CHAPTER-2

Phytochemical Analysis and Antibacterial Activity of Nigella sativa L. Seed in Various Germination Phases

“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.”

~Albert Einstein
2.1 Rationale

Infectious diseases are responsible for one-half of all deaths in tropical countries. In US 8% of the deaths are occurring by Infectious diseases (Demissew and Dagne, 2001). The increase is attributed to increase in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections (Pinner et al., 1996). Bacteria show behavior that indicates intelligence and they are acting together throughout the world to counter the antibiotics we have invented to kill them off. Bacteria, it turns out, are inextricably intertwined with the formation of the human species and the health of the Earth. $10 billion was spent on antibiotics in the U.S. every year. Many bacteria have become increasingly resistant to these drugs and that many drugs are outdated as soon as they are released for use.

Nowadays, multiple drug resistance (MDR) has been developed due to the indiscriminate use of commercial antimicrobial drugs in the treatment of infectious diseases. In addition to this problem, antibiotics are sometime associated with adverse effects on the host including hypersensitivity, immuno suppressant and allergic reactions. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases especially from medicinal plants with no side effects (Manandhar et al., 1987). Antimicrobial agents of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Crag et al., 1999; Lide et al., 1992).

The medicinal power of plants lies in phytochemical constituents that cause definite pharmacological action on human body (Chopra et al., 1986; Akinmoladun et al., 2007). Phytochemicals are natural compounds occur in plants such as medicinal plants, vegetables and fruits that work with nutrients and fibers to act against diseases or more specifically to protect against diseases. Phytochemicals are mainly divided into two groups (Krishnaiah et al., 2009); primary and secondary
constituents according to their activity in plant metabolism. Primary constituents contain common sugars, amino acids, proteins and chlorophyll while secondary constituents comprise of alkaloids, flavonoids, saponins, tannins, phenolic compounds and many more (Bentley et al., 1966; Cordell et al., 1981; Edeoga et al., 2005; Krishnaiah et al., 2007). Majority of phytochemicals have been known to bear valuable therapeutic activities such as insecticidal (Kambu et al., 1982) antibacterial, antifungal (Lemos et al., 1990), anti-constipative (Ferdous et al., 1992) spasmolytic (Sontos et al., 1998), antiplasmodial (Benoitvical et al., 2001) and antioxidant (Vardar-unlu et al., 2003) activities etc. The plants thus find their medicinal value due to respective phytochemical constituents they contains.

Keeping in view the importance of medicinal plants and their phytochemicals present study was aimed to investigate the phytochemicals content and antimicrobial activity of *Nigella sativa* L. seed in various germination phases on clinical bacterial strains isolated from human patients.

### 2.2. REVIEW OF LITERATURE

Present study is focused on those bacteria which are highly responsible for liver infections and diseases. Bacterial strains used in present study were isolated from human patients who are harmful for liver or cause liver damage and liver failure.

The liver plays an important role in host defense against invasive microorganisms. The effect of microbial pathogens on the liver can vary to the highest degree with a wide variety of manifestation from asymptomatic increases in amino-transaminases, acute liver failure, hepatic fibrosis and cirrhosis. In evaluating the liver manifestations of a potential infectious pathogen, diagnosis of some of the less common infectious pathogens is dependent on a high level of suspicion and recognition of some of the key diagnostic clues (Talwani et al., 2011).
2.2.1. Bacteria vs liver diseases

Bacteria are microorganisms that are most common organisms on the planet. Bacterial diseases occur when pathogenic bacteria get into the body and begin to reproduce and crowd out healthy bacteria or grow in normally sterile tissues in body. Harmful bacteria may also emit toxins that damage the body (Williams, 2011). A liver abscess occurs when bacteria destroy hepatic tissue and produced a cavity. These cavities fill with infectious organisms, liquelled liver cells and with leukocytes. Necrotic tissue then walls off the cavity from the rest of the liver. The bacteria may reach the liver through the blood or bile ducts. A liver abscess most often comes from infection of the bile ducts caused by gallstones, infections in the intestines, or appendicitis. Bacteria may also enter during a direct trauma to the liver or during procedures involving the liver (Better Health Research, 2011). Bacterial infection could be responsible for some cases of chronic liver disease (Olga Ananieva et al., 2002) such as infectious canine hepatitis, canine herpesvirus, leptospirosis, abscesses, histoplasmosis, coccidiomycosis and toxoplasmosis. Bacterial infection is responsible for up to a quarter of the deaths of patients with liver disease (Sloth et al., 1970; Powell et al., 1971; Correia et al., 1971; Rimola et al., 1984).

Bacteremia is the presence of viable bacteria in the blood stream. There are few prospective surveys of the incidence of bacteremia among patients with liver diseases. Patients with chronic active hepatitis or primary biliary cirrhosis are rarely affected by bacteremia (Mistilis et al., 1970; Kirk et al., 1980; Crowe et al., 1980). In contrast, bacteremia was documented in 7 to 20% of patients admitted to hospital with cirrhosis (Javaloyes de et al., 1984).
Table 2.1: Types of organisms responsible for bacteremia and chronic liver diseases (Jones et al., 1967; Wyke et al., 1982).

<table>
<thead>
<tr>
<th>Type of organisms</th>
<th>Chronic liver disease (%)</th>
<th>Fulminant hepatic failure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Streptococci Group B haemolytic</td>
<td>–</td>
<td>13</td>
</tr>
<tr>
<td>Pneumoniae</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Viridans</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Clostridium welchii</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Other gram positive sp.</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Gram negative</td>
<td>42</td>
<td>39</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Klebsiella pneumoniaae</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Other gram negative sp.</td>
<td>16</td>
<td>-</td>
</tr>
</tbody>
</table>

The mortality for bacteremia in patients with decompensates alcoholic cirrhosis is about 70% compared with 30% for cases with compensated liver disease (Clumeck et al., 1979). Gram-negative organisms are most frequently isolated from patients with decompensate cirrhosis (Table 2.1), while Gram-positive organisms are more common in patients without hepatic decompensate.
2.2.2 Gram-positive and Gram-negative bacteria responsible for liver abscess

2.2.2.1 Staphylococcus aureus

*Staphylococcus aureus* (*S. aureus*) is an anaerobic Gram-positive coccal bacterium. It is frequently found as part of the normal skin flora on the skin and nasal passages. It is estimated that 20% of the human population are long-term carriers of *S. aureus* (Kluytmans, 1997). According to the study of Kang *et al.*, (2010) *S. aureus* increasingly is recognized as an important pathogen in patients with chronic liver diseases. Pyogenic liver abscesses usually develop secondary to biliary tract and intra-abdominal infections and members of the Enterobacteriaceae family are usually implicated as the etiologic agents. Staphylococcal bacteria are ubiquitous, normal inhabitants of the skin and mucous membranes, and the bacteria require a break in those protective layers for infection to occur. That proved *S. aureus* cause liver infections in human as well as in avian. *S. aureus* can cause serious disease in humans both as a wound infection and as a source of food poisoning (Calnek *et al.*, 1997).

