CHAPTER 3

To study the in vitro anti-oxidant, anti-inflammatory and anti-cancer activity of the norabogori extract

3.1 Introduction

The neutralization of free radicals by antioxidants, which are present in some foods, may help to mitigate some of the harm that they can do. The antioxidant nutrients are among these. Numerous epidemiological studies have confirmed the associations between diet and disease. [1]. The health-promoting properties of fruits are due to the presence of some secondary metabolites such as phenols that have aroused substantial attention due to their protective potential against diseases [2]. The pathophysiology of various health issues is thought to include oxidative stress heavily [3]. Antioxidant-rich foods can lower the risk of numerous diseases. Adequate levels of antioxidants in the diet stimulate immune processes and enhance cellular defences in the right ways [4].

To evaluate the antioxidant qualities of food, several methods were devised. These tests vary in terms of radical generation, target molecules, and how the end points are evaluated. Since antioxidants can exert their effects in vivo via a variety of routes, measuring antioxidant activity required a multifactorial strategy based on the application of many analytical techniques. As a result, picking an appropriate assay is crucial, and in practice, many test protocols are used to assess the antioxidant activity of the relevant samples [5,6]. Research on vegetable and fruits sources for novel antioxidants has been encouraged by the increased interest in replacing synthetic antioxidants with natural ones. Antioxidants are frequently required to stop the deterioration of other oxidisable products, such as cosmetics and pharmaceuticals, since oxidation processes are not just a problem for the food business.

Antioxidants assist in preventing inflammatory reactions from occurring when they are not necessary. Consequently, because of their function in the body, antioxidants also exert anti-inflammatory characteristics. Macrophage cells, which act as the first line of defense in infected cells, produce inflammatory mediators and phagocytose infections to prevent their proliferation and spread. Pathogen-associated chemicals, like lipopolysaccharides (LPS), start an inflammatory response when pathogens invade cells. Cytokines such tumor necrosis (TNF-α), interleukin (IL-1), and prostaglandin (PGE2) are crucial in this process because they help to normalize ROS levels [7].

Traditional medicines in diverse cultures are based on medicinal plants, which have been studied for their pharmacological properties. Research has led to the discovery of numerous important new phytochemicals that have the potential to treat and manage diseases [8]. This includes the discovery of paclitaxel (Taxol), an anticancer drug derived from the bark of the Himalayan Yew tree [9], as well as the antineoplastic and anti-diabetic properties of the berberine isoquinoline alkaloid found in plants of the Berberidaceae family [10] and the antimalarial properties of artemisinin [11], to name a few.

*Prunus persica is o*ne of the most popular fruit trees [12]. Because of its bioactive components (phenols and carotenoids), peach fruit is well known for both its nutritional value and medicinal capabilities [4,12-17]. Therefore, efforts have been made to estimate the bioactivity and functionality present in the various peach cultivars in order to assess the potential of peach fruits.

Several studies examined the bioactive components of *P. persica* utilizing various solvents for the extraction process. Since it offers a high recovery of antioxidant chemicals, methanol was among them the most frequently employed substance [4,12-18]. On the other hand, ethanol is less efficient than methanol when it comes to extracting vegetable and fruits phenolic components. However, because ethanol is safe and acceptable for human use, it is crucial to promote the assessment of ethanol extract's potential as a useful source of bioactive chemicals. The pharmaceutical and food sectors could find alternative value-added ingredients with the help of the generated scientific knowledge in order to create new functional intermediates or goods with positive health effects.

Prunus persica L. Batsch fruits plant is found in many regions around the world including India, China, North Africa, South Africa, and Europe. Various parts of this plant have been assessed for their therapeutic effects on in-vitro assay conditions. Some of these examples are its leaves against breast cancer cell line (MDA-MB-231) and cervical cancer cell line (HeLa) [19], seed and kernels extracts for anti-ageing, anti-biofilm properties, protection against oxidative stress, cancer cell proliferation, inhibition of type II diabetes, Alzheimer's disease and obesity [20], prokinetic activity of the flower extract and its possible mechanism of action in rats [21], fruits methanol extract on lipopolysaccharide-stimulated glial cells and effects of root extract on inhibition of the liver cancer cell HepG2 growth by inducing cell cycle arrest and migration suppression [22]. So, the plant from different regions have highly potential therapeutic properties.

