Chapter 2

General Materials and Methods
Potato is a starchy, tuberous food crop belonging to the genus *Solanum*. The word "potato" may refer either to the plant itself or to the edible tuber (Raben *et al*., 1994; Dita Rodriguez *et al*., 2006). The edible portion of potato is its tuber which is morphologically an underground stem (Cassells and Kowalski, 1998). Potato plants are herbaceous perennials that grow about 60 cm (24 in) high, depending on variety, with the leaves dying back after flowering, fruiting and tuber formation (Malik and Tufail, 1984; Nunn and Qian, 2011). It is one of the most important vegetable crops in the world (Herriott *et al*., 1990; Walker *et al*., 1999). Potato plants are attacked by several plant pathogens causing serious diseases during the growing season (Christ and Haynes, 2001; Mirkarimi *et al*., 2013). In potatoes early blight disease occurs in most of production areas almost every year although it has a significant effect on yield only when frequent wetting of the foliage favours early and rapid symptom development (Teng and Bisonette, 1985; Nachmias *et al*., 1988; Waals *et al*., 2004; Pasche *et al*., 2004, 2005; Dey and Das, 2015).

*Alternaria solani* is the causal agent of early blight and important foliar pathogen of potato worldwide (Wharton and Kirk, 2007). Susceptibility to infection is primarily determined by the age of the host plant (Nachmias *et al*., 1988; Rotem, 1998). The primary infection occurs on older foliage early in the season and then the inoculum spreads to immature surfaces, such as young tubers (Folsom and Bonde, 1925; Boiteux *et al*., 1995). Early maturing varieties are more susceptible, and may show severe defoliation (Holm, 2000; Yanar, 2011). The wide and indiscriminate use of chemical fungicides has been the cause of the appearance of resistant plant pathogens, leading to the occurrence of serious diseases (Harrison and Venette, 1970; Heaney *et al*., 2000; Kumar and Schweizer, 2005). Synthetic fungicides not only impose adverse effects on ecosystems, but have also created a possible carcinogenic risk and toxicological problems (Cameron and Julian, 1984; Osman and Al-Rehiayam, 2003; Shiva *et al*., 2004; Masuduzzaman *et al*., 2008; Siva *et al*., 2008; Gurjar *et al*., 2012).

Due to the damage caused by plant diseases, continuous research is essential for developing new control methods to increase or even maintain current levels of crop production (Treadway, 1998; Runyoro *et al*., 2006; Jabeen *et al*.
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Nowadays, the natural products and medicinal plants are a subject of great global interest for the discovery of new antimicrobial agents (Gupta and Briyal, 2004; Sashikala et al., 2009; Abdallah, 2011; Hajra et al. 2011). One of the main procedures used in search of new biologically active substances is the systematic screening of plant extracts for their antimicrobial activity (Tuzun & Klopper, 1995; Sharma et al., 2011). This procedure has been a source of useful agents to control the microbial survival (Salvat et al. 2001). Plant products of recognized antimicrobial spectrum could appear in food conservation systems as main antimicrobial compounds or as adjuvant to improve the action of other antimicrobial compounds (Arora and Kaur, 1999; Simões et al., 2003; Kagale et al., 2004, Maya and Thippanna, 2013).

In the present study, antifungal activity of herbal formulation from Cassia fistula fruit pulp extract in combination with various elicitors like neem, mustard and coconut oil cake and binders like cow dung, guar gum and gum acacia has been assayed against Alternaria solani.

**Test Fungus**

*Alternaria solani* infects stems, leaves and fruits of potato (*S. tuberosum*), tomato (*Solanum lycopersicum* L.), eggplant (*S. melongena* L.), bell pepper and hot pepper (*Capsicum* spp.), and other members of the Solanum family (Nachmias et al., 1988; Holm, 2000). Distinguishing symptoms of *A. solani* include leaf spot and defoliation, which are most pronounced in the lower canopy. In some cases, *A. solani* may also cause damping off (Nachmias et al., 1988; Rotem 1998; Sharma, 2001).

