CHAPTER 4

In Vivo Anti-Inflammatory and Analgesic Activities of Nigella sativa L. Seed in Various Germination Phases

“The good thing about science is that it's true whether or not you believe in it.”

~Neil deGrasse Tyson
4.1 Rationale

Throughout the world inflammatory diseases are becoming common in aging society. Recent studies indicate that the mediators and cellular effectors of inflammation are important constituents of the local environment of tumors (Mantovani et al., 2008). Inflammation is self-protective mechanism of the body to eliminate the injurious stimuli as well as begin the healing process for the tissue. However, if runs unchecked, leads to onset of diseases such as vasomotor rhinnorrhoea, rheumatoid arthritis, and atherosclerosis (Henson et al., 1989). Inflammation can increase pain independent of the direct experience of pain. Pain can be defined as a somatic sensation of acute discomfort a symptom of some physical hurt or disorder, or even emotional distress. Pain can be classified into two types: acute pain and chronic pain. Acute pain “is a body’s warning of present damage to tissue or disease. It is often fast and sharp followed by aching pain. It is short-term pain or pain with easily identifiable causes. Chronic pain is a pain that last much longer than pain normally would with a particular injury. Chronic pain can be constant or intermittent and generally harder to treat than acute pain. Pain can also be grouped by its source and related pain detecting neurons such as cutaneous pain somatic pain, visceral pain and neuropathic pain (Niv et al., 2007).

The drugs which are used presently for the management of pain and inflammatory conditions are either narcotics or non narcotics (NSAIDs), and have known toxic and lethal effects. It is alleged that current drugs available such as opioids and NSAIDs drugs are not helpful in all cases of inflammatory disorders, because of their side effects, less effectiveness and economy (Ahmadiani et al., 1998; Gambhire et al., 2009). Development of newer anti-inflammatory compounds possessing no side effect still remains a challenge to the scientific community. On the contrary, herbal medicines with good absorption, less toxicity, and easy availability have been used since ancient times. Natural products in general and medicinal plants in particular, are believed to be a key source of new chemical
substances or phytoconstituents with potential therapeutic efficacy (Ameh et al., 2009).

The present study was undertaken to investigate the anti-inflammatory and analgesic effects of *Nigella sativa* L. (*N. sativa*) methanol extracts of successive germination phases by using *in vivo* and *in silico* techniques. Anti-inflammatory drug target receptors were draw out from literature survey for *in silico* study and docking studies were carried out with the active constituents of *N. sativa* to identify the possible leads using AutoDock 4.2 software.

4.2. REVIEW OF LITRATURE

4.2.1. Non-steroidal anti-inflammatory drugs (NSAIDs): Drugs to control inflammation and pain

Non-steroidal anti inflammatory drugs (NSAIDs) have been commonly used in both human and veterinary medicine for reduction or treatment of pain and inflammation in different arthritic and postoperative conditions due to their anti-inflammatory, antipyretic, and analgesic activities. Phenylbutazone, diclofenac, aspirin, indomethacin, meloxicam and some other NSAIDs are being used as therapeutic measures for pain, inflammation and fever. NSAIDs exert their anti-inflammatory effect through inhibition of prostaglandin G/H synthase or cyclooxygenase, which is the enzyme catalyzing the transformation of arachidonic acid to prostaglandins and thromboxanes. This enzyme has two recognized forms: Cyclooxygenase-1 (cox-1) and Cyclooxygenase-2 cox-2 (Vane et al., 2003).

Although they are classified as mild analgesics, NSAIDs have a more significant effect on pain resulting from the increased peripheral sensitization that occurs during inflammation and leads nociceptors to respond to stimuli that are normally painless. In particular, it is believed that inflammation leads to a lowering of the response threshold of polymodal nociceptors (Fitzgerald, 2003).
Figure 4.1: Mechanism of selective and non selective NSAIDs (Vane et al., 2003; Fitzgerald, 2003).

NSAIDs wield their antipyretic effect by inhibition of prostaglandin E2 (PGE2) synthesis, which is responsible for triggering the hypothalamus to increase body temperature during inflammation (Fitzgerald, 2003).

4.2.2. Adverse effects of NSAIDs

The widespread use of NSAIDs has meant that the adverse effects of these drugs have become increasingly prevalent. The main adverse drug reactions (ADRs) associated with NSAIDs relate to gastrointestinal (GI) effects, renal effects of the agents and hepatic toxicity.

4.2.2.1. Gastrointestinal (GI) toxicity

The main target of NSAID toxicity is gastrointestinal tract (GIT). It has also been estimated that one third of the cost of treating arthritis patients relates to treatment of the side effects of NSAIDs. Approximately 1,07,000 patients are hospitalized annually for NSAIDs related gastrointestinal (GI) complications, and at least 16,500 death occur among arthritis patients alone by NSAID using. Indian
studies have shown that NSAIDs are among the most common drugs responsible for adverse drug reactions seen in clinical practice (Doomra et al., 2001).

**Non selective property induced GI ulcerations**

Prostaglandins (PGs) have long been known to be mucous protective and ulcer healing agents. Prostaglandins protect GI mucosa by forming a cytoprotective layer and increasing the secretion of bicarbonate ions that neutralize the gastric acidity. All therapeutically useful NSAIDs act by inhibiting the synthesis of PGs (Tamblyn et al., 1997). Cyclooxygenase has two isoforms, one constitutive (COX-1) and another inducible (COX-2). NSAIDs are now divided into selective (those inhibiting COX-2) and non-selective (inhibiting both COX-1 and COX-2). Conventional NSAIDs cause non-selective inhibition of cyclooxygenase, which leads to reduction in bicarbonate secretion and reduced mucous production (Raskin et al., 1999). Prolonged contact with most NSAIDs can result in ulceration due to caustic properties. They can result in oesophagitis and even strictures (Vikas Dhikav et al., 2003).

