. solanacearum*

On the basis of efficacy of plant extracts under *in vitro* and *in vivo* conditions, five organic plant extracts (peppermint, eucalyptus, ageratum, guava and deodar) were selected for fractionation. Fractionation of plant extracts was done by thin layer chromatography which yielded several bands indicating the presence of a variety of inhibitory components in each extract. TLC is used for separation of mixtures and identification of constituents using many solvents. In the present study methanol: chloroform (10:90) solvent system was used to separate the components. Ahmed *et al* (2002) reported TLC as a useful technique for separation and identification of constituents using suitable solvent for resolution of maximum number of constituents in an extract in one single step.

Further, the efficacy of fractionated components of plant extracts was tested by contact bioautography method, and the development of clear cut zones of inhibition on the seeded media indicated the presence of the antibacterial

compounds in the plant extracts. Anjana *et al.* (2008) analyzed the chemical constituents of bark of *Syzygium cumini*, leaves of *Lawsonia inermis* and fruits of *Terminalia bellerica* by TLC-bioautography. Bioautography is the quicker method to get information about antimicrobial activities of substances separated from a mixture.

Further research could be undertaken in future to detect and identify the actual antibacterial compounds (active ingredients) responsible for inhibition of the test bacterium. Such an identification of active ingredients can be useful in preparation of formulations which would be useful in developing an eco-friendly practice for the management of bacterial wilt disease in future.

Summary

Chapter-VI

SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* (Smith 1986) Yabuuchi *et al.,* 1996 is a severe and devastating disease of solanaceous crops in the tropical, sub tropical and warm temperate regions of the world. The disease is well established in the mid hill sub – humid areas of Himachal Pradesh causing losses in yield up to 100 per cent. The bacterium is highly variable and is very difficult to manage because of its wide host range and exceptional ability to survive in the roots of non host plants and in soil. All the commercial cultivars including hybrid varieties are highly susceptible and chemical control is not feasible.

Aqueous and organic plant extracts of 14 botanicals were evaluated *in vitro* and *in vivo* along with five essential oils under *in vitro* conditions against *R. solanacearum* with the objective of identifying additional components for inclusion in the integrated management system for bacterial wilt. The present study indicated the potential of aqueous and organic plant extracts and the essential oils in the management of bacterial wilt.

In vitro evaluation of 14 plant species against tomato isolate of *R .solanacearum* was done by paper disc, spectrophotometeric and plate count methods. Irrespective of the method of evaluation, the extracts of peppermint and eucalyptus were found most inhibitory against the bacterium at 100 and 50 per cent concentrations, followed by the extracts of ageratum and guava. Their

efficacy decreased with the decrease in concentration. Streptocycline was more inhibitory than all the plant extracts at both 100 and 50 per cent concentrations whereas organic extracts of peppermint at both the concentrations, and the eucalyptus, ageratum and guava at 100 per cent concentration and aqueous extracts of eucalyptus at 100 per cent concentration were more inhibitory than copper oxychloride.

Six out of 14 aqueous and organic extracts found inhibitory under *in vitro* conditions and were also evaluated under *in vivo* conditions and the survivability of seedlings after 60 and 30 minutes dipping in the respective extract was determined. Irrespective of the aqueous or organic solvent used, the extracts of eucalyptus and peppermint were found most inhibitory against the *R. solanacearum*, although the organic extracts were comparatively more inhibitory than aqueous under *in vitro* as well as *in vivo* conditions.

Among the essential oils used, terpinol was the most inhibitory to *R. solanacearum* at all three concentrations (100, 50 and 25 percent) followed by citronella, lemon grass and palmarosa oils, though they were not evaluated under *in vivo* conditions in the present study. The neem oil formulation (Max neem oil) made up of neem kernels was inhibitory at 100 per cent concentration. Interestingly, the essential oils terpinol and citronella were more inhibitory than test chemical streptocycline at all the three concentrations, whereas oils of terpinol, citronella, lemon grass and palmarosa were more inhibitory than copper oxychloride at all the three concentrations.

Based on their efficacy, fractionation of organic plant extracts of peppermint, eucalyptus, ageratum, guava and deodar by TLC revealed the presence of a variety of inhibitory components in each extract. The antibacterial activity of fractionated components of each plant extract was tested by contact bioautography method and development of clear cut zones of inhibition indicated the presence of antibacterial components in the plant extracts against *R. solanacearum.*

The laboratory evaluation of plant extracts and essential oils revealed encouraging result, indicating their potential use in the management of bacterial wilt in future. Further studies may involve testing the efficacy of essential oils (terpinol, citronella, lemon grass and palmarosa oils) by conducting elaborated pot and field experiments and precise identification of the antibacterial compounds responsible for inhibition of test bacterium. Their usefulness for suppressing the bacterial wilt under pot and field conditions will ultimately depend upon isolation and precise identification of specific antimicrobial substances contained in different plant parts and their diffusability into the soil.

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Appendix

APPENDIX-I

Composition of Kelman's (1953) TZC media

Peptone	10 g
Casamino acid	1 g
Glucose	5 g
Agar	20 g
Distilled water	1000 ml

To the molten medium add sterile 2, 3, 5 triphenyle tetrazolium chloride to obtain final concentration 0.006 g/l.

TZC Broth

Peptone	10 g
Casamino acid	1 g
Glucose	5 g
Distilled water	1000 ml