Plate 2.1: CT and MRI on Axial (A) and coronal (B) demonstrate a multiseptated abscess by *S. aureus* in liver of 20 years old man. (Dogan *et al.*, 2009).
2.2.2.2 *Escherichia coli*

*Escherichia coli* (*E. coli*) is a common Gram negative bacteria responsible for intra-abdominal infections often result from a perforated viscous (e.g., appendix, diverticulum) or may be associated with intra-abdominal abscess, cholecystitis and ascending cholangitis. Patients with diabetes mellitus are also at high risk of developing pylephlebitis of the portal vein and liver abscesses. Abscesses are often polymicrobial and *E. coli* is one of the more common Gram-negative bacilli observed together with anaerobes (Madappa, 2011).

Cholecystitis and cholangitis result from obstruction of the biliary system from biliary stone or sludge leading to stagnation and bacterial growth from the papilla or portal circulation in liver. When bile flow is obstructed colonic organisms including *E. coli* colonize in the jejunum and duodenum. Interestingly partial obstruction is more likely than complete obstruction to result in infection, bacteremia, bactibilia, and gallstones. The patient may have only low-grade fever, generalized malaise and anorexia. In the postoperative patient who may have a distended and tender abdomen clinical diagnosis of *E. coli* intra-abdominal abscess may be difficult (Kappeli, 2011). *E. coli* liver abscess is seen in the image below.

**Plate 2.2:** *E. coli* liver abscess (Kappeli, 2011).
2.2.2.3 Pseudomonas aeruginosa

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative opportunistic pathogen that evokes respiratory, urinary tract, skin infections and gastrointestinal disorders associated with enter colitis and bowel perforation. *P. aeruginosa* frequently causes septicemia in immune compromised patients and a high incidence of *P. aeruginosa* bacteremia was observed in patients with impaired barrier function of the liver (Pollack, 1990; Korvik *et al*., 1991). *P. aeruginosa* is the fourth most common cause of primary hospital-acquired Gram-negative bacteremia and is usually associated with high resistance to antibiotic treatment and high mortality rates. *P. aeruginosa* bacteremia clinically resembles other forms of Gram-negative sepsis with common symptoms fever, hypotension, refractory shock, adult respiratory distress syndrome and renal failure. However, jaundice appears to occur more often than in other forms of Gram-negative sepsis (Pollack, 1990). The two bacterial products most likely implicated in the systemic toxicity of *P. aeruginosa* are its lipopolysaccharide (LPS) Pseudomonas exotoxin A (PEA) (Cross *et al*., 1980). Hepatic injury due to systemic inflammatory processes has been reported to occur in the pathophysiology of septic shock (Cowley *et al*., 1982). LPS from Gram-negative bacteria have been intensively studied with respect to their capacity to induce shock by stimulation of monocytic cells. PEA inhibits protein synthesis in mammalian cells especially in hepatocytes (Pavlovskis *et al*., Iglewski *et al*., 1977).

**Plate 2.3:** Hepatotoxicity of *P. aeruginosa*.

**A:** Electron micrographs of mouse livers treatment with PEA. Liver tissue containing an apoptotic cell with typical condensed chromatin at the nuclear membrane.

**B:** Proposed sequential stimulation of the immune system by PEA resulting in hepatotoxicity, liver damage, and death following administration to mice (Pavlovskis *et al*., Iglewski *et al*., 1977).
2.2.2.4 *Klebsiella pneumonia*

*Klebsiella pneumoniae (K. pneumoniae)* is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic and rod shaped bacterium found in the normal flora of the mouth, skin and intestines (Ryan, 2004). *K. pneumoniae* can produce infection at a variety of sites, with the risk being increased in patients with impaired host. It is also associated with a community-acquired primary invasive liver abscess syndrome. In addition to liver abscess, some patients develop metastatic infection at other sites.

*K. pneumoniae* primary liver abscess has been variably defined in the literature. Primary liver abscess was defined as *K. pneumoniae* liver abscess occurring in the absence of predisposing intra-abdominal factors such as hepatobiliary disease, colorectal disease, a history of intra-abdominal surgery, trauma (Fang *et al.*, 2007) or as a monomicrobial *K. pneumonia* isolate (Yu *et al.*, 2006).
While polymicrobial liver abscess are usually secondary to hepatobiliary disease or intra-abdominal infection (Wang et al., 1998). Most cases of *K. pneumoniae* primary liver abscess particularly those associated with metastatic infection have been reported in Taiwan and are community-acquired.

**Plate 2.4:** Computed tomography (CT) scans of abdomen showing multiple liver abscesses in *K. pneumoniae* infection (Fang et al., 2007).

*K. pneumoniae*, presenting in 50–88% of pyogenic liver abscesses (Chang et al., 1995; Wang et al., 1998). Approximately 3–7.8% of cases involving this organism have distant metastases to the eye via the bloodstream causing septic endophthalmitis (Cheng et al., 1991). *K. pneumoniae* isolated in community-acquired pneumonia is increasingly found in primary pyogenic liver abscesses. Melissa et al., reported that presence of *mag A* in *K. pneumoniae* has been implicated in hypermucoviscosity and virulence of liver abscess isolates. The K2 serotype has also been strongly associated with hyper virulence (Melissa et al., 2009).
2.2.2.5 *Proteus mirabilis*

*Proteus mirabilis* (*P. mirabilis*) is part of the *Enterobacteriaceae* family. It is a small gram-negative bacillus and a facultative anaerobe rod shaped bacterium. *P. mirabilis* is one of the most common gram-negative pathogens encountered in clinical specimens and can cause a variety of community or hospital-acquired illnesses including urinary tract, wound and bloodstream infections. *P. mirabilis* compromises the care of many patients undergoing long-term indwelling bladder catheterization. It forms crystalline bacterial biofilms in catheters which block the flow of urine, causing either incontinence due to leakage or painful distention of the bladder due to urinary retention. *P. mirabilis* is the micro-organism after *E. coli* which is most frequently associated with urinary tract infection (UTI) particularly in the elderly. However, unlike *E. coli* which is usually confined to the bladder *P. mirabilis* appears to have a special predilection for the upper urinary tract. This may lead to stone formation and acute pyelonephritis (Neal Guentzel, 1996).