### 4.2.2.2. Renal toxicity

COX-1–related prostaglandins are largely constitutive and responsible for maintaining the integrity of the gastrointestinal mucosa, platelet adhesion, and acid secretion. In general, COX-1 functions in the control of renal hemodynamic and the glomerular filtration rate (GFR); COX-2 functions affect salt and water excretion, although there is some overlap. This separation of COX-mediated functions in the kidney is based in part on the physiologic/anatomic distribution of COX-1 compared to COX-2, blockade of either or both of these enzymes can have, therefore, different effects on renal function (Nantel et al., 1999; Schnermann et al., 1999). However, renal syndromes associated with the use of nonselective NSAIDs and COX-2–selective inhibitors can be either prostaglandin-dependent (i.e. functional) or prostaglandin-independent (i.e. anatomic). NSAID-induced decreases in PGE2 can increase sodium and water re-absorption and can produce some weight gain and
occasionally edema. NSAIDs can significantly decrease renal blood flow, with resultant acute renal failure. NSAIDs can be the cause of acute renal failure, exacerbation of renal insufficiency, hyperkalemia and interstitial nephritis (Murray et al., 1990).

4.2.2.3 Hepatotoxicity

NSAIDs are consumed massively worldwide and along with antimicrobial agents, are the most frequent causes of drug induced liver injury (Andrade et al., 2005; Sgro et al., 2002; Hussaini et al., 2007). Indeed, roughly 10% of total drug-induced hepatotoxicity is NSAIDs related. Long-term usage of NSAIDs at doses sufficient to suppress inflammation has been implicated in serious adverse effects mainly involving the gastrointestinal tract, kidneys, liver, haemovascular system, and the lungs (Acker et al., 1995; Gay et al., 1990).

Clinical trials were done with hospitalized patients, which demonstrated that the increased risk of acute symptomatic hepatitis in patients using NSAID therapy is approximately two-fold (Garcia et al., 1994). Indeed, a number of diseases treated with NSAIDs can themselves be connected with mild or severe liver enzyme abnormalities, and these must be distinguished from the cases of NSAID hepatotoxicity (Mandell et al., 1999). Earlier all of the NSAIDs have been implicated in causing liver injury and tend to be hepatocellular in nature: the mechanism is thought to be immunological idiosyncrasy (Zimmerman 1990; Rabinovitz et al., 1992). Diclofenac, and particularly sulindac, are reported to be more commonly associated with hepatotoxicity (Bjorkman et al., 1998; Walker et al., 1997). There are two main clinical patterns of hepatotoxicity due to NSAIDs (Rabinovitz et al., 1992), the first is an acute hepatitis with jaundice, fever, nausea, greatly elevated transaminases and sometimes eosinophilia. The alternative pattern is with serological (ANF-positive) and histological (periportal inflammation with plasma and lymphocyte infiltration and fibrosis extending into the lobule) features of chronic active hepatitis.
Ethno-botanical research done in last few decades have revealed the anti-inflammatory and analgesic properties of plants cited in the traditional literature. Lot of compounds were characterized from plants. The research into plants with alleged folkloric use as pain relievers and anti-inflammatory agents is definitely a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs with no side effects.

4.2.3 Anti-inflammatory and analgesic activities of *N. sativa* L.

*N. sativa* L. regarding its therapeutic use, 1400 years ago Prophet Muhammad (Peace and Blessings be upon Him) acclaimed that 'the black seed is a cure for all the diseases'. Detailed scientific enquiries proved that *N. sativa* have an immense array of diverse pharmacological activities. Extracts obtained from the seeds of *N. sativa* are used as a spice or remedy for the treatment of various inflammatory diseases. Al-Shebani investigated the antinociceptive activity of the watery suspension of powdered *N. sativa* seeds in mice. Which implicate an opioid activity of *Nigella* seeds constituents (particularly thymoquinone/TQ) participating in its analgesic activity which characterized by inhibitory effect on nociceptive system and / or inflammatory mediators (Al-Shebani *et al.*, 2009).

Analgesic and anti-inflammatory effects of *N. sativa* seed polyphenols were studied in mice and rats using the acetic acid-induced writhing, formalin, light tail flick, carrageenan-induced paw edema, and croton oil-induced ear edema tests. In the acetic acid-induced writhing test, oral administration of *N. sativa* polyphenols decreased the number of abdominal constrictions. Both oral and intraperitoneal administration of *N. sativa* polyphenols significantly suppressed in a dose-dependent manner the nociceptive response. *N. sativa* polyphenols inhibited paw edema in a dose-dependent manner. These results suggest that *N. sativa* polyphenols have analgesic and anti-inflammatory effects (Ghannadi *et al.*, 2005). The anti-inflammatory activity of *Nigella* seed oil has also been evaluated using carrageenan-induced paw edema in rats and croton oil-induced ear edema in
mice by Hajhashemi and colleagues in 2004. The aqueous and methanolic extracts of *N. sativa* showed analgesic effect in mice as it produced significant increases in reaction times in the hot plate and pressure tests (Al-Naggar *et al.*, 2003). The aqueous extract also has an anti-inflammatory effect as demonstrated by its inhibitory effects on carrageenan-induced paw edema in mice (Al-Naggar *et al.*, 2003).

TQ obviously has an important role in these pharmacological effects (El-Gouhary *et al.*, 2005). Thymoquinone has been reported to have potent superoxide anion scavenging ability and to inhibit iron-dependent microsomal lipid peroxidation. This is promising considering the fact that superoxide reacts with protein and non-protein sulfhydryls and polyunsaturated fats and initiates specific reactions, thus damaging cells and causing inflammation. Meanwhile free radical oxidative stress is implicated in many inflammatory diseases. Therefore, it is reasonable that the anti-inflammatory activities of thymoquinone are attributed to its antioxidant effect. Interestingly, it was found that the whole oil had both antioxidant and anti-eicosanoid effects greater than thymoquinone, the oil's active constituent.

The anti-inflammatory effect of thymoquinone was supported by its ability to attenuate allergic airway inflammation by inhibiting Th2 cytokines and eosinophil infiltration into the airways and goblet cell hyperplasia. Attenuation of airway inflammation occurred concomitant to inhibition of COX-2 (cyclogenase) protein expression and prostaglandin D2 production in a mouse model of allergic airway inflammation induced with ovalbumin (Boskabady *et al.*, 2004).
4.3 MATERIALS AND METHODS

4.3.1 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.

