**Summary and Conclusion**

In India, approximately 95% of population is mainly dependent on traditional medicine. Large numbers of medicinal plants have curative properties to treat various diseases. Many drugs from natural products are used for treating various ailments including cancer.

Free radicals play a main role that causes many diseases including cancer. Antioxidants can eliminate these free radicals and prevent the oxidation of important cellular components and are used as additives to diets. One of the best natural sources of antioxidants are the medicinal plants.

The incidence of cancer is increasing day by day all over the world, the reason being uncontrolled cell division or failure of apoptosis. Disturbance of apoptotic pathway contributes to an imbalance between life and death of cells, which leads to cancer development. Cancer cells also encounter a physiologically ubiquitous cellular program that aims to eliminate damaged or abnormal cell. Thus, it is essential that cancer cells acquire instruments to circumvent programmed cell death.

Among the different types of cancers, breast cancer is one of the leading cancers among Indian women. Several forms of therapies are used to treat cancer such as radiation therapy, hormonal therapy, chemotherapy and biological therapies which are very expensive, causes many severe side effects and chances of relapse are more. Hence, natural sources have been used for the treatment of cancer and to reduce the risk of side effects.

*Nigella sativa*, is a well known medicinal plant and is distributed in different parts of the country. The seeds of this plant are useful in treating various types of cancer, gastrointestinal disorders as well as skin or respiratory ailments. Thymoquinone, one of the major active components of *Nigella sativa* seeds,
showed significant toxicity towards pancreatic, lung, colon, uterine and leukemic carcinomas, hepatotoxicity, anti-inflammatory activity, antioxidant activity, antidiabetic, antiulcer, antimicrobial, antiparasitic and also decreased chemically induced nephrotoxicity.

The study was carried out in four distinct phases. In phase I, the cytotoxic activity and apoptosis influencing activity of thymoquinone in an eukaryotic model organism *Saccharomyces cerevisiae* was analysed. In phase II, the anticancer activity of thymoquinone was tested using breast cancer cell lines – a hormone receptor positive cell line MCF-7 and triple negative cell line MDA MB 231 and this was compared with normal breast cell line HBL-100. In phase III, cell cycle arrest and mechanism of cell death was assessed. The stages of distribution cell death were assessed by Annexin V/FITC and PI. In order to identify the expression level of PARP, a nuclear DNA-binding protein that detects DNA strand breaks and functions in base excision repair, its expression was studied using q-RT PCR. In last phase, molecular docking studies were carried out using Schrödinger software version 9.0, to find the interaction between the ligand namely thymoquinone with cancer targets and apoptotic proteins.

In Phase I, the cytotoxic effect of thymoquinone was analysed using primitive eukaryotic model organism *Saccharomyces cerevisiae* in the presence and absence of an oxidative stress inducing agent hydrogen peroxide (200μM) and the cytotoxic activity was assessed by using MTT, SRB and LDH release assays. The morphological and nuclear changes characteristic of apoptotic cell death was analysed by using various staining techniques such as Giemsa, EtBr, AO/EtBr, PI, DAPI and the apoptotic ratio was also calculated.

The results of the viability and cytotoxicity assays showed that, the exposure of *S. cerevisiae* cells to H₂O₂ induced a steep decrease in the viability of cells. When it was co-administered along with thymoquinone, the percent viability was significantly increased, which was evidenced by MTT and SRB assays. The results of the LDH release assay revealed that there was a drastic reduction in the percent LDH release when treated with thymoquinone both in the presence and absence of H₂O₂.
The characteristic features of apoptotic cell death was visualised using results of various staining techniques. It showed that thymoquinone by itself did not induce any cytotoxicity in yeast cells. Additionally, it protected yeast cells from hydrogen peroxide induced cell death and also showed a significant decrease in the apoptotic ratio. The amount of DNA damage was assessed by diphenyl amine method. The results of the DNA damage showed that thymoquinone protected the yeast cells from oxidative damage and when treated along with oxidizing agent, a further decrease was found in DNA damage compared to oxidative stress inducing agent H₂O₂ alone treated group. Hence the results showed that thymoquinone protected normal cells S. cerevisiae from oxidative damage.