The causal pathogen of early blight *Alternaria solani*, belongs to the subdivision Deuteromycotina; class Hyphomycetes and family Dematiaceae. It has a polycyclic life cycle (Wharton and Kirk, 2007). There is no known sexual stage and hence it is classified as a Deuteromycetous fungus (Ellis and Gibson, 1975; Alexopolous and Mims, 1993). The genus *Alternaria* is a large and important group of plant pathogenic fungi, which cause a significant number of important diseases (Boiteux et al., 1995). The fungus is readily cultured on potato dextrose agar media where it produces a deeply pigmented grayish-brown to black
Colonies (Fig. 2.1 A). The mycelium is usually slight brown, slender, branched and septate (O’Brien and Rich, 1976) (Fig. 2.1 B). Unbranched conidiophores, made up of dark cells, which are broader than the vegetative hyphae, bear chains of obclavate, muriform, light or dark brown coloured conidia of varying sizes in a chain (Folsom and Bonde, 1925; Wharton and Kirk, 2007; Yanar, 2011). The beaked conidia are transversely as well as longitudinally septate (Dube, 1990) (Fig. 2.1 C). Presence of septate conidia is the identifying character for this fungus (Ellis and Gibson, 1975; Alexopolous and Mims, 1993; Pandey et al., 2003).

*Alternaria solani* spores are universally present in fields where host plants have been grown (Nachmias et al., 1988). Spores require presence of free water for germination. Spores are unable to infect a perfectly dry leaf (Heaney et al., 2000; Pandey 2007). *Alternaria solani* spores germinate within half an hour to 2 hours over a wide range of temperatures at 26.6-29.4°C (80-85°F) (Holm, 2000). Another 3 to 12 hours are required for the fungus to penetrate the plant depending on temperature (Wharton and Kirk, 2007). After penetration, lesions may form within 2–3 days or the infection can remain dormant awaiting proper conditions [15.5°C, 60°F] and extended periods of wetness. *Alternaria solani* sporulates best at about 26.6°C (80°F) when abundant moisture (as provided by rain, mist, fog, dew, irrigation) is present (Rotem 1998; Harrison and Venette, 1970). Infections are most prevalent on poorly nourished or otherwise stressed plants (Chaerani and Voorrips, 2006).

Primary infection begins from inoculum that survived the winter either in the soil or from plant debris or inoculum from other hosts (Ellis and Gibson, 1975; Folsom and Bonde, 1925). When the potato foliage comes in contact with the contaminated soil or plant debris it forms a lesion (Boiteux et al., 1995). This lesion then produces spores which are responsible for secondary infection. Spore formation is optimal with foliage that alternates between wet and dry conditions (Dube, 1990; Wharton and Kirk, 2007). Such is the case with overhead irrigation systems or frequent dew or fog. Spores that are formed can be dislodged by wind and rain and are carried to foliage and soil within that field as well as to surrounding fields (Holm, 2000; Yanar, 2011).
Infection spreads to tubers through wounds during or after harvest (Pscheidt and Stevenson, 1988). This occurs when a wounded tuber comes in contact with contaminated soil or plant material (Wharton and Kirk, 2007. Moisture on tuber surfaces and warm environments favor infection (Shtienberg et al., 1990; Franc, 1995). In order to help decrease the spread of disease store tubers in a room with forced air ventilation in order to decrease moisture that is needed for spore germination (Dube, 1990; Pasche et al., 2004, 2005). Also storage temperatures should be below 41°F in order to slow the development of disease (Shtienberg et al., 1990; Franc, 1995). The disease does not spread from tuber to tuber in storage; however it may seem that they do because it may take weeks or months for lesions to appear infection (Waals et al., 2004; Pandey, 2007; Yanar, 2011).

**Isolation, Identification and Development of Pure Culture of Test Fungus**

Infected leaves of potato having typical leaf spot symptoms were collected from the nearby fields of Udaipur (Saveenakheda, Manwakheda etc) in the month of January 2013. The infected parts of leaves were cut into small pieces, surface sterilized with 0.1% mercuric chloride solution for 30 seconds, washed three times by distilled water and transferred onto Petri plates containing solid PDA (Potato dextrose agar) media. The inoculated plates were incubated at 25°C for 4-6 days (Simmons, 2007; Phalisteen et al., 2008). Identification of fungus was done by Dr. T. Prameela Devi (Head, Division of Plant Pathology, Indian Type Culture Collection (ITCC), IARI, New Delhi, India, with I.D. No. 9944.15).