4.3.2 Germination of N. sativa seeds

Seed lots used for different experiments showed germination capacities ranging from 80 to 98%. The seeds were surface sterilized with 0.1% HgCl₂ 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.

4.3.3 Harvest of germinated seeds

The germinated seeds of different days were harvested with a 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 radical emerged from the testa. All the samples were stored at -80°C in a deep freezer until used further.
4.3.4 Preparation of distilled extracts

The samples of seed and germinated phases 5\textsuperscript{th}, 7\textsuperscript{th} and 11\textsuperscript{th} 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\textdegree C until use.

4.3.5 Animals

Male Wistar rats, weighing 150 - 200 g, were purchased from Central Drug And Research Institute (CDRI), Lucknow, India and housed in a temperature controlled room (22±2\textdegree C) with a 12 hour light-12 hour dark cycle and allowed free access to a standard rat chow and filtered tap water for 7 days for acclimatization. The study received the approval of the Institutional Animal ethics Committee (IAEC) of Era’s Lucknow Medical College & Hospital. Animals were cared for in accordance with the internationally accepted principles for laboratory animal use and care and the procedures followed were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). They were housed under controlled conditions of temperature of 23±20C, relative humidity of 30–70% and 12 h light–12 h dark cycle. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment. All animals were fed with sterile commercial pelleted rat chow supplied by Hindustan Lever Ltd. (Mumbai, India) and had free access to water. Animals were kept under fasting for overnight and weighed before the experiment.
Plate 4.1 Rats in cages housed in a temperature controlled room.

4.3.6 Animal handling and care

Rats were usually held by the tail and handled in different ways for various procedures, i.e., for feeding by gastric intubation, marking rats for identification, blood retrieval etc. Rats were taken out of their respective cages by gripping them by the tail. It might be necessary to calm them down by gently patting them till they are still. The tail was held tightly close to the anal orifice to keep the mouse under control while the middle finger and thumb of the left hand is gently moved up from its rear end to the junction between its neck and head. The rats were then held by gripping the skin on their neck right behind the ears between the middle finger and the thumb while the index finger is used to gently pull the skin from the tip of the snout in a swift but firm motion. This method ensured that the mandible is pulled down and the maxilla held firmly in place, and the mouth of the mouse is held wide open baring its teeth. The tail was
usually trapped between the ring finger and little finger of the left hand restricting all possible movements that might interfere with the procedure.

4.3.7 Oral administration of doses

Procedure

Doses were administered to the rats orally by the Gavage method. An 18 G ball tipped needle known as the Gavage Canula was fitted onto a one milliliter syringe and used for administering the respective doses on the skin on their neck right behind the ears between the middle finger and the thumb while the index finger was used to gently pull the skin from the tip of the snout in a swift but firm motion. This method ensured that the mandible was pulled down and the maxilla held firmly in place, and the mouth of the mouse was held wide open baring its teeth. It also prevented the mouse from interfering while the dose was being administered to it. The needle was inserted through the pharynx into the oesophagus while the mouse was in the act of swallowing. Once the needle went inside the esophagus the dose was injected. This method prevents the mouse from regurgitating the dose.

Plate 4.2: Gastric intubation using a gavage canula.
4.3.8 Drugs and chemicals

Indomethacin, Jagsonpal Pharmaceuticals, Gurgao, India.

4.3.9 Acute toxicity studies

The acute oral toxicity test of the extract was carried out by using Wistar rats of either sex weighing between 150-200g. The methanolic extracts of N. sativa from different germination phases were administered orally to overnight fasted animals at the dose of 250 mg/kg, 500 mg/kg, 1000 mg/kg, 3000 mg/kg and 5000 mg/kg of body weight. After administration of the extracts, the animals were observed continuously for the first two hours, for any toxic manifestation. Thereafter, observations were made at regular intervals for 48 hours. Further the animals were kept under investigation up to a period of 2 weeks for mortality and general behavior.

4.3.10 Anti-inflammatory activity

The rats were divided into six groups containing six rats in each group (one control, one standard & four test groups) Paw oedema was induced by injecting 0.1ml of 5% suspension of kaolin into the sub plantar tissues of the left hind paw of each rat (Lorenz, 1961; Wagner-Jauregg et al., 1964). Methanol extracts of N. sativa from different stages of germination (Seed, 5th day, 7th day and 11th day germination seed extract respectively) were administered orally in doses of 1 g/kg b.w. 1hr prior to kaolin administration in test groups. Indomethacin (10 mg/kg b.w) was given to standard group.

Control group: 1ml distilled water

Standard group: Indomethacin (10 mg/kg)

Test group 1: seed extract (1 g/kg b.w.)

Test group 2: 5th day extract (1 g/kg b.w.)
Test group 3: 7th day extract (1 g/kg b.w.)
Test group 4: 11th day extract (1 g/kg b.w.)

Percentage inhibition of oedema = \[ \frac{V_c - V_t}{V_c} \] \times 100

Where, \( V_c \) is the inflammatory increase in paw volume in control group of animals and \( V_t \) is the inflammatory increase in paw volume in drug-treated animals. The paw volume was measured at 0, 1, 3, 6 and 18 hrs after induction of inflammation using plethismometer. Anti-inflammatory activity was measured as the percentage reduction in oedema level when drug was present, relative to control (Duffy et al., 2001).

Plate 4.3: During anti-inflammatory test injection of kaolin into the sub plantar tissues of hind paw and plethysmometer.
4.3.11 Analgesic study

Hot plate method

The analgesic activity of the extracts was measured by hot-plate method (Eddy et al., 1957). The rats were divided into six groups containing six rats in each group (one control, one standard & four test groups). Methanol extracts of Nigella sativa at different stages of germination (Seed, 5th day, 7th day and 11th day germination seed extract respectively) were administered orally in doses of 1 g/kg b.w. Indomethacin (10 mg/kg b.w) was given to standard group.

Control group: 1ml distilled water

Standard group: Indomethacin (10 mg/kg)

Test group 1: seed extract (1 g/kg b.w.)