In Phase II, the apoptosis inducing effect and the anticancer activity of thymoquinone was assessed using two breast cancer cell lines, namely ER⁺ non-invasive breast cancer cell line MCF-7, which is specific to mammary epithelium, and, triple negative, highly invasive breast cancer cell line MDA MB 231, derived from an aggressive breast cancer in which estrogen receptors (ER⁺), progesterone receptors (PR⁺), and Her 2 receptors (HER 2') are absent. A non-tumorigenic breast cancer cell line HBL-100 was also used for comparison as untransformed cells. The effect of thymoquinone was also compared with the standard chemotherapeutic drugs that are currently used for the treatment, namely tamoxifen for MCF-7 and HBL-100 cells and etoposide for MDA MB 231 breast cancer cells.

The dose and time period of exposure was optimized in both the non-cancerous and cancerous cell lines. The non-cancerous cell line, HBL-100, and cancerous cell lines, MCF-7 and MDA MB 231, were exposed to varying concentrations of thymoquinone ranging from 25µM to 400µM for different time periods namely 6 hours, 12 hours, 18 hours, 24 hours, 36 hours and 48 hours and the cell viability was assessed using MTT assay.

The results of cytotoxicity testing showed that thymoquinone did not inhibit the cell proliferation drastically in non-cancerous HBL-100 breast cancer cells and showed significant cytotoxicity in MCF-7 and MDA MB 231 in a dose and time dependent manner. In HBL-100 cells a lower dose of 25µM and lesser incubation period of 12 hours showed minimal cytotoxic effect and also contained a large
Summary and Conclusion

In vitro and in silico characterization of the anticancer activity of thymoquinone against breast cancer

number of surviving cells and this dose and time was used for further assays. The results of MTT assay in ER positive breast cancer cell line MCF-7 showed that, 50% cytotoxicity was observed at a concentration of 25 µM at 24 hours incubation period. In the case of triple negative breast cancer cell line MDA MB 231, a similar trend was observed. Hence, the optimal dose of 25 µM and the optimal time period of 24 hours was used for consecutive experiments in both the cancerous cell lines.

The extent of cytotoxicity induced by thymoquinone in the presence and the absence of a standard chemotherapeutic drugs tamoxifen/etoposide in the three cell lines were studied using MTT, SRB and LDH release assays. The results showed that treatment with thymoquinone alone reduced the viable cells in both cancerous cells MCF-7 and MDA MB 231. When it was treated along with the standard drugs tamoxifen/etoposide, the percent viability was further decreased in cancer cells. In the non-cancerous cells, the viability was significantly more when compared to drug alone treated group. It was observed that the decrease in viability induced by thymoquinone was more towards actively proliferating cancerous cells than the non-cancerous cell line, iterating its anticancer effect.

The above findings suggested that thymoquinone induced cell death in cancer cells and showed minimal toxicity in non-cancer cell line tested. In order to find out the nature of the cell death induced by thymoquinone, various morphological and nuclear changes associated with apoptosis were assessed by various staining techniques namely Giemsa, EtBr, AO/EtBr, PI, DAPI.

The results of the various staining techniques revealed that, the treatment with thymoquinone showed differential response in both breast cancer cell lines MCF-7 and MDA MB 231 and non-tumourogenic cell line HBL-100. When treated along with the chemotherapeutic drugs tamoxifen/etoposide, there was a significant hike in the number of cells committed to apoptosis as compared to the groups treated with the chemotherapeutic drug alone. This effect was more pronounced in triple negative cells compared to ER positive MCF-7 cells. The results showed that, in thymoquinone alone treated group, the number of cells undergoing apoptosis was more in MDA MB 231 and MCF-7 breast cancer cell lines, whereas the extent of apoptosis was less in HBL-100 breast cells. When the cells were treated with
thymoquinone along with the standard drugs tamoxifen/etoposide, similar results were noticed.

The extent of DNA damage was also assessed by comet assay. The results showed that exposure to thymoquinone, along with the chemotherapeutic drugs, caused an increase in the number of comet bearing cells in breast cancer cell lines MCF-7 and MDA MB 231 and showed minimal DNA-damaging effect in HBL-100 cell line compared to the corresponding drug alone treated groups.

Percentage of apoptosis was measured by Annexin V/FITC and PI staining. More number of apoptotic cells was observed in standard drug tamoxifen/etoposide treated group in all the three cell lines. On treatment with thymoquinone alone the extent of apoptosis induction was reduced in HBL-100 non-cancerous cell line and more in cancerous cell line MCF-7 and MDA MB 231. When thymoquinone was treated along with the standard drugs the number of cells committed to apoptosis were reduced in HBL-100 and found to be increased in both the cancer cell lines.