Serial dilution and single spore inoculation method (Prescott et al., 1993; Yanar, 2011) were used to develop subculture of the *Alternaria solani* on Potato dextrose agar (PDA) medium. The culture was maintained on maintenance media *i.e.* Potato dextrose agar medium. Pure culture of test pathogen was stored on agar slants at 4°C and regularly sub cultured after every 7 days. Characters of *Alternaria solani* shows in table 2.1. Compositions of agar media used for fungus isolation is listed in annexure 1.
**Test Plant Material**

*Cassia fistula* (Linn.) belongs to family Fabaceae and Sub–family Caesalpinioideae. It is a semi wild plant very known for its medicinal properties (Khare, 2007). It is commonly known as Amaltas or “Indian Laburnum” and has been extensively used in Ayurvedic system of medicine for various ailments known for its beautiful bunches of yellow flowers (Fig. 2.2 A) (Prashanth *et al.*, 2006 Bhalerao and Kelkar, 2012).

It is a deciduous tree with greenish grey bark, 23-40 cm long compound leaves and 5-12 cm long 4-8 leaflets pairs. It is used in traditional medicine for several indications (Gupta, 2010). The fruit is cylindrical pod and bears many seeds embedded in black sweet pulp separated by transverse partitions (Fig. 2.2 B). (Bhalerao and Kelkar, 2012). The long pods which are green, when unripe, turn black on ripening after flowers are shed (Prashanth *et al.*, 2006). Pulp is dark brown in colour, sticky, sweet and mucilaginous, with characteristic odor. (Fig. 2.2 C). (Danish *et al.*, 2011; Bhalerao and Kelkar, 2012).

The tree is 6-9 m high; trunk straight; branches spreading, slender; main rhachis pubescent; stipules minute, linear-oblong (Nadkarni, 2009; Gupta, 2010). Leaflets ovate or ovate-oblong, acute, bright green and glabrous above, paler and silvery pubescent beneath when young, the midrib densely pubescent on the underside, base cuneate; main nerves numerous, close, conspicuous beneath; petiolules 6-10 mm long, pubescent or glabrous (Prashanth *et al.*, 2006; Danish *et al.*, 2011). Flowers in lax racemes 30-50 cm long; pedicels 3.8-5.7 cm. long, slender, pubescent and glabrous. Calyx 1 cm long divided to the base, pubescent; segments oblong, obtuse; corolla 3.8 cm across, yellow; stamens all antheriferous (Kirtikar and Basu, 1984; Gupta and Mazumdar, 2010).

The pods are pendulous, cylindrical, nearly straight, smooth, shining, brown-black, indehiscent, with numerous (40-100) horizontal seeds immersed in a dark coloured sweetish pulp (Gupta and Mazumdar, 2010). Seeds broadly ovate, 8mm. long, slightly less in breadth, and 5mm thick. The fruit pods are 40-70 cm
long and 20-27mm in diameter, straight or slightly curved, smooth but finely striated transversely, the striations appearing as fine fissures (Kirtikar and Basu, 1984; Khare, 2007). The rounded distal ends bear a small point marking the position of the style. The dorsal suture appears as a single vascular strand and the ventral suture as two closely applied strands. Internally the pod is divided by thin, buff coloured, transverse dissepiments at intervals of about 0.5cm. (Satyavati and Sharma, 1989). Each compartment contains one seed which is flat, oval, reddish brown with a well marked raphe. The seed contains a whitish endosperm in which the yellowish embryo is embedded (Kirtikar and Basu, 1984; Gupta, 2010).