Test group 2: 5th day extract (1 g/kg b.w.)

Test group 3: 7th day extract (1 g/kg b.w.)

Test group 4: 11th day extract (1 g/kg b.w.)

Plate 4.4: During analgesic test rat on hot plate.
The animals were positioned on Eddy’s hot plate kept at a temperature of 55±0.5°C. The reaction time was taken as the interval from the instant animal reached the hot plate until the moment animal licked its feet or jumped out. A cut off period of 15s was observed to avoid damage to the paw. The reaction time was recorded before and after 0, 30, 60 and 90 min and 120 min following administration of test or standard drug (Eddy et al., 1957).

4.3.12 Statistical analysis

Statistical significance was determined by One Way Analysis of Variance (ANOVA) followed by Dunnet’s t-test to compare group means. The level of significance was $P < 0.001$.

4.3.13 *In silico* anti-inflammatory and analgesic study of active constituents of *N. sativa*.

4.3.13.1 Selection of anti-inflammatory drug target receptors

Membrane Protein Prostaglandin H2 synthase-1 or cyclooxygenases-1(COX-1) and Prostaglandin H2synthase-2 or cyclooxygenases-2(COX-1) is formed in many different cells which is responsible for the physiological production of prostaglandins (Kiefer et al., 2004). Prostaglandins are signaling molecules and are chemical messengers of inflammation and pain (Sruthi et al., 2012). Hence inhibition of this enzyme stops prostaglandin production and in turn regulation of inflammatory activities. The 3-dimensional structure of cyclooxygenase1 (COX-1) and cyclooxygenase1 (COX-2) used for the docking study was retrieved from protein data bank PDB ID: 3N8V, 5COX respectively.

4.3.13.2 Ligand preparation

3D structures of active constituents of *N. sativa*, Thymoquinone (CID: 10281), thymol (CID: 6989), carvacrol (CID: 10364) and alpha hederin (CID:
73296) and standard drug indomethacine (CID: 3715) were downloaded from Pubchem.

4.3.13.3 Molecular docking using AutoDock

All the ligands were docked to the enzyme (Cox-1 and Cox-2) using ‘AutoDock 4.2’. For energy minimization of the ligands molecules, MMFF94 force field was used. Gasteiger partial charges were added to the ligands atom. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculation was carried out on the protein molecule. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools (Michel et al., 1999). Prior to docking conserved water molecules were duly added to the binding pocket in ordered to mimic the in vivo environment. Affinity (grid) maps of 40 x 40 x 40 Å grid points were generated with the help of Auto grid program aimed to target grid co-ordinates in proximity with the anionic sub site of the catalytic site (CAS) of COX-1 and COX-2. The values of x, y and z co-ordinates for targeting the active site were taken as 21.087, -39.12 and 25.098 for cyclooxygenase1; 56.24, 38.19 and 68.34 for cyclooxygenase2 respectively. AutoDock parameter set and the distance dependent dielectric functions were used in calculation of the van der Waals and the electrostatic terms, respectively. Docking simulation was performed using the ‘Lamarckian genetic algorithm’ and the ‘Solis and Wets local search method’. Torsion of the ligands molecules was set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. The population size was set to 150. The final figures were generated with the help of Discovery Studio Visualizer (Accelrys). AutoDock results were analyzed to study the interactions and the binding energy of the docked structure.
4.4 RESULTS AND DISCUSSION

4.4.1 Acute Toxicity Studies

Acute toxicity studies were carried out to evaluate the toxicity of the different extracts, using Wistar rats. No death was observed till the end of the study. The extract was found to be safe up to the dose of 5000 mg/kg, hence 1/5th of the tested dose, 1000 mg/kg dose was chosen as the experimental dose.

4.4.2 Effect of *N. sativa* treatment on kaolin induced paw oedema in rats

The acute anti-inflammatory activity of *N. sativa* extracts during different phases of germination was measured plethysmographically using kaolin as inflammatory agent, keeping indomethacin (10mg/kg b.w) as reference standard. All tested extracts of *N. sativa* (1g/kg b.w) during different phases of germination showed significant reduction in paw oedema in comparison to control (P<0.001).
Table 4.1: Anti-inflammatory effect of *N. sativa* extracts of different germination phases against kaolin induced paw oedema in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Volume of paw oedema after drug administration (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
</tr>
<tr>
<td>Control</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg)</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Test group 1</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Test group 2</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Test group 3</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>Test group 4</td>
<td>0.20±0.02</td>
</tr>
</tbody>
</table>

* Data are expressed as Mean ± S.E.M of six animals in each group. **Values significantly differ from the control, \(^aP<0.001\) vs Control and \(^bP<0.001\) vs indomethacin group. ***Test group 1: seed extract, Test group 2: 5\(^{th}\) day extract, Test group 3: 7\(^{th}\) day extract, Test group 4: 11\(^{th}\) day extract.
Table 4.2: % inhibition in inflammation after administration of *N. sativa* extracts against kaolin induced paw oedema.

<table>
<thead>
<tr>
<th>Groups</th>
<th>% inhibition in paw oedema after drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hrs</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg)</td>
<td>50.84</td>
</tr>
<tr>
<td>Test group 1 (Seed)</td>
<td>40.67</td>
</tr>
<tr>
<td>Test group 2 (5th day)</td>
<td>66.10</td>
</tr>
<tr>
<td>Test group 3 (7th day)</td>
<td>64.4</td>
</tr>
<tr>
<td>Test group 4 (11th day)</td>
<td>49.15</td>
</tr>
</tbody>
</table>

Figure 4.2: Anti-inflammatory effect of *N. sativa* extracts of different germination phases against kaolin induced paw oedema.
Figure 4.3: % inhibition in inflammation after administration of *N. sativa* extracts against kaolin induced paw oedema.