The aim of this study is to evaluate the norabogori (*Prunu spersica* L. Batsch) fruit extract of Assam, India for its anti-inflammatory activities and analyze its causing genes. Above that, we have also explored its antioxidant activities and anti-cancer potential on human ovarian cancer cell line.

3.2 Materials And Methods:

3.2.1 Chemicals and Reagents

The cell culture specific chemicals and reagents were purchased from Sigma Aldrich (St Louis, MO, USA). Other required chemicals were procured from Himedia (Mumbai, India), Thermo Scientific, MA, USA, Merck Millipore (MA, USA) and penicillin/streptomycin was brought from Life Technologies (Gibco, USA).

3.2.2 Sample preparation

Norabogori fruits were procured from the nearby area of Tezpur University, Tezpur, Sonitpur, Assam. The fruits were washed properly under tap water and then finally rinse them with distilled water. The fruits were deseeded and cut in thin slices to prepare for drying. The slices were spread on a tray and kept for drying in freeze dryer at -80°C for 12h. After drying the fruit slices were powdered and kept in an airtight container at-20℃ until further investigation.

3.2.3 Purification of Norabogori extract:

Column chromatography was used to obtain polyphenols rich Norabogori fruit extract. Amberlite XAD-7HP resin (75 g) was soaked in ethanol for 10 min. The resin was packed into a 30 × 2 cm glass column [23]. The extract collected was dried and kept at -20℃ for further analyses.

3.2.4 Antioxidant activity:

3.2.4.1 DPPH free radical scavenging activity

The ability of the antioxidant to scavenge the DPPH cation radical is the basis of the Brand-Williams et al. (1995) method, which was used to test the DPPH radical scavenging activity of the Norabogori extract [24]. This procedure doesn't cause oxidative chain reactions or interact with free radical intermediates; instead, it assesses a molecule's ability to donate hydrogen. When DPPH interacts with an antioxidant that has hydrogen donation capabilities, it

is reduced and transforms into a stable diamagnetic molecule. When it accepts an electron or a free radical species, it loses its absorption, which causes a discoloration from purple to yellow. 2 mL of a 0.1 mM DPPH solution in ethanol was added to 4 mL of sample in the concentrations of 50, 100, 150, 200, 250, and 300 g/mL. The concentration variant set was created with the idea that there should be a correlation between the concentrations utilized and the activity displayed under different assay settings. The tubes were incubated for 10 minutes in the dark, the absorbance at 517 nm was measured, and ethanol was used in place of DPPH solution for the blank. As a control, the absorbance of the DPPH solution incubated with BHT was used. [25]. After 30 minutes of incubation in the dark, the % inhibition of the DPPH radical scavenging activity was determined colorimetrically at 517 nm using the formula below.

% DPPH radical scavenging activity = (Abs control−Abs test sample)/Abs control X 100%

3.2.4.2 ABTS Radical Cation Decolorization Assay

Through the interaction of ABTS+ with potassium persulfate, this approach is involved in the creation of the blue/green ABTS+ chromophore. Antioxidant chemicals compete with ABTS+ and reduce color production when present. Similar to the previous method, ABTS solution (7 mM ABTS and 2.6 mM potassium persulphate in a 1:1 ratio, allowed to stand in the dark at room temperature for 16 h before use) and phosphate buffer (100 mM, pH 7.4) were added to 4 mL of sample in a series of concentrations of 50, 100, 150, 200, 250, and 300 g/mL, and the absorbance was measured at 734 nm. BHT was utilized as the control, and the blank contained a buffer and DW [26,27].