There was an increase in the number of TUNEL positive cells in the group treated with thymoquinone alone compared to drug the treated groups. On the other hand, the DNA damage was significantly high in standard drug alone treated group in both cancerous and non-cancerous cell line. In HBL-100 cells, however, the DNA damage was found to be comparatively less, as indicated by a decrease in the number of TUNEL positive cells.

In phase III, cell cycle analysis showed that thymoquinone arrested growth of MCF-7 and MDA MB 231 at sub-G₀ phase. To obtain further evidence on the stage of cell death by apoptosis, distribution of apoptotic cells was identified by flow cytometry using PI and Annexin V-FITC staining. Treatment with thymoquinone induced cell death in late apoptotic stage compared to standard drugs which induced cell death at early apoptotic stage.

The results of qRT-PCR showed that in the case of HBL 100 cells the expression level of PARP gene was found to be increased in thymoquinone and tamoxifen treated group compared to the standard drug alone treated group. Exposure to thymoquinone alone showed that the PARP gene expression was
found to be similar to that of control in HBL-100 non-cancerous cell line. Whereas in cancerous cell line MCF-7 and MDA MB 231 the PARP gene expression was downregulated in thymoquinone alone treated group and this was further reduced on co-administration with standard drugs.

*In silico* docking will help us to understand the interaction between the ligand (thymoquinone) and receptors for cancer targets (p53, LOX, PPAR, ER beta and Tubulin) and apoptotic proteins (TRAIL-R, Bcl-2, MDM2, Bak and Bax) based on their binding mode. Studies were done using Schrödinger Drug Design software.

The ligand, thymoquinone was docked to the prepared proteins with Glide in standard precision mode. Thymoquinone was subjected to ADME profiling using QikProp 3.0 module of Schrodinger Drug Design Suite. The crystal structure of the receptors was derived from protein data bank (PDB) and used as targets for docking. The 3D structure of the ligand was obtained from Pubchem.

The results of phase IV revealed that thymoquinone possessed good absorption and distribution as revealed by ADME studies. The ligand, thymoquinone was docked to the prepared proteins with Glide, and a correlation was calculated by the Glide score. To predict the results, parameters like Glide score, Glide energy, H-bonds and good Van der Waals interactions were used for assessing the binding affinity of ligand towards receptor. The ligand showed efficient docking score with all the apoptotic target proteins involved in the extrinsic pathway of apoptosis and cancer target proteins too. These results suggested that thymoquinone can act as an effective anticancer agent.

To summarize, the results of current study revealed that thymoquinone is fairly non-toxic to non-cancerous cells (both *S. cerevisiae* and HBL-100 breast cell line). It showed considerable cytotoxicity towards breast cancer cells, affecting both ER positive and triple negative cells.

Moreover it rendered protection to HBL-100 cells indicating it is less toxic to untransformed cells which is a sure advantage over standard chemotherapeutic drugs which cannot differentiate cancer and untransformed cells. This was the significant finding of our research. Chemotherapeutic drugs commonly employed for
treatment are receptor specific. Our study showed that thymoquinone was effective against both ER positive and triple negative breast cancer cells lacking any receptor suggesting that it can be employed in developing drugs to target both types of cancers.

In both ER positive MCF-7 cells and Triple negative MDA MB 231, thymoquinone exhibited significant toxicity and its effect being more than standard drug used. This suggests that thymoquinone mediates its action both receptor mediated and receptor independent mechanism too.

In non-cancerous breast cells, the extent of toxicity was less compared to cancerous cells. Thus, this effect can be exploited to develop drugs using thymoquinone to treat cancer both alone and in combination with standard chemotherapeutic drugs. As thymoquinone is a phytocompound the major drawback of cancer therapy namely chemo resistance exhibited towards standard drugs can be overcome.

**Suggestions for future research**

1. The anticancer activity of thymoquinone can be tested using other types of breast cancer cell lines.
2. The anticancer effect of thymoquinone can be assessed *in vivo* using experimental animals.
3. The biomarkers responsible for the differential response can be identified for targeted therapy.
4. The molecular mechanism underlying apoptotic cell death can be further probed.
5. The ability of thymoquinone to influence the expression of other related gene targets can be assessed.