The root is prescribed as a tonic, astringent, febrifuge and strong purgative. Extract of the root bark with alcohol can be used for back wart fever (Patil, 2012). The roots are used in chest pain, joint pain, migraine and blood dysentery. The extract of the root lowered the blood sugar level up to 30 per cent (Bhalerao and Kelkar, 2012). Root is useful in fever, heart diseases, retained excretions and biliousness (Mohd. et al., 2011). The aqueous extract of the root bark exhibits anti-inflammatory activity. The root is used in cardiac disorders biliousness, rheumatic condition, haemorrhages, wounds, ulcers and boils and various skin diseases (Khare, 2007). Cassia fistula leaf paste mixed with mixed with coconut oil is used to treat burnt skin (Nadkarni, 2009; Danish et al., 2011).

The stem bark is used against amenorrhoea, chest pain and swellings (Patil, 2012). The bark possess tonic and antidysenteric properties, it is also used for skin complaints, the powder or decoction of the bark is administered in leprosy, jaundice, syphilis and heart diseases (Mohd. et al., 2011). The leaf extracts reduced mutagenecity in E. coli. The leaves are laxative and used externally as emollient, a poultice is used for chilblains, in insect bites, swelling, rheumatism and facial paralysis (Gupta, 2010; Danish et al., 2011). Leaves posses anti periodic and laxative properties, and are used in jaundice, piles, rheumatism ulcers and also externally for skin eruptions, ring worms, eczema (Satyavati and Sharma, 1989; Gupta, 2010)

The drug is used as analgesic as an antipyretic, it is a remedy for malaria and fever. It is also applied in blood poisoning, anthrax and antidysenteric, leprosy and antidiabetic, for the removal of abdominal obstruction (Nadkarni, 2009). The
extract of the flower inhibited the ovarian function and stimulated the uterine
function in albino rats. Fruits are used in the treatment of diabetes, antipyretic,
abortifacient, demulcent, lessens inflammation and heat of the body; useful in
chest complaints, throat troubles, liver complaints, diseases of eye and gripping
(Prashanth et al., 2006; Satyavati and Sharma, 1989). The fruit pulp is used for
constipation, colic, chlorosis and urinary disorders. The seeds are emetic, used in
constipation and have cathartic properties. The seeds are slightly sweet and
possess laxative, carminative, cooling, improve the appetite and antipyretic
activity (Patil, 2012). They are useful in jaundice, biliousness, skin disease and in
swollen throat. Seed powder is used in amoebiasis (Khare, 2007; Gupta, 2010;
Danish et al., 2011; Bhalodia et al., 2012).

The antimicrobial activities of Cassia fistula plant parts have been studied
earlier by many scientists (Phongpaichit et al., 2004; Prashanth et al., 2006;
Sharma et al., 2006; Abubacker et al., 2008; Hajra et al., 2011; Bhalodia and
Shukla, 2011; Bhalodia et al., 2012; Hada and Sharma, 2015a).

Collection and Identification of Plant Material

The healthy, infection free, mature pods of Cassia fistula were collected in
the month of May, 2013 from the campus of University College of Science,
Mohanlal Sukhadia University, Udaipur, Rajasthan, India. The herbarium
specimen was identified by Dr. Amit Kotia, Department of Botany, University of
Rajasthan, Jaipur, Rajasthan, India, where it was deposited as a specimen voucher
number RUBL 211505. The pods were shade dried at room temperature and
broken with the help of a pestle to extract out the pulp. The pulp was grounded in
an electrical grinder after removal of the seeds from the pulp. The ground material
was passed through sieve of mesh size 60 to obtain a fine powder which was used
to prepare the extract.

Elicitors

Elicitors in plant biology are extrinsic, or foreign, molecules often
associated with plant pests, diseases or synergistic organisms. Elicitor molecules
can attach to special receptor proteins located on plant cell membranes (Kimaru
et al., 2004; Sheikh et al., 2012). These receptors are able to recognise the molecular pattern of elicitors and trigger intracellular defence signalling via the Octadecanoid pathway (Vidhyasekaran, 1988; Bhalodia and Shukla, 2011). This response results in the enhanced synthesis of metabolites which reduce damage and increase resistance to pest, disease or environmental stress (Bektas & Eulgem, 2015). Oil cakes act as elicitors and enhance antimicrobial activity of plant extracts. Oil cakes is one of the natural organic fertilizers with high nitrogen content, which is the residues of neem seeds, mustard, peanut seeds, sesame, coconut etc. after oil extraction process of the processing plant (Rao et al., 2003; Kimaru et al., 2004; Bektas & Eulgem, 2015).