3.2.4.3 IC_{50} Value Calculation

The interpolation of the curves displayed for percentage inhibition against the individual concentrations allowed for the determination of the PPEs' concentration needed to bring about 50% of scavenging (IC50). According to the IC50 value, the tested preparation must contain 50% of the inhibitor's total amount in order to demonstrate the desired action. The ideal chemical concentration that must be delivered in order to achieve the desired function must be precisely measured. These figures may be helpful when these polyphenols are processed further for use in creating functional meals.

3.2.5 Cell lines and culture conditions

The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (ATCC, USA) and was maintained in complete RPMI 1640 medium (Himedia, India) supplemented with 10% fetal bovine serum (Life Technologies, Gibco, USA) and penicillin/streptomycin (100 units/mL) (Life Technologies, Gibco, USA). Trizol and c-DNA conversion kit was obtained from Thermo. Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma Aldrich, USA.

3.2.6 Cytotoxicity assay

The in vitro cytotoxicity of Norabogori extract was assessed in this experiment. This was accomplished using a THP-1 cell line and the MTT assay (3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) [28]. MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) must be converted by living cells into formazan crystals in order to detect cell growth. The enzyme mitochondrial succinate dehydrogenase converts MTT to formazan, which can be used to determine whether a cell is alive or dead. A slightly modified version of the protocol supplied by Mosmann et al. was used for the experiment. [29]. Around 5 x 10^3 THP-1 monocytes cells were seeded in 96 well plate and differentiated to macrophages with 5 ng/mL PMA for 48 h. Cells were allowed to rest for 24 h in complete RPMI medium and treated with different concentration of extract for 24 h for testing cell viability using MTT assay. The media was removed carefully and MTT dissolving solution was added and absorbance was taken at 590 nm wavelength using UV-Vis spectrophotometer (Multiscan Go, ThermoScientific). [30]

3.2.7 Effect of Norabogori extract on pro-inflammatory marker:

3.2.7.1 Cell Treatments

After finding the IC50 value, compounds were further tested for their anti- inflammatory activity. Around $0.5 \times 10^6 - 0.75 \times 10^6$ THP-1 cells/well in 6-well plate mm dish were differentiated to macrophages with 5 ng/mL PMA for 48 h. Cells were allowed to rest for 24 h in complete RPMI medium and pre-treated with different concentration of extract for 4h followed by induction with lipopolyssachride b4 (LPS) for 2 h. All the treatments were done in 1% FBS-containing RPMI medium. Gene expression study was done for pro-Inflammatory genes like TNF-alpha, IL-1 beta, Cox-2 to study the anti-inflammatory action of the compounds [31].

3.2.7.2 RNA isolation and Semi Quantitative-PCR

Total RNA was isolated and quantified from the cells using Trizol reagent as per the manufacturer's protocol. RNA was converted to c-DNA using verso c-DNA kit (Thermo Scientific, USA). Gene-specific primers for various inflammatory genes were used to study mRNA expressions. The genes and their primer sequences are as follows: COX-2 (78 bp) forward 5′-GTTCCAGACAAGCAGGCTAATA-3′, reverse 5′ -CCACTCAAGTGTTGCACATAATC-3′; IL-1 beta (125 bp) forward 5′-GGTGTTCTCCATGTCCTTTGTA-3′, reverse 5′GCTGTAGAGTGGGCTTATCATC- 3′; TNF-alpha (106 bp) forward 5′-CCAGGGACCTCTCTCTAATCA-3′, reverse 5′-TCAGCTTGAGGGTTTGCTAC-3′, and Beta Actin (429 bp) forward 5′- CTCGCCTTTGCCGATCC -3′, reverse 5′-GAAGGTCTCAAACATGATCTGG -3′. Resulting PCR products obtained was resolved on a 1.5% agarose gel, and the intensity of the bands was measured using NIH Image J Software. [32]