2.2.3 Antimicrobial activity of *Nigella sativa* Linn.

Several investigations have been directed towards *N. sativa* antibacterial properties (Voravuthikunchai *et al.*, 2005; Nazma Ara *et al.*, 2006; Salman *et al.*, 2008; Hannan *et al.*, 2008; Suresh Kumar *et al.*, 2010). *N. sativa* exhibited strong antimicrobial activity against *Salmonella typhi*, *P. aeruginosa* and others. The essential oil has been shown to have activity against Gram-positive and Gram-negative bacteria. However, sensitivity against Gram-positive bacteria such as *S. aureus* and *Vibrio cholerae* was found to be stronger. Bacteria like *S. aureus*, *S. pyogenes* and *S. viridans* are more susceptible to *N. sativa*. In an in-vitro study, volatile oil showed activity comparable to ampicillin. The activity of the volatile oil also extended to drug-resistant strains of *Shigella spp*, *V. cholerae* and *E. coli* and was found to have a synergistic action with streptomycin and gentamycin (Chopra *et al.*, 1958).
Methicillin resistant *S. aureus* (MRSA) continues to be one of the commonest pathogens encountered in clinical as well as laboratory practice. It has become a major health problem worldwide. Newer antimicrobials agents are urgently needed to combat this problem MRSA resistance to various anti-staphylococcal agents. In the back-drop of this difficult situation *N. sativa* extract showed anti-staphylococcal activity (Hannan et al., 2008). The in-vitro antimicrobial activity of the volatile oils of *N. sativa* L. seeds was reported against fifteen pathogenic microbial strains including Gram-positive bacteria represented by *S. aureus* and Gram-negative bacteria represented by *P. aeruginosa*, *E. coli* and pathogenic yeast *C. albicans* (Hanafy et al., 1991; Nazma Ara et al., 2005).

*N. sativa* essential oil was studied for antibacterial activity against various clinical isolates of bacteria resistant to a number of antibiotics in varying concentrations by disc agar diffusion technique using impregnated filter paper discs on inoculated Mueller Hinton agar plates. The oil showed pronounced dose-dependent antibacterial activity which was more against Gram-positive than Gram-negative bacteria. Among Gram-positive bacteria tested *S. aureus*, *S. epidermidis*, other coagulase negative Staphylococci and *S. pyogenes* were sensitive to the oil and *E. faecalis*, *S. agalactiae* were resistant. Out of 144 strains tested, most of which were resistant to a number of antibiotics, 97 were inhibited by the oil of black cumin. The essential oil of the *Nigella* seeds have also dose-dependent antibacterial effects on Gram positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria (Hosseinzadeh, et al., 2007). The volatile oil of *N. sativa* seeds, prepared by steam distillation was proved to be more effective against many strains of bacteria, including those known to be highly resistant to drugs (Salman et al., 2008).
2.3 MATERIALS AND METHODS

2.3.1 Collection of *N. sativa* seeds

Seeds of *N. sativa* were procured in September, 2010 from a herbal shop in Lucknow, India and authenticated by a botanist at National Botanical Research Institute, Lucknow. A voucher specimen of the seeds was kept in the museum of the Department for future reference.

2.3.2 Germination of *N. sativa* seeds

Germination was done according to the method of Ahmad *et al.*, (2010a). Seed lots used for different experiments showed germination capacities ranging from 80 to 98%. The seeds were surface sterilized with 0.1% HgCl$_2$ for 3 min. They were rinsed thoroughly with double distilled water and soaked in de-ionized water for 30 min. For germination of seeds, they were placed on four folds of damp filter paper at 25°C and incubated in dark till the initiation of sprouting after which they were placed at a light intensity of 100 µmol m$^{-2}$ s$^{-1}$ (that was measured by LI-190SA quantum Sensor, Li-COR Co., USA) and a 14/10 h (day/night) photoperiod till the complete plantlet with two leaves were obtained. The complete germination took eleven days with emergence of epicotyl, hypocotyl, roots and green leaves. Germination, defined as 1 mm radicle emergence, was followed for 11 days. No contamination by microorganisms was observed during this time period.

2.3.3 Harvest of germinated seeds

The germinated seeds of different days were harvested with sterilized forceps and were kept on blotting sheet to remove excess water. The germinated seeds collected for different experiments were used immediately for preparing extracts. Seeds were considered to be germinated after the radicle emerged from the testa. All the samples were stored at -80°C in a deep freezer until used further.
Plate 2.5: Harvested germinated seeds of *N. sativa*.

2.3.4 Preparation of distilled extracts

The samples of seed and germination phases of 3rd, 5th, 7th, 9th, and 11th day were shade-dried and ground to a fine powder. The powder (20gm) was extracted with 200 ml methanol solvent for 48 h in order to extract bioactive compounds using soxhlet apparatus (AOAC method 1980). The extracts were filtered using Whatman filter paper (No.1) and methanol was evaporated using rotary distillation apparatus to obtained pure extract. Oily fraction of extracts was stored at 4°C until use.

Plate 2.6: Preparation of distilled extracts using soxhlet apparatus.
2.3.5 Clinical bacterial strains used for the study

The clinical bacterial isolates used in study are listed in Table 2.2. These isolates were collected from Era’s Lucknow Medical College and Hospital U.P. (India) from various patients. They were authenticated by Dr. Vineeta Mittal, MD, Department of Microbiology, Era’s Lucknow Medical College, Lucknow.

**Table 2.2:** Clinical bacterial strains isolated from human patient used in the present study and their sources.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Organism</th>
<th>Patient code</th>
<th>Disease</th>
<th>Age/gender</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>U/397</td>
<td>Intestine infection</td>
<td>17 yr/M</td>
<td>Urine</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em></td>
<td>P/312</td>
<td>Stomach ulcer/ infection</td>
<td>26yr/M</td>
<td>Ascitec fluid</td>
</tr>
<tr>
<td>3</td>
<td><em>K. pneumoniae</em></td>
<td>P/299</td>
<td>wound at surgical sites</td>
<td>50yr/F</td>
<td>Pus</td>
</tr>
<tr>
<td>4</td>
<td><em>P. aeruginosa</em></td>
<td>P/322</td>
<td>Jaundice</td>
<td>1day/F</td>
<td>CBF</td>
</tr>
<tr>
<td>5</td>
<td><em>P. mirabilis</em></td>
<td>U/401</td>
<td>Cystitis</td>
<td>51yr/M</td>
<td>Urine</td>
</tr>
</tbody>
</table>

2.3.6 Inoculum preparation

The collected microorganisms were maintained at 4°C on nutrient agar slants. Active cultures for each bacterial species were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth. The inoculated tubes were incubated without agitation for 24 hrs at 37°C. The cultures were diluted with fresh nutrient broth to achieve optical densities corresponding to $10^6$cfu/mL (Duraipandiyan *et al.*, 2006).
2.3.7 Qualitative study of phytochemicals of \textit{N. sativa} during germination

The phytochemical properties (saponin, tannin, alkaloids, polyphenols, sterols, flavonoids) were determined by the methods of Sofowora (1993), Trease and Evans (1983) and Evans and Brightman (1980).