**Collection of Elicitors**

Various elicitors like neem, mustard and coconut oil cake were procured from the local market Udaipur.

**Binders**

A binder is any material or substance that holds or draws other materials together to form a cohesive whole mechanically, chemically, or as an adhesive (Sravani et al., 2014). Often materials labeled as binders in different proportions or uses can have their roles reversed with what they are binding. Guar gum is primarily the ground endosperm of guar beans. The guar seeds are dehusked, milled and screened to obtain the guar gum (Sravani et al., 2014; Zandraa et al., 2015). Acacia gum is used widely in the pharmaceutical industry as a demulcent and in the cooking industry to give body and texture to processed food products (Duke, 1985). It is also used to stabilize emulsions (Dobelis, 1986). Cow dung is traditionally used as organic fertilizer and binder also in Indian sub-continental farming for centuries. The addition of cow dung increases the mineral status of soil, enhances resistance of plant against pests and diseases (Matsuzaki et al., 1998; Naskar & Ray, 2003).

**Collection of Binders**

Cow dung was collected from a cow farm at Titardi, Udaipur, aseptically in sterile poly bags and taken to the laboratory for analysis. Guar gum and gum acacia (HIMEDIA) of laboratory grade was used in this experiment.
Research Design

The study was divided in two different stages; *in vitro* and *in vivo*. Antifungal activity of *Cassia fistula* fruit pulp extract, elicitors and binders individually and in combination was established *in vitro* against *Alternaria solani*. The *in vivo* studies were designed on the basis of results obtained from *in vitro* studies. Composition of all reagents used for *in vitro* studies is given in Annexure 2.

The plan of work was as follows:

- **Preparation of extracts and phytochemical analysis of extracts**

  Plant extracts were prepared by both cold extraction as well as hot extraction methods (Harborne, 1984). Phytochemistry of plant extracts was studied by rapid qualitative tests (Kokate, 1990). Partial purification of active fractions of chloroform extract and identification of active molecule was done by using different chromatographic methods such as TLC fingerprinting, Column chromatography and Gas chromatography Mass spectrometry (GC-MS) (Anitha and Miruthula, 2014). The details of the experiments are given in chapter 3.

- **In vitro Assay of antifungal activity of crude extract, partially purified and column fractions**

  Antimicrobial assay was done by Poison food technique (Groover and Moore, 1962). Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) was determined by method suggested by Collee *et al.*, (1996), Aboaba *et al.*, (2006). Experimental details are given in chapter 4.

- **Study of Effect of *Cassia fistula* fruit pulp extract on Cytomorphology of Test Fungus**

  Effects of selected fruit pulp extract on various morphological and cytological parameters such as mycelium width and conidia size were studied (Goel and Sharma, 2013) and the details are given in chapter no.5.

- **In vitro Assay of antifungal activity of Elicitors and Binders**

  Antifungal activity of various elicitors like neem, mustard and coconut oil cake and binders like cow dung, guar gum and gum acacia was done by Poison food technique (Groover and Moore, 1962). The details of the experiment are given in chapter 6.
• **Preparation of Herbal formulation and their *in vitro* antifungal activity**

Different ratios of herbal formulation were prepared from *Cassia fistula* fruit pulp extract in combination with neem oil cake and cow dung. Antifungal activity of these ratios was done by poison food technique (Groover and Moore, 1962) against *Alternaria solani*. Experimental details are given in chapter 7.

• ***In vivo* study of preventive/protective effect of herbal formulation on early blight disease of Potato**

The most effective herbal formulation ratios were assayed for *in vivo* study of preventive/protective effect using potato as test crop and *Alternaria solani* as test pathogen.

The preventive effect was measured as a function of change in following parameters: plant height, number of leaves/plant, total tuber weight/pot, tuber size, number of tubers /pot and disease incidence (Jan *et al.*, 2003; Ganie *et al.*, 2013). Experimental details are given in chapter 8.