The increase in paw volume after 1\textsuperscript{st}, 3\textsuperscript{rd}, 6\textsuperscript{th} and 18\textsuperscript{th} hour was calculated as percentage compared with volume measured immediately after the injection of kaolin in each rat. The result of anti-inflammatory studies was presented as mean ± SEM. The values obtained showed a significant reduction in the growth of oedema in the hind paw of the rats. Kaolin induced oedema significantly in all control rats. After 1 hr administration of kaolin three times increase in oedema. Kaolin induced long time oedema in rats. All tested extracts of *N. sativa* from different germination phases as well as indomethacin (standard drug) showed inhibition in inflammation in rats. The percentage inhibition was higher in 5\textsuperscript{th} day germination extract (1g/kg) followed by 7\textsuperscript{th} day and Indomethacin (10 mg/kg) group. Extract of 5\textsuperscript{th} day germination caused 72.5% inhibition at 18 hrs while this was 51.25%, 46.25% in indomethacin group and seed extract group respectively at the same time (Table 4.2 and Figure 4.3). So, extracts of germination stages showed higher inhibition of inflammation than non-germinated seed extracts (Table 4.1 and 4.2). The extracts of different germination phases of *N. sativa* showed inhibition of the kaolin-induced rat paw oedema in a time-dependent manner throughout the duration of the study. The
extracts of germination stages significantly (P < 0.001) inhibited formation of oedema in rat paw than Indomethacin and seed extract throughout the duration of the study.

4.4.3 Effect of *N. sativa* extracts on analgesic activity in rats during hot plate test

The analgesic activity of *N. sativa* extracts during different phases of germination was measured by hot plate method keeping indomethacin (10mg/kg b.w) as reference standard. All tested extracts of *N. sativa* (1g/kg b.w) of different germination phases showed significant increased (P<0.001) in the reaction time. The increase in latency period at different time points significantly differed (P<0.01) compared to control values within the same drug treated groups. The increase in the reaction time was time-dependent and differed significantly among the groups of rats receiving different germination extracts and indomethacin (Table 4.3).

**Table 4.3:** Analgesic effect of *N. sativa* extracts of different germination phases during hotplate test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean latency (s) before and after drug administration</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Control</td>
<td>2.62±0.41</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg)</td>
<td>2.62±0.30</td>
</tr>
<tr>
<td>Test group 1</td>
<td>2.61±0.36</td>
</tr>
<tr>
<td>Test group 2</td>
<td>2.62±0.36</td>
</tr>
<tr>
<td>Test group 3</td>
<td>2.59±0.40</td>
</tr>
<tr>
<td>Test group 4</td>
<td>2.60±0.44</td>
</tr>
</tbody>
</table>

*Data are expressed as Mean ± S.E.M of six animals in each group. **Values significantly differ from the control, <sup>a</sup>P<0.001 vs Control and <sup>b</sup>P<0.001 vs
indomethacin group. *** Test group 1: seed extract, Test group 2: 5th day extract, Test group 3: 7th day extract, Test group 4: 11th day extract.

Figure 4.4: Analgesic effect of *N. sativa* extracts of different germination phases during hot plate test.

The result of analgesic studies was presented as mean±SEM. The values obtained showed a significant increase in reaction time. All the rats showed a reaction time of 2-3 seconds on hot plate before administration of any drug. The reaction time was increased in the groups receiving *N. sativa* extracts from different germination phases and indomethacin. However, compared to control group, significant increase was seen at 60 and 90 minutes in the entire test group. All tested extracts of *N. sativa* from different germination phases as well as indomethacin (standard drug) showed increase in reaction time in hot plate test for rats. The reaction time was higher in 5th day germination extract (1g/kg) followed by 7th day and Indomethacin (10 mg/kg) group. Extract of 5th day germination increased reaction time of rats on hot plate up to 14.99s at 90 min This was 12.98s and 10.89s in indomethacin group and seed extract group respectively at the same time. So,
extracts of germination stages showed higher analgesic effect than non-germinated seed extract (Table 4.3).

The extracts of germination stages significantly (P < 0.001) showed increase in reaction time than indomethacin and seed extract throughout the duration of the study. The onset of action was seen at 60-90 minutes in all the test groups. This showed that the drug takes around 1 hour time in being absorbed passage through the liver and reaching the systemic circulation and CNS. The peak effect occurred at 90 minutes and the analgesic effect decreased at 120 minutes probably because of metabolism and elimination of the drug.

4.4.4 *In silico* anti-inflammatory and analgesic study of active constituents of *N. sativa*.

All the ligands (active constituents of *N. sativa* such as thymoquinone, thymol, carvacrol and alpha-hederin) were docked to the enzyme (Cox-1 and Cox-2) using ‘AutoDock 4.2’. For energy minimization of the ligands molecules. Results are summarized in table 4.4.
Table 4.4: Binding energies and interacting amino acids of cyclooxygenase enzymes with ligands during docking.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyclooxygenase-1</th>
<th>Cyclooxygenase-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE (kcal/mol)</td>
<td>IC (K_i)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>-6.40</td>
<td>140.22</td>
</tr>
<tr>
<td>Thymoquinone</td>
<td>-4.47</td>
<td>526.27</td>
</tr>
<tr>
<td>Thymol</td>
<td>-3.89</td>
<td>229.14</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>-4.11</td>
<td>225.20</td>
</tr>
<tr>
<td>Alpha-hederin</td>
<td>-6.82</td>
<td>635.37</td>
</tr>
</tbody>
</table>

*BE: Binding energy, **IC: Inhibition constant.
Figure 4.5: Interaction of indomethacin docked to the catalytic site of Cox-1 enzyme. The ligand indomethacin has been shown in ball and stick representation.

Figure 4.6: Interaction of thymoquinone docked to the catalytic site of Cox-1 enzyme. The ligand thymoquinone has been shown in ball and stick representation.
Figure 4.7: Interaction of thymol docked to the catalytic site of Cox-1 enzyme. The ligend thymol has been shown in ball and stick representation.

Figure 4.8: Interaction of carvacrol docked to the catalytic site of Cox-1 enzyme. The ligend carvacrol has been shown in ball and stick representation.
**Figure 4.9:** Interaction of alpha-hederin docked to the catalytic site of Cox-1 enzyme. The ligand alpha-hederin has been shown in ball and stick representation.