2.3.7.1 Test for Sterols

\textbf{Salkowski reaction}

0.5 mg of each residue of each extract was taken in 2ml of chloroform and 2ml of concentrated sulphuric acid was added from the side of the test-tube. The test-tube was shaken for few minutes. The development of red colour in the chloroform layer indicated the presence of sterols.

2.3.7.2 Tests for Alkaloids

\textbf{Hager’s reagent}

A saturated aqueous solution of picric acid was employed for this test. When the test filtrate was treated with this reagent, an orange yellow precipitate was obtained, indicating the presence of alkaloids.

\textbf{Wagner’s reagent (Iodine-potassium iodine)}

1.27gm of iodine and 2gm of potassium iodide were dissolved in 5ml of water and the solution was diluted to 100ml with water. When few drops of this reagent were added to the test filtrate, a brown flocculent precipitate was formed indicating the presence of alkaloids.

\textbf{Mayer’s reagent (Potassium mercuric iodide reagent)}

1.36 gm of mercuric chloride was dissolved in 60 ml of distilled water. Both the solutions were mixed and diluted to 100 ml with distilled water. To the test filtrate and a few drops of the above reagent were added in a watch glass. Formation of cream coloured precipitate showed the presence of alkaloids.
2.3.7.3 Tests for Tannins

The test residue of each extract was taken separately in water, warmed and filtered. Tests were carried out with the filtrate using following reagents.

**Ferric chloride reagent**

5% w/v solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution were added to the above filtrate. Dark green colour was obtained which showed the presence of tannins.

**Lead acetate test**

10% w/v solution of basic lead acetate in distilled water was added to the test filtrate. Precipitation was obtained which showed the presence of tannins.

2.3.7.4 Tests for Saponins

**Foam test**

Few mg of test residue was taken in a test tube and shaken vigorously with a small amount of sodium bicarbonate and water. Froth is obtained which means saponins were present.

2.3.7.5 Tests for Phenolic compounds

**Ferric Chloride test**

Extract was taken in water and warmed; 2 ml of ferric chloride (FeCl₃) was added. The formation of green colour solution indicates the presence of phenolic compounds.

**Lead Acetate test**

Extract was added to 2 ml of lead acetate. The formation of precipitate indicates the presence of phenolic compounds.
2.3.7.6 Tests for Flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample.

a) 5ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H$_2$SO$_4$. A yellow colouration observed in each extracts which indicate the presence of flavonoids. The yellow colouration disappeared on standing.

b) Few drops of 1% ammonia solution were added to a portion of each filtrate. A yellow coloration was observed indicated the presence of flavonoids.

c) A portion of the powered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1ml of dilute ammonia solution. A yellow coloration was observed which indicate a positive test for flavonoids.

2.3.7.7 Test for Terpenoids

5 ml of each extract was mixed in 2 ml of chloroform and concentrated H$_2$SO$_4$ (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed which showed positive results for the presence of terpenoids.

2.3.7.8 Test for cardiac glycosides

5 ml of each extracts treated with 2 ml of glacial acetic acid with one drop of ferric chloride solution and 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates deoxysugar characteristics of cardenolides. A violet ring appeared below the brown ring while in the acetic acid layer a greenish ring formed just gradually throughout thin layer which confirmed presence of glycosides.
2.3.8 Study of Phytochemicals by Thin Layer Chromatography

(Wagner, and Bladt, 1996)

2.3.8.1 Preparation of TLC plate

Silica gel slurry was prepared by macerating silica gel powder (TLC grade, HIMEDIA) with water. The slurry was prepared in a mortar and was thoroughly ground to a fine paste according to the thickness of the plate required for the experiment. It was then poured uniformly on the glass plate which served as matrix and kept on a flat platform to settle before keeping in an oven for activation.

2.3.8.2 Activation of TLC plate

The plates prepared above were kept in an oven at a temperature of 100-110°C for 2-3 hours for activation. During this period the water present in the slurry evaporates rendering it suitable for the experiment.

2.3.8.3 Equilibration of TLC plate

The TLC tank is equilibrated with the developing solvent for one hour by using 100 ml of solvent. The solvents were mixed in a beaker in a definite ratio according to the nature of the solute which is to be separated and then poured into the tank.

2.3.8.4 Loading of the sample

The dried TLC plate was marked using a marker pen, a line was drawn along the plate 2 cm from one edge. The samples were spotted along this line. The samples were loaded by resuspending the dried samples in appropriate solvent then vortexed well and spun down to the bottom of the tube and loaded with a capillary tube evenly along the line.
2.3.8.5 Running the Sample

The tank was opened and the plate was gently kept into tan. Samples ran with the solvent and the plate was removed when it reached the solvent front line.

2.3.8.6 Analysis of the data

The data were analyzed by spraying with color reagent. Non-radioactive samples were sprayed with appropriate stain and visualized as directed in the protocol. The compounds which give fluorescence in the UV range were visualized in UV light on a transilluminator whereas other components were easily seen as bands in visible light. The solute front and the solvent front were recorded and the $R_f$ value was calculated from this data.

2.3.8.7 TLC study of sterols

The sterols were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and $R_f$ values of these spots were recorded under visible light after spraying the plates with anaisaldeyde-sulphuric acid reagent and heating (100°C/6 min).

2.3.8.8 TLC study of alkaloids

The alkaloid spots were separated using the solvent mixture chloroform and methanol (15:1). The colour and $R_f$ values of the separated alkaloids were recorded both under ultraviolet (UV-254 nm) and visible light after spraying with Dragendorff’s reagent.
2.3.8.9 TLC study of phenols

The phenols were separated using chloroform and methanol (27:0.3) solvent mixture. The colour and \( R_f \) values of these phenols were recorded under visible light after spraying the plates with Folin-Ciocalteu’s reagent heating at 80°C/10min.

2.3.8.10 TLC study of flavonoids

The flavonoids spots were separated using chloroform and methanol (19:1) solvent mixture. The colour and \( R_f \) values of these spots were recorded under ultraviolet (UV-254 nm) light.