• ***In vitro* study of effect of various physical factors on extract and herbal formulation efficacy**

Study of effect of various physical factors such as pH, sunlight and temperature was studied by exposing the extract to varying conditions of these physical factors (Lee *et al.*, 2004) and then assaying the antifungal activity once again (Groover and Moore, 1962). Experimental details are given in chapter 9.

**Standard Antifungal Compounds Used For the Study**

Mancozeb and Bavistin were used as standard compounds and the sensitivity of *Alternaria solani* was assayed against both these compounds. In case of *in vivo* studies mancozeb was used as a standard because it shows good antifungal activity against *Alternaria solani* and it is presently recommended for farmers (Choulwar, 1989).

**Statistical Analysis**

All the data obtained were subjected to the statistical analysis. Arithmetic mean, standard deviation (SD), standard error for the mean (SEm), critical
difference (CD) and coefficient of variation (CV) were analyzed. Experiment was conducted by Completely Randomized Block Design (CRD). Mean data were used to compare the efficacy of treatments (Panse and Sukhatma, 1989).

**Instruments and Techniques Used**

Solid and liquid media used for the study were sterilized in an autoclave (Yorco Scientific Instruments, India) at 121°C, 15 lb for 15 min. pH of the medium was set by digital pH meter (Systronics 335, India). Hot air oven (Yorco Scientific Instruments, India) was used for sterilizing glassware like petridishes, test tubes etc. Sterilization was accomplished by exposure of items to 150° C - 180° C for 2 to 4 hours. Hot plate (Remi) was used for dissolving the solid material. Inoculation was done with nichrome wire loop (calibrated to deliver 10 μl approximately) under aseptic condition of stericlean horizontal laminar flow bench (Deepak Meditech, India, Model No. DMI 88). Aerobic incubation was done in Biological oxygen demand (BOD) incubator (Super Deluxe, Yorco Scientific Industries Pvt. Ltd.) at optimum temperature and time required. Digital balance (Sartorius Model number GE 412) was used for weighing of material.

Microbial cells were counted by haemocytometer (Rohem Instruments, India Model No. B5 748 Silverlite ISI 0269) and microbial cell size was measured by ocular micrometer (Sigma Inc., Japan) calibrated previously with stage micrometer. Microscopy was done by monocular microscope (Olympus, Germany) as well as binocular microscope whereas trinocular microscope (Olympus, Germany) was used for microscopic photography work. Photography work was done by digital camera (Olympus, Japan, Model No. BX51).

Partially purified fractions in different organic solvents were prepared in glass soxhlet assembly. The extract was vacuum dried in JSWG vacuum evaporator.

Precoated silica gel 60 F254 TLC plates (E-merck) of uniform thickness (20mm x 20mm) and column chromatography was used for separation of secondary metabolites according to colour of bands. UV Fluorescence Analysis Cabinet (Macro Scientific works(R), New Delhi) was used for observed of thin layer chromatography plates. Water bath (Yorco Universal Water Bath, Yorco Scientific Indus. Pvt. Ltd.) was used for maintaining the temperature.
Table 2.1: Identifying Characters of *Alternaria solani*

<table>
<thead>
<tr>
<th>Colony Morphology on PDA</th>
<th>Mycelium</th>
<th>Conidiophores</th>
<th>Conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark black to brown, circular</td>
<td>Brown, septate, branched</td>
<td>Dark, unbranched, broader than vegetative hyphae, bearing chains of conidia</td>
<td>dark brown, transversely and longitudinally septate, have a distinct beak</td>
</tr>
</tbody>
</table>
Fig. 2.1: Cultural and Morphological Characteristics of *Alternaria solani*

A: *Alternaria solani* colony on Potato dextrose agar

B: Mycelium of *Alternaria solani* (at 400 X)

C: Conidia of *Alternaria solani* (at 400 X)
Fig. 2.2: *Cassia fistula* Plant and its Fruit

A: Whole plant of *Cassia fistula*

B: Fruit (Pod) of *Cassia fistula*

C: Broken fruit with pulp of *Cassia fistula*