**Figure 4.10:** Interaction of indomethacin docked to the catalytic site of Cox-2 enzyme. The ligand indomethacin has been shown in ball and stick representation.
Figure 4.11: Interaction of thymoquinone docked to the catalytic site of Cox-2 enzyme. The ligand thymoquinone has been shown in ball and stick representation.

Figure 4.12: Interaction of thymol docked to the catalytic site of Cox-2 enzyme. The ligand thymol has been shown in ball and stick representation.
**Figure 4.13:** Interaction of carvacrol docked to the catalytic site of Cox-2 enzyme. The ligand carvacrol has been shown in ball and stick representation.

**Figure 4.14:** Interaction of alpha-hederin docked to the catalytic site of Cox-2 enzyme. The ligand alpha-hederin has been shown in ball and stick representation.
Results showed that the active site of cyclooxygenase-1 (Cox-1) was found to interact with indomethacin through the amino acid residues ARG120, LEU92, LEU112, VAL116, LEU115, VAL119, TRP100, PRO84, ILE89 and LEU93 (Figure 4.5). The free energy of binding and estimated inhibition constant (K_i) were found to be -6.40 kcal/mol and 40.221 µM respectively. One oxygen atoms of indomethacin (O1) was found to be involved in polar interactions with amino acid residues ARG120 of Cox-1 enzyme while eight carbon atoms named C1, C4, C8, C7, C14, C16, C17 and C19 were observed to make hydrophobic interaction with amino acid residues like LEU92, LEU112, LEU115, VAL116 and VAL119 of Cox-1 enzyme. π-π interaction was observed where atoms C14, C16 and C18 interacted with amino acid residue TRP100 of Cox-1 enzyme.

Cox-1 interact with thymoquinone (TQ) through amino acid residues ARG120, LEU93, LEU112, LEU115, VAL116 and LEU357 (Figure 4.6). Free energy of binding and estimated inhibition constant (K_i) were found to be -4.47 kcal/mol and 526.270 µM respectively. One oxygen atom named O2 of thymoquinone was observed to make polar bond involving one amino acid residue ARG120 of Cox-1 enzyme. Carbon atoms C1-C9 were involved in hydrophobic interaction with amino acid residues LEU93, LEU112, LEU115, VAL116 and LEU357 of Cox-1 enzyme.

Cox-1 was found to interacts with thymol through amino acid residues ARG120, GLU524, VAL119 and LEU123 (Figure 4.7) and free energy of binding and estimated inhibition constant (K_i) were found to be -3.89 kcal/mol and 229.200 µM respectively was observed. Two oxygen and two hydrogen atoms of thymol named O1 and H1 were observer to be involved in polar interaction with amino acid residues ARG120 and GLU524; and three carbon atoms namely C6, C9 and C3 were found to make hydrophobic interaction with amino acid residues VAL119 and LEU123 of the enzyme.

Carvacrol interacts with Cox-1 through amino acid residues ARG83, ARG120, GLU524, VAL119, LEU123 and PRO86 (Figure 4.8). Free energy of binding and estimated inhibition constant (K_i) were found to be -4.11 kcal/mol and
225.200 µM respectively. Three hydrogen atoms named H1 and two oxygen atoms (O1) of carvacrol were found to be involved in polar interaction with amino acid residues ARG83, ARG120 and GLU524 of Cox-1 enzyme. There were five carbon atoms named C6, C4, C2, C9 and C10 observed to be made hydrophobic interaction with amino acid residues VAL119 and LEU123 of Cox-1 enzyme.

Alpha-hederin interacts with Cox-1 through amino acid residues ARG79, ARG83, VAL119, ILE89, LEU93, LEU112, LEU115, VAL119, LEU123, TRP100, ARG83 and ASN122 (Figure 4.9). Free energy of binding and estimated inhibition constant (K_i) were found to be -6.82kcal/mol and 635.371 µM respectively. One oxygen atom named O3 of alpha-hederin was found to make polar interaction with amino acid residue ARG79 of cyclooxygenase1 enzyme. Fourteen carbon atoms namely C26, C41, C25, C7, C40, C36, C37, C31, C34, C2, C10, C11, C21 and C30 were observed to make hydrophobic interaction involving amino acid residues ILE89, LEU93, LEU112, LEU115, VAL119 and LEU123 as well as two hydrogen atoms of ligand named H5 and H6 make cation-π interaction with amino acid residue TRP100 of Cox-1 enzyme.

‘Van der Waals’, ‘hydrogen bond’ and ‘desolvation’ energy components for indomethacine, thymoquinone, thymol, carvacrol and alpha-hederin interaction with Cox-1 were found to be -7.48, -4.69, -4.30, -4.23 and -9.14, and kcal/mol respectively, while the ‘electrostatic’ energy component was found to be -0.07, -0.08, -0.20, -0.09 and -0.07kcal/mol respectively. Total interaction surface area for Cox-1 complexes with different ligands such as indomethacine, thymoquinone, thymol, carvacrol and alpha-hederin were found to be 937.265, 591.38, 639.304, 601.223 and 1452.88 Å² respectively.

Docking results showed cyclooxygenase-2 (Cox-2) was found to interact with indomethacine through the amino acid residues SER38, TYR55, ASN68, PRO40 and GLU67 (Figure 4.10). Free energy of binding estimated inhibition constant (K_i) were found to be -4.78 kcal/mol and 110.850 µM respectively. Two oxygen atoms of indomethacin named O1 and O4 interacts with amino acid residue SER38 of Cox-2 enzyme by hydrogen bonding. Three carbon atoms of indomethacin named C1 and
C4 were observed to make hydrophobic interaction with amino acid residues TYR55 of enzyme. Two oxygen atoms named O3 involved in polar interaction with amino acid residues namely TYR55 and ASN68 of the enzyme. π-π interaction was found between carbon atoms C2, C3, C12 and C14 with amino acid residue TYR55 of Cox-2 enzyme.