2.3.8.11 TLC study of glycosides

The glycosides were separated using Ethyl acetate, methanol and water (80:10:10) solvent mixture. The colour and \( R_f \) values of these spots were recorded by observing under ultraviolet (UV-254 nm) light.

2.3.9 Liquid Chromatography Electrospray ionization Mass Spectrometry (LC-ESI-MS) study of \( N. \ sativa \) extracts from different germination phases.

LC-ESI-MS experiments was performed on thermo Finnigan LCQ advantage max ion trap mass spectrometer having Finnigan Surveyor HPLC system connected to it. The column was thermo ODS-2, 250 X 4.5, 5µm and solvent was eluted as given gradient programme at 800µl/min. solvent system was MeOH, H2O, CAN and 5mM AcNH₄. The 10µl sample was introduced into the ESI source through Finnigan Surveyor autosampler. The mass spectra was scanned in the range 150-1000 Th and the maximum ion injection time was set 150nS. Ion spray voltage was set at 5.3 KV and capillary voltage 40 V. The MS scan run upto 55min.
2.3.10 Determination of *in vitro* anti-microbial effect on *N. sativa*

2.3.10.1 Broth dilution assay

The minimum inhibitory concentration (MIC) values were determined by using a modified macro-broth dilution technique (Ibrahim *et al.* 1997). Overnight culture of bacteria grown in nutrient both cultures were diluted 100 folds in NB (100μl bacterial cultures in 10ml nutrient broth (NB) which contained $10^5$ cfu of bacteria). Gradually increasing volumes of the extracts were added to test tubes containing the bacterial cultures to know the inhibitory concentration in a particular tube inhabiting the bacterial growth. The tubes were incubated at 37°C for 18-24 hours. The tubes were examined for visible turbidity and optical density of cultures was determined at 620nm using NB as control. The lowest concentration that inhibited visible growth of the tested organisms was recorded as the MIC.

2.3.10.2 Agar well diffusion assay

The agar well diffusion method was used to test the antimicrobial effects of *N. sativa* crude extracts in different stages of germination. (Perez *et al.*1990; Okeke *et al.* 2001). All media plates (9 cm in diameter) were prepared with nutrient agar. After agar solidification, the well (7 mm in diameter) was cut from the agar to produce a total of three wells per agar plate. Different doses of extract (25, 50, 75 μg/well) were used for test. Standard antibiotics streptomycin (30 μg), ciprofloxacin (10 μg), doxycycline (30 μg), ampicillin (10 μg) and ofloxacin (5 μg) (HIMEDIA) were used as positive control. 100 μl ($10^5$ cfu) of each diluted microbial suspension were inoculated on nutrient agar plates using sterile cotton swab. The extracts and positive control (streptomycin, ciprofloxacin, doxycycline, ampicillin and ofloxacin) were added separately to each well of agar plate and allowed to diffuse at room temperature for 15-20 min. After incubation at 37°C for 24h, all plates were examined for zones of growth inhibition and the diameter of these zones was measured. The assay was repeated three times for each extract. The antimicrobial
effects were recorded as the mean diameter of the resulting inhibition zones of growth in millimeter.

2.4 RESULTS AND DISCUSSION

2.4.1 Phytochemical analysis of *N. sativa* extracts in different germination phases

The complete germination of seed took eleven days with emergence of epicotyl, hypocotyl, roots and green leaves. Germination, defined as 1 mm radicle emergence, was followed for 11 days. No contamination by microorganisms was observed during this time period. Further, Phytochemical analysis carried on extracts from different germination phases of *N. sativa* showed the presence of some bioactive compounds. In the seed (non-germinated), 3rd, 5th, 7th, 9th and 11th day extracts of germination phases.

**Table 2.3:** Screening of phytochemicals in methanolic extracts of *N. sativa* from different germination phases.

<table>
<thead>
<tr>
<th>Days</th>
<th>Sterols</th>
<th>Alkaloids</th>
<th>Tannins</th>
<th>Saponins</th>
<th>Phenols</th>
<th>Flavonoids</th>
<th>Terpenoids</th>
<th>Glycoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>+++</td>
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<td>+</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>5</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
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<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>9</td>
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<td>+++</td>
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<td>+++</td>
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<td>++</td>
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<tr>
<td>11</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Data is a mean of three replications.*
Plate: 2.7: Different stages during germination of *N. sativa* seed.
Plate: 2.8: Successive stages of *N. sativa* seed germination (in large).

The qualitative analyses of phytochemicals present in the methanolic extract of *N. sativa* seed showed the presence of sterols, alkaloids, saponins, phenols, flavonoids, terpenoids and cardiac glycosides. No effect of germination was observed on the presence of sterols, phenols and cardiac glycosides. There was a slight increase in the alkaloid content from 3rd to 7th day of germination was observed. Our study is the confirmation of other studies in which three types of alkaloids were isolated from the defatted seeds of *N. sativa* (Atta-ur-Rahaman et al., 1985; Atta-ur-Rahaman et al., 1992). Alkaloids have been defined as a cyclic organic compound having nitrogen in a negative oxidation state, which has limited distribution in living organism (Hodnick et al., 1988). They are well known for their CNS activities (Yamamoto and Gaynor, 1980) and play some metabolic roles and control development in living (Edeoga et al., 2006).

Sterol was present equally in non germinated seed as well as all days of germination. Sterol was shown to reduce carcinogen-induced cancer of the colon in a rat model as well as exhibiting anti-inflammatory, anti-pyretic and insulin-releasing properties (Gupta et al., 1980). A slight decrease was observed in tannins from 5th to 7th day while a higher quantity was observed on primary and late phase of germination. Tannins are basically used for the treatment of inflammation, leucorrhoea, gonorrhea, burn, piles, diarrhea and as antidote in the treatment of alkaloidal poisoning (Mattila et al., 2007).
A clear decrease in saponins was observed from 7th day, higher amount of saponins was observed till 5th day of germination. The major saponins in the defatted seeds of \textit{N. sativa} is the glycoside \(\alpha\)-hederin or helixin or melanthin which on acid hydrolysis releases its sugar arabinose and gives the aglycone hederagenin (or melanthigenin) or caulosapogenin (Ansari \textit{et al.}, 1988; Kumara \textit{et al.}, 2001). The saponins are naturally occurring surface active glycosides. Many pharmacological activities have been reported about saponins such as antibiotic antifungal, antiviral, hepatoprotective, anti-inflammatory and anti-ulcer as well as chemoprotective activities (Ireland \textit{et al.}, 1986; Fukuda \textit{et al.}, 1985; Sindambwe \textit{et al.}, 1998).

A decrease in phenols and terpenoids contents was observed in 3rd day and 7th to 11th day extracts respectively. While continuously increased in phenols from 5th to 11th day was observed. Phenols are very wide spread in nature. They range from simple structures having a simple aromatic ring to highly complex polymeric structures and often exist in glycosidic forms (Crag \textit{et al.}, 1999). It has been reported that the changes in the primary metabolic pathways during various phases of \textit{N. sativa} seed germination lead to the changes in the concentration of carbohydrates and proteins. It was observed that the seeds had maximum amount of soluble sugars at 10 days, fructans at 8 days and proteins at 5 days of germination (Ahmad \textit{et al.}, 2010b).

Photochemical study showed that flavonoids and cardiac glycosides were equally present all the days of germination in higher amount. Increase in flavonoids, phenols, and in cardiac glycosides was observed as the germination proceed. Flavonoids are class of secondary plant phenolics with significant antioxidant and chelating properties. They are categorized into various subclasses including flavones, flavonols, flavonones, isoflavonones, isoflavonoids, anthocyanidins and catechins (Cushnie \textit{et al.}, 2005; Yaqin \textit{et al.}, 2005). They show anti-allergic, anti-inflammatory (Oakenfull \textit{et al.}, 1986) antimicrobial and anticancer activities (Zhang \textit{et al.}, 2001; Ali, 2005).
Secondary metabolites mean they are organic compounds that have no direct involvement with the growth or development of plants. The effect of flavonoids on plants growth which is known as at least partly indirect and associated with the action of auxins. It was reported that flavonoids can improve the blood circulation and lower the blood pressure (Hostettmann et al., 1995). In addition to primary metabolism which enables the growth, development and reproduction of a plant organism a secondary metabolism occurs in plant cells. The main secondary metabolites in plants are glycosides, terpenoids, steroids, tannins and phenolic compounds. Secondary metabolites are involved in an important biological and pharmacological activity such as anti-oxidative, anti-allergic, antibiotic, hypoglycemic and anti-carcinogen (Katalinic et al., 2004).

The presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols etc. showed remedial properties in N. sativa. Thus the preliminary screening tests for bioactive compounds may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development.

2.4.2 Thin layer chromatography for phytochemicals of N. sativa extracts in different germination phases.

Qualitative analysis of phytochemicals of N. sativa during different germination phases showed the significant presence of metabolites. These metabolites further analyzed by thin layer chromatography.

There were four bands with \( R_f \) value 0.16 cm, 0.38 cm, 0.11cm and 0.22 (with green, blue, yellow and pale green colour respectively) for alkaloids in N. sativa seed extracts sample from germination phases during thin layer chromatography were observed under Ultra violet (254 nm) light. The data of qualitative separation of alkaloids is tabulated in table 2.4.
Table 2.4: Phytochemical analysis of *N. sativa* methanolic extracts of different germination phases by thin layer chromatography.

All bands appeared in all tested samples except 3rd day of germination. Fluorescence was observed high in band with *R*ₚ value 0.38 in 5th day germination sample. It may be 5-methoxy-NN-dimethyltryptamine (Culvenor *et al.*, 1964). 5-methoxy-N, N-dimethyltryptamine (5-MeO-DMT) belongs to a group of naturally
occurring psychoactive indolealkylamine drugs (Shen et al., 2010). Antihypoxic, anti-ischemic and anti fungal activities have been reported for some alkaloids of *N. sativa* (Ali et al., 2001).

Two different spot with *R*<sub>f</sub> value 0.08 and 0.16 (green and blue colour respectively) were observed for phenols in all tested sample of *N. sativa* from germination phases by thin layer chromatography after spraying phenol reagent. Spot with *R*<sub>f</sub> value 0.08 of green color was observed only in seed and 3<sup>rd</sup> day germination sample. While another spot with *R*<sub>f</sub> value 0.16 of blue color was present in 5<sup>th</sup>, 7<sup>th</sup>, 9<sup>th</sup> and 11<sup>th</sup> day sample of germination (Table 2.4 and Plate 2.5).

There were seven different fluorescent bands with different *R*<sub>f</sub> value 0.03, 0.16, 0.23, 0.30, 0.33, 0.36 and 0.80 (with green, blue and orange color) for flavonoids in all tested sample of *N. sativa* from germination phases by thin layer chromatography. Band of *R*<sub>f</sub> 0.03 was seen in all samples except 11<sup>th</sup> day of germination. There were three common spots with *R*<sub>f</sub> 0.16, 0.36 and 0.80 of green, blue and orange color respectively in seed sample and 5<sup>th</sup> day germination. Two bands with *R*<sub>f</sub> 0.23 and 0.30 of blue color was observed in 5<sup>th</sup>, 7<sup>th</sup> and 9<sup>th</sup> day of germination, band of *R*<sub>f</sub>=0.23 was also present in 3<sup>rd</sup> day of germination. In of 5<sup>th</sup> and 11<sup>th</sup> day of germination a band of *R*<sub>f</sub> 0.33 was also seen which was absent in other tested samples of seed and germination phases.

Two different glycosides with similar green fluorescence were observed during germination of *N. sativa* seeds (Table 2.5). There were two bands of different *R*<sub>f</sub> values, band of *R*<sub>f</sub> 0.14 was observed in all tested samples while another band of *R*<sub>f</sub> 0.20 was see only in germination phases of *N. sativa*. The data of TLC confirmed the presence of phytochemicals (alkaloids, phenolic compounds, flavonoids and glycosides) in all samples of germination phases. It was concluded that there are numbers of different flavonoids, phenolic compounds, alkaloids and glycosides present in *N. sativa* during germination.
Plate 2.9: TLC results of *N. sativa* extracts from different germination phases.

TLC for alkaloids

TLC for phenols

TLC for flavonoids

TLC for glycosides
2.4.3 LC-ESI-MS study of different extracts from germination phases of *N. sativa* seed.