3.2.8 Anti-cancer activity of Norabogori extract by MTT assay:

With the help of an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric experiment, the anti-cancer efficacy of Norabogori extract was evaluated. $1x10^4$ cells/well in 100 µl of RPMI-1640 supplemented with 10% (v/v) FBS were seeded onto 96-well plates. The cells were treated with serum-free RPMI-1640 that contained varied doses of norabogori extract after 24 hours of incubation at 37°. A further 24 hours of incubation were followed by the addition of 50 l of MTT (5 mg/ml in PBS) to each well. The cells were cultured for two hours at 37 °C. After the media was removed, the cells were exposed to 100 l of dimethyl sulfoxide (DMSO) for 5 min. After that, the optical density was assessed at 550 nm using a microplate reader. The fraction of viable cells was used to determine cell viability. [33]

3.2.9 Statistical Analysis

Calculation of mean values, standard deviation, and plotting of curves were expressed as average of three replicates. Statistical significance was calculated by p value and a p value of 0.05 was considered as statistically significant for all analysis. Statistical significance was set at 95% confidence.

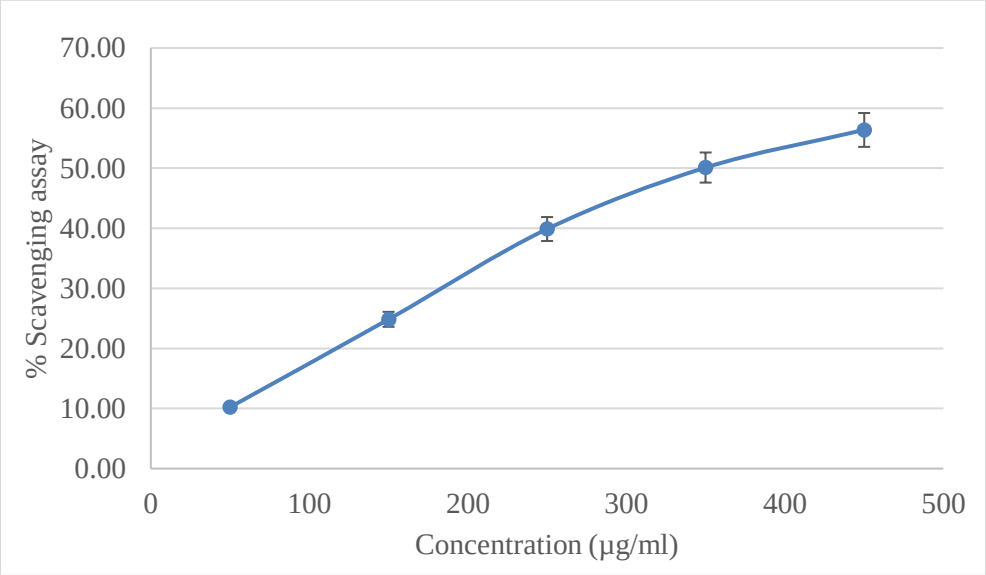
3.3 Results:

3.3.1 Antioxidant activity of norabogori fruit extract:

3.3.1.1 DPPH free radical scavenging activity

An indicator of antioxidant activity in a plant or food product is the measurement of DPPH radical scavenging activity. The amount of antioxidants present has a significant impact on the effectiveness of DPPH radical scavenging activity. Antioxidant activity increases with antioxidant concentration. DPPH free radical scavenging activities of the norabogori fruit extract and BHT standard are shown in Fig.3.1 The norabogori fruit extract was showing highest activity of 50.31% at 300 µg/mL and was close to the reference BHT standard 56.37%. The IC_{50} value of the fruit extract and BHT are 288.52µg/mL and 366.7 µg/mL respectively which are well within the concentrations taken for analysis.

Loizzo et al. (2015), Dhingra et al., and Christabel et al., also reported the anti-oxidant activity evaluation by DPPH assay [34-36]. Loizzo et al. worked on different parts of the peach plant like pulp, peel and seed ethanolic extracts and Dhingra et al., and Christabel et al. worked on different fraction of peach leaves. They revealed the values to be in the similar trend with our findings.