Cox-2 and TQ interact through the amino acid residues TYR385, SER530, VAL349, LEU352, PHE381, LEU384, SER353 and VAL523 (Figure 4.1). Free energy of binding and estimated inhibition constant (Ki) were found to be -6.90 kcal/mol and 100.070 µM respectively. One oxygen atom of thymoquinone named O1 was observed to make polar bond with amino acid residue TYR385. There were ten carbon atoms C1, C3, C4, C5, C7, C9 and C10 of indomethacin observed to make hydrophobic interaction with amino acid residues VAL349, LEU352, PHE381, LEU384 and TYR385 of Cox-2 enzyme. Hydrogen bonding was found between O1 with amino acid residue TYR385 of Cox-2 enzyme.

Cox-2 and thymol interaction took place through amino acid residues VAL349, LEU352, LEU384, TRP387, MET522, SER353, VAL523 and ALA527 (Figure 4.12). Free energy for binding and inhibition constant were found to be (Ki) -5.17 kcal/mol and 162.110 µM respectively. Neither hydrogen bond nor polar interactions were found between Cox-2 and thymol. Only polar and π-π interactions were found between Cox-2 and thymol, where polar interaction was seen between carbon atoms C3, C6 and C10 of thymol with amino acid residues VAL349, LEU352, LEU384, TRP387 and MET522 of enzyme. One carbon atom C9 involved in π-π interaction with amino acid residue TRP387 of Cox-2 enzyme.

Cox-2 and carvacrol interacted through amino acid residues ASN375, PHE209, ILE377,ALA378, PHE381, PHE529 and LYS532 (Figure 4.13). Free energy for binding and inhibition constant were found to be -6.11 kcal/mol and 105.300 µM respectively. In case of Cox-2 and carvacrol interaction one oxygen and one hydrogen atom O1 and H1 made polar interaction involving amino acid residue ASN375 of the enzyme. Eleven carbon atoms C1, C3, C6, C9, C2, C10 and C4 of Carvacrol were observed to make hydrophobic interaction with amino acid residues
PHE209, ILE377, ALA378, PHE381 and PHE529 of enzyme. Four carbon atoms namely C6, C9, C2 and C7 were involved in $\pi-\pi$ interaction with amino acid residues PHE381 and PHE529 of Cox-2 enzyme.

Alpha-hederin interacted with Cox-2 through amino acid residues SER38, GLU67, PRO40, TYR55, ASN68, LYS166, GLN54, VAL165 and GLU465 (Figure 4.14). Free energy for binding and inhibition constant were found to be -4.90 kcal/mol and 255.56 $\mu$M respectively. One oxygen atom of alpha-hederin O4 made hydrogen bond with amino acid residue VAL228 of the enzyme. Three hydrogen H1, H6 and one oxygen atom O2 of alpha-hederin were involved in polar interaction with amino acid residues ARG120, TYR355 and TYR385 of Cox-2 enzyme. Fourteen carbon atoms namely C22, C24, C29, C30, C9, C21, C12, C20, C1, C2, C4, C18, C19, and C11 were observed to interact hydrophobically with amino acid residues PHE205. Five hydrogen atoms H1, H2 and H3 made cation-$\pi$ interaction with amino acid residues TYR385, TRP387 and PHE518 of the enzyme.

‘Van der Waals’, ‘hydrogen bond’ and ‘desolvation’ energy components for indomethacine, thymoquinone, Thymol, Carvocrol and alpha-hederin interaction with Cox-2 enzyme were found to be -5.93, -6.18, -5.69, -6.65 and -6.13 kcal/mol respectively while the ‘electrostatic’ energy component was found to be -0.12, -0.03, -0.18, -0.06 and -0.09 kcal/mol respectively. Total interaction surface area for indomethacine, thymoquinone, thymol, carvocrol and alpha-hederin interaction to make Cox-2 and legend complexes were found to be 572.029, 371.119, 371.065, 374.426, and 774.991 Å$^2$ respectively.

Pain and inflammation are associated with pathology of various clinical conditions like arthritis, cancer and vascular diseases (Collier et al., 1968). In various traditional medical systems, a number of natural products are used to reduce the symptoms of inflammation. The methanolic extract of $N$. sativa in different phases of its germination exhibited a significant anti inflammatory activity. After the administration of kaolin plasma leukotriene (LT) C$_4$-like and prostaglandin (PG) E2-like activities were increased (Melli, 1988). Kaolin induced oedema appears to have a significant prostaglandin component since large amounts of prostaglandin-like
materials production in kaolin blebs and indomethacin reduced the kaolin induced paw oedema (Lewis et al., 1976). Indomethacin is a nonselective inhibitor of cyclooxygenase (COX) 1 and 2 enzymes that participate in prostaglandin synthesis from arachidonic acid. Prostaglandins are hormone-like molecules normally found in the body, where they have a wide variety of effects, some of which lead to pain, fever, and inflammation. In the study, injection of commonly used NSAIDs (indomethacin) was observed to significantly reduce inflammatory indices while histamine and 5- hydroxytryptamine (5-HT) were not effective. This observation is consistent with those of others (Gemmell et al., 1979; Masso et al., 1993) that PGs are the major mediators of kaolin-induced inflammation. The researchers suggested that kaolin-induced inflammation should be used as a model of inflammation for assessing the efficacy of NSAIDs and other drugs acting via the same mechanism. The advantages of kaolin induced inflammation compared to other model of inflammation like carrageenan are its longer duration of inflammation and being a clay mineral, it is unlikely to have anti-genicity or to cause hypersensitivity reactions. It may therefore be suggested that the extracts of different germination phases of *N. sativa* remission of kaolin-induced rat paw oedema observed through inhibition of prostaglandins biosynthesis. Several investigations have been directed towards *N. sativa* anti-inflammatory activity (Ghannadi et al., 2005).