**Figure 2.1:** LC-ESI-MS mass spectrum of seed (0\textsuperscript{th} day) extract.
Figure 2.2: LC-ESI-MS mass spectrum of 5th day germination phase extract.
Figure 2.3: LC-ESI-MS mass spectrum of 7\textsuperscript{th} day germination phase extract.
Figure 2.4: LC-ESI-MS mass spectrum of 11\textsuperscript{th} day germination phase extract.
Table 2.5: LC-ESI-MS analysis of *N. sativa* extracts of different germination phases.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular weight</th>
<th>m/z</th>
<th>M⁺+1</th>
<th>M⁺+Na</th>
<th>M⁺+K</th>
<th>0ᵗʰ Day (seed)</th>
<th>5ᵗʰ Day</th>
<th>7ᵗʰ Day</th>
<th>11ᵗʰ Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene</td>
<td>136</td>
<td></td>
<td></td>
<td></td>
<td>177</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Magnoflorine</td>
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<td>342</td>
<td></td>
<td></td>
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<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Kaempferol</td>
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<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>β-pinene</td>
<td>136</td>
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<td></td>
<td></td>
<td>177</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>β-myrcene</td>
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<td></td>
<td></td>
<td>177</td>
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<td>++</td>
<td>++</td>
<td>+</td>
</tr>
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<td>γ-Terpinene</td>
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<td></td>
<td></td>
<td>177</td>
<td>+</td>
<td>++</td>
<td>++</td>
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<td>p-cymene</td>
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<td>+</td>
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<td>Ethyl Heptanoate</td>
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<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>α-longipinene</td>
<td>204</td>
<td>205</td>
<td></td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Estragole</td>
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<td></td>
<td>187</td>
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<td>+++</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>L-Carvenol</td>
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<td>177</td>
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<td></td>
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<td>+++</td>
<td>+++</td>
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<tr>
<td>Thymol</td>
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<td>+++</td>
<td>++</td>
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<tr>
<td>Carvacrol</td>
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<td>151</td>
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<td></td>
<td></td>
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<td>+++</td>
<td>++</td>
<td>++</td>
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<td>Thymoquinone</td>
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<td>+++</td>
<td>++</td>
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<tr>
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<td>+++</td>
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<td>++</td>
</tr>
<tr>
<td>Longifolene</td>
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<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>α-Hederin</td>
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<td></td>
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<td>+++</td>
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<tr>
<td>Dihydrocarvone</td>
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<tr>
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<td></td>
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<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>A-Linolenic Acid</td>
<td>278</td>
<td>279</td>
<td></td>
<td></td>
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<td>+++</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>6-Camphenol</td>
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<td>153</td>
<td></td>
<td></td>
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<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rutin</td>
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<td>610</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Quercetin</td>
<td>302</td>
<td></td>
<td></td>
<td></td>
<td>342</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*‘+’ sign denotes presence of compounds.

*‘-‘ sign denotes absence of compounds.
The data of LC-MS analysis showed the presence of an array of wide spectrum of compounds in *N. sativa* methanolic extracts during germination stages. The reported compounds are mentioned in Table 2.5 (More + sign denotes the higher peak). All the reported compounds in the samples were generally low molecular weight compounds. During 5\textsuperscript{th} day of germination the abundance of all the compounds increased.

2.4.4 **Antibacterial activity of *N. sativa* extracts from various germination phases against clinical bacterial strains**

Antibacterial activity of *N. sativa* seed was evaluated in various germination phases against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis*) clinical bacterial strains isolated from pus, urine, ascitec fluid and cerebrospinal fluid of various patients. The minimum inhibitory concentration (MIC) values were determined by using a modified macro-broth dilution technique. The agar well diffusion method was used to test the antimicrobial effects of *N. sativa* extracts. Some broad spectrum antibiotics were used as positive control. All the extracts from germination phases gave excellent antibacterial results against tested clinical bacterial strains. Results indicated that different extracts of *N. sativa* showed different degrees of growth inhibition depending on the day of germination and bacterial strains.
Table 2.6: Minimum inhibitory concentration of *N. sativa* methanolic extracts from different germination phases on clinical bacterial isolates.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Organism</th>
<th>Minimum Inhibitory Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day of germination</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>1.6±0.09</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em></td>
<td>1.5±0.11</td>
</tr>
<tr>
<td>3</td>
<td><em>K. pneumoniae</em></td>
<td>1.3±0.10</td>
</tr>
<tr>
<td>4</td>
<td><em>P. aeruginosa</em></td>
<td>1.3±0.12</td>
</tr>
<tr>
<td>5</td>
<td><em>P. mirabilis</em></td>
<td>1.0±0.05</td>
</tr>
</tbody>
</table>

*Data is a mean ± SD of three replications.*

**Technique used was broth dilution assay.**

Figure 2.5: Minimum inhibitory concentration of *N. sativa* methanolic extracts from different germination phases on clinical bacterial isolates.
The minimum inhibitory concentration of the germinating seed extracts were moderate (0.80 µg/ml) for *E. coli*, *S. aureus* and *K. pneumoniae* whereas it was maximum for *P. aeruginosa* (1.0 µg/ml) and least for *P. mirabilis* (0.60 µg/ml). From these data, it could be said that the extracts of *N. sativa* during various germination phases were effective in inhibiting *P. mirabilis* at very low concentration (MIC, 0.60 µg/ml) but they were most effective against Gram-positive bacteria *S. aureus* (5th and 11th day inhibition zone, 34mm) as it showed maximum zone of inhibition (Table 2.6 and Figure 2.5).
Table 2.7: Zone of Inhibition shown by methanolic extracts of *N. sativa* from different germination phases.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Organism</th>
<th>Zone of inhibition (mm)</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>12±0.24</td>
<td>12±0.1</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em></td>
<td>20±0.16</td>
<td>32±0.24</td>
</tr>
<tr>
<td>3</td>
<td><em>K. pneumoniae</em></td>
<td>28±0.32</td>
<td>25±0.41</td>
</tr>
<tr>
<td>4</td>
<td><em>P. aeruginosa</em></td>
<td>22±0.42</td>
<td>18±0.35</td>
</tr>
<tr>
<td>5</td>
<td><em>P. mirabilis</em></td>
<td>22±0.51</td>
<td>15±0.51</td>
</tr>
</tbody>
</table>

*Data is a mean of three replications. **“-” No inhibition observed. ***ST: Streptomycin (30 μg), CF: Ciprofloxacin (10 μg), DO: Doxycycline (30 μg), AM: Ampicillin (10 μg), OF: Ofloxacin (5 μg), **** Technique used was agar well diffusion assay. ***** Extracts (75μg/well).*
Figure 2.6: Antibacterial activity of *N. sativa* extracts of different germination phases.

In this study, we investigated the antibacterial effects of methanolic extracts of successive stages of the germinating seed on Gram-positive and Gram-negative clinical bacterial isolates collected from different pathological samples. The results of antibacterial test is presented in Table 2.6 and 2.7 which indicated that different germination extracts of *N. sativa* showed different degrees of growth inhibition depending on the day of germination and bacterial strains.

The general trend of the inhibition of bacterial strains by the extracts was the increase in the activity from 0 day reaching maximum on 5\textsuperscript{th} day and then showed decline again. On 11\textsuperscript{th} day the inhibitory activity was equal to that of 5\textsuperscript{th} day extract in case of *S. aureus*, *P. aeruginosa* and *P. mirabilis*. The 9\textsuperscript{th} and 11\textsuperscript{th} day extracts of germinating seeds did not show the inhibition of *E. coli*. Maximum sensitivity was shown by *S. aureus* (5\textsuperscript{th} and 11\textsuperscript{th} day inhibition zone, 34mm) followed by *P. aeruginosa* (5\textsuperscript{th} and 11\textsuperscript{th} day inhibition zone, 30mm) and *P. mirabilis* (5\textsuperscript{th} and 11\textsuperscript{th} day inhibition zone, 30mm). *K. Pneumonia* showed moderate sensitivity (5\textsuperscript{th} day inhibition zone, 28mm) and *E. coli* was
the least sensitive organism towards these extracts (5th day inhibition zone, 13mm). From the above results, it could be said that the extracts showed day-dependent activity and the 5th day extract of germinating seed was most effective in inhibiting the growth of bacterial pathogens isolated from human patients (Table 2.7).

The minimum inhibitory concentration of the germinating seed extract was moderate (0.80 µg ml⁻¹) for E. coli, S. aureus and K. pneumoniae whereas it was maximum for P. aeruginosa (1.0 µg ml⁻¹) and least for P. mirabilis (0.60 µg ml⁻¹). From these data, it could be said that the extracts of N. sativa during various germination phases were effective in inhibiting P. mirabilis at very low concentration (MIC, 0.60 µg ml⁻¹) (Table 2.6) but they were most effective against S. aureus (5th and 11th day inhibition zone, 34mm) as it showed maximum zone of inhibition (Table 2.7 and Figure 2.6).

All the pathogenic organisms were resistant towards ciprofloxacin (10 µg), doxycycline (30 µg), ampicillin (10 µg) and ofloxacin (5 µg) and sensitive towards streptomycin (30 µg). The discs of these standard antibiotics (HIMEDIA) served as positive controls in the experiment. The extracts were more effective on Gram-positive S. aureus (5th and 11th day inhibition zone, 34mm) as compared to Gram-negative E. coli (5th day inhibition zone, 13mm), K. Pneumonia (5th day inhibition zone, 28mm), P. aeruginosa (5th and 11th day inhibition zone, 30mm) and P. mirabilis (5th and 11th day inhibition zone, 30mm) (Table 2.7).

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which have been found in vitro to have antimicrobial properties. The extracts of seeds of N. sativa in different germination stages have exposed the presence of sterols, alkaloids, tannins, saponins, phenols, flavonoids, terpenoids and cardiac glycosides in most of the samples (Table 2.4). An interesting consequence is that these compounds are potent bioactive compounds that could be used for therapeutic purpose or which are precursors for the synthesis of useful drugs (Sofowora, 1982).
Level of antimicrobial activities of the methanolic extracts on clinical bacterial strains was compared with the chemical composition of extract to determine the chemical composition activity relationship of extract. The alkaloid and saponins content showed a decrease while the phenol, tannin and flavonoids contents have showed an increase with germination. High tannin and flavonoids content might also be responsible for the antibacterial activity in later stages of germination.

Recent studies have shown that the secondary metabolite content varies during germination of seeds. A significant antibacterial effect of *Allium roseum* L (bulb, leaf, seed and flower) extracts on *S. aureus*, *B. subtilis*, *B. cereus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. typhimurium* and *C. albicans* strains has been shown (Najjaa, 2009). *N. sativa* antibacterial properties are well known from previous study (Voravuthikunchai et al., 2005; Salman et al., 2008; Hannan et al., 2008; Suresh Kumar et al., 2010). Kamal et al., (2010) reported that *N. sativa* extracts during germination produce antimicrobial activity against a broad range of microbes and especially on multiple antibiotic resistant bacteria. The preliminary assessment of the *in vitro* antimicrobial effects of different germinating stages of *N. sativa* extracts revealed some basic outcomes in the present study. First, the methanol extracts of *N. sativa* showed good inhibitory effect against Gram-positive and Gram-negative clinical bacterial strains during germination phases as compared to seed extract, the extracts showed highest activity from 5th day to 11th day of germination.

The activity exerted by the methanolic extracts may be due to the presence of some active phytoconstituents such as thymol and thymoquinone. Thymol is a phenolic alcohol present in the essential oil of *N. sativa* (Randhawa and Al-Ghamdi, 2002) that has been reported to possess antibacterial activity (Karapinar and Aktug, 1987). Thymol and thymoquinone is present in the methanol soluble portion of *N. sativa* oil (Abou Basha et al., 1995). In a study by Kahsai (2002), thymoquinone present in volatile oil obtained from the crude extract exhibited remarkable inhibition of the growth of various strains of bacteria. Thymoquinone is present in the methanol soluble portion of *N. sativa* oil (Abou
Basha et al., 1995) and thus will be extracted in methanolic extract of seed also. Results of LC-MS showed many active constituent were present in germination phases extracts which may be responsible for this activity.

Finally the extracts of germination phase revealed good inhibitory effect when compared with the standard antibiotics and non-germinated seed extracts. These antibiotics are the inhibitors of cell wall synthesis, the cross-linking of different peptidoglycan strands etc. The extracts of N. sativa showed the activity against standard antibiotics resistant bacteria from 5th to 11th day of germination. This might be due to the presence of metabolites in methanolic extract on these days of germination which could act by cell wall synthesis inhibition, by inducing changes in membrane structure, by inhibiting bacterial protein synthesis or by binding to ribosomal 50S subunit and interfering with the peptidyl transferase activity. The illustration of the exact mechanism of inhibition by the extracts needs further investigation.

2.5 CONCLUSION

Qualitative analyses of phytochemicals present in the methanolic extract of N. sativa seed showed the presence of sterols, alkaloids, saponins, phenols, flavonoids, terpenoids and cardiac glycosides. This is first LC-ESI-MS study on N. sativa during germination as well as the alterations in the antimicrobial properties against clinical bacterial isolates from human patients. LC-ESI-MS analysis showed the presence of an array of wide spectrum of compounds in N. sativa methanolic extracts during germination stages. N. sativa seed extracts during various germination phases possesses potential antimicrobial activity against several multidrug resistant clinical bacterial isolates. Antimicrobial activity was higher in 5th day germination extract as showed in results. High metabolic activity and highest contents of secondary metabolites during germination might be responsible for the antibacterial activity. N. sativa can be used as alternative antibiotic during germination phases.
Plate 2.10: Zone of inhibition by antibiotics during antibacterial test on clinical bacterial strains.
Plate 2.11: Zone of inhibition by *N. sativa* extracts of germination phases during antibacterial test on clinical bacterial strains.
2.6 REFERENCES


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