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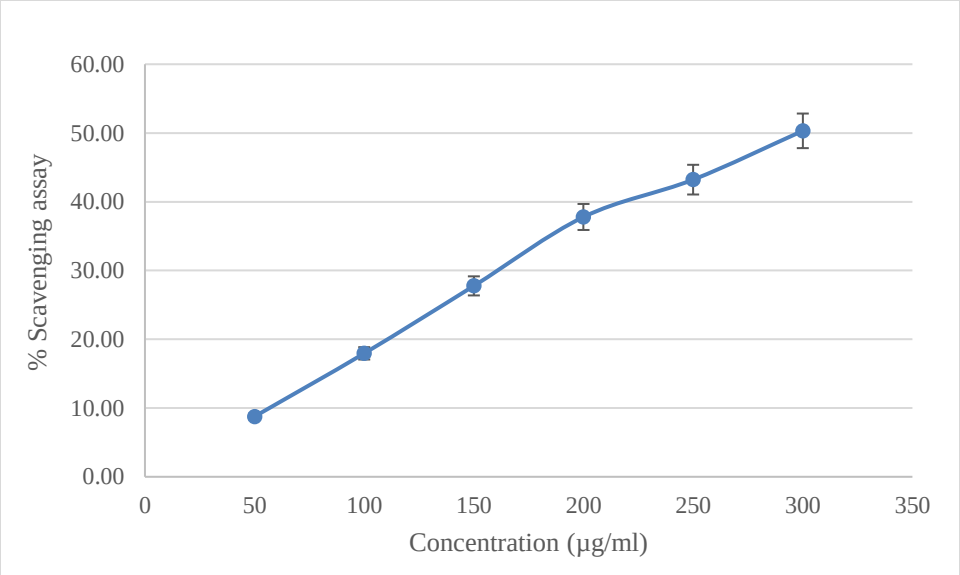

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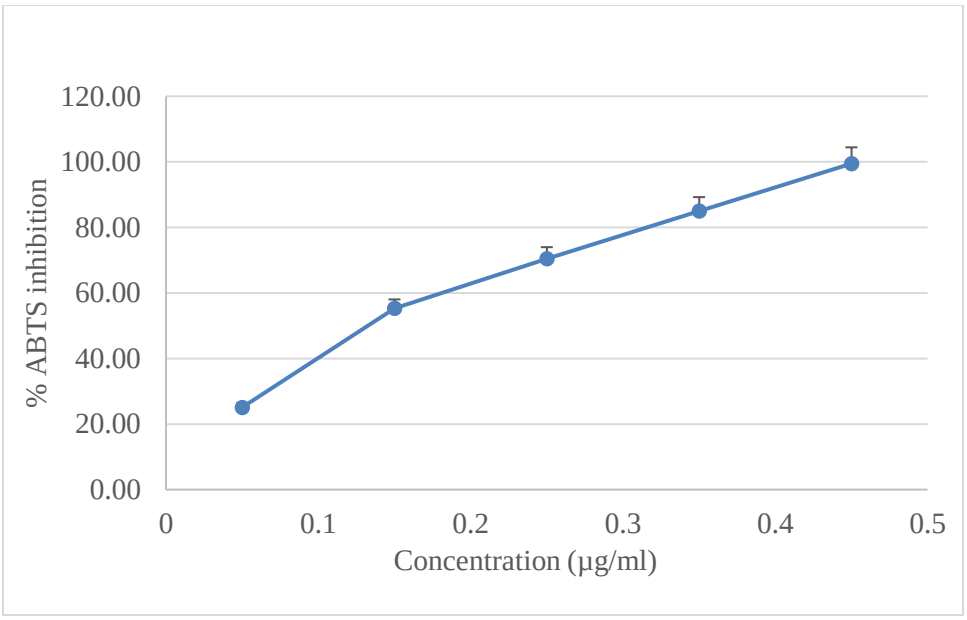
Fig.3.1 DPPH free radical scavenging activity assay of a) BHT standard, b) Norabogori fruit extract

3.3.1.2 ABTS Scavenging Activity

ABTS radical decolorization activities of the norabogori extract and the BHT standard against the respective concentrations are presented in Fig.3.2. The results indicate that ABTS scavenging activity of the sample under study increased