In Present *in vivo* study oral administration of methanolic extracts of *N. sativa* from different germination phases showed higher analgesic as well as anti inflammatory activity compared to seed extract and indomethacin. As shown in results (Table 4.1 and 4.2) during anti-inflammatory test extracts reduced paw oedema up to 72 % comparison than control. Extract of 5\textsuperscript{th} day germination caused 72.5% inhibition at 18 hrs while this was 51.25%, 46.25% in indomethacin group and seed extract (non-germinated seed) group respectively at the same time (Table 4.2 and Figure4.3). During analgesic test extracts from germination phases increased latency period on hot plate and reduced pain. In both test 5\textsuperscript{th} day germinated extract showed highest activity followed by 7\textsuperscript{th} day of germination. Activity was high in germination extracts when compared than non-germinated seed of *N. sativa*. This
may be because of higher metabolic rates during germination which increased the production of secondary metabolites such as thymoquinone, thymol and carvacrol as explained in chapter one and two using LC-ESI-MS results which showed higher amount of these metabolites. Thymoquinone and thymol both are the alcohol soluble phenolic compounds (Abou Basha et al. 1995), which are probably responsible for the analgesic and anti-inflammatory activities. Thymol is a promising compound to be used in treatment of inflammatory processes as well as wound healing. It showed helpful effects in controlling the inflammatory processes present in many infections (Riella et al., 2012; Braga et al., 2006). Carvacrol and alpha-hederin also cause anti-inflammatory and analgesic effects by reducing the production of inflammatory mediators (Li et al., 2003; Gepdiremen et al., 2005; Lima et al., 2013). Carvacrol inhibited production of prostaglandin E(2) catalyzed by COX-2, and showed anti-inflammatory potential of this compound due to the inhibition of inducible COX-2 isoform (Landa et al., 2009).

These active compounds (thymoquinone, thymol, carvacrol and alpha-hederin) of N. sativa may be responsible for analgesic and anti-inflammatory effects. The purpose of structure-based in silico study in this respect was to discover whether these compounds are potential to be used as COX-2 inhibitors. Docking was performed to the binding pockets of both COX-1 and COX-2 enzymes, to examine their selective character on COX-2 keeping indomethacin as standard.

Cyclooxygenase (COX) enzyme plays an important role in inflammatory response, i.e catalyze the prostaglandins biosynthesis. Two isoforms, known as COX-1 and COX-2, have similar amino acid residues composition and hydrophobic channel as binding pocket (Fabiola et al., 2001). The use of non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of inflammation and pain is often accompanied by adverse gastrointestinal and renal side effects because of non-selective inhibition of both COX-1 and COX-2 (Kurumbail et al., 1996). The anti-inflammatory activities of these drugs are mediated by the inhibition of cyclooxygenases (COXs), which catalyze the bioconversion of arachidonic acid to prostaglandins (Surh et al., 2001). However, the inhibition of COXs may lead to
undesirable side effects. The constitutively expressed COX-1 isozyme is produced in a variety of tissues and appears to be important for the maintenance of physiological functions such as gastro protection and vascular homeostasis (Kiefer et al., 2004). On the other hand, the COX-2 isozyme is induced by mitogenic and pro-inflammatory stimuli, suggesting the involvement of this isozyme in inflammatory processes. Therefore, the selective inhibition of COX-2, but not COX-1 is useful for treating inflammation and inflammation-associated disorders (Sruthi et al. 2012).

In this study active compounds of *N. sativa* thymoquinone, thymol, carvacrol and alpha-hederin were investigated whether these compounds are potential to be used as COX-2 inhibitors through structure-based *in silico* study. Based on the docking study result it was apparent that thymoquinone, thymol, carvacrol and alpha-hederin showed significant binding affinity towards Cox-2 enzyme.

The free energy for binding of these compound with COX-1 enzyme was higher than indomethacin (-6.40 kcal/mol), for thymoquinone, thymol and carvacrol it was -4.47, -3.89, and -4.11 kcal/mol respectively, that means these compounds not show a good binding affinity for Cox-1 enzyme but indomethacin. While the free energy for binding of these compounds with COX-2 enzyme was lower than indomethacin (-4.78 kcal/mol), for thymoquinone, thymol, carvacrol and alpha hederin it was -6.90, -5.17, -6.11 and -4.90 kcal/mol respectively, that means these compounds showed a good binding affinity towards Cox-2 enzyme than indomethacin. These results suggest that all these active constituents of *N. sativa* significantly inhibit Cox-2 enzyme more than cox-1, that a sign of reduced toxicity of these compounds as anti-inflammatory agents. The $K_i$ values of the ligands (thymoquinone, thymol, carvacrol and alpha-hederin) interaction with COX-2 are smaller than COX-1 (Table 4.4) which means that the ligands better interacted with COX-2 (Nyi et al., 2013). Binding $K_i$ value of indomethacin with Cox 1 (40.22 µM) was less than Cox 2 (110.22 µM) enzyme. This means indomethacin binds better with Cox-1 in comparison with Cox-2. Among all compounds thymoquinone and carvacrol were on first position as Cox-2 inhibitor followed by thymol and alpha-hederin on the basis of free energy for binding and inhibition constant ($K_i$). Another
recent study reported that *N. sativa* and thymoquinone may be an effective treatment for rheumatoid arthritis (Hawkey *et al.*, 2000). This comes as no surprise, since it has been shown that thymoquinone is an inhibitor that is more potent than indomethacin of COX-2-catalyzed PGE2 production (Abdel-Fattah *et al.*, 2000). These *in vivo* and *in silico* studies showed that *N. sativa* demonstrate anti-inflammatory and analgesic effect activities possibly through inhibition of Cyclooxygenase enzyme especially Cox-2.

### 4.5 CONCLUSION

To the best of our knowledge, no earlier study has been performed on anti-inflammatory and analgesic activity of *N. sativa* in different germination phases and this is being reported for the first time. The methanolic extracts of germinative phases of *N. sativa* showed significant anti-inflammatory and analgesic activity as compare to seed extract and standard drug indomethacin. The extracts showed highest anti-inflammatory and analgesic activity from 5th day to 11th day of germination especially in 5th day germinative extract. This is first *in silico* study with active compounds of *N. sativa* thymoquinone, thymol, carvacrol and alpha-hederin to investigate whether these compounds are potential to be used as COX-2 inhibitors. All these active constituents bind significantly with Cox-2 compared with Cox-1 enzyme. High metabolic activity and higher contents of secondary metabolites during germination phase of seed might also be responsible for the anti-inflammatory and analgesic activity. Since, *N. sativa* may be used to develop analgesic and anti-inflammatory herbal drugs in germination phases of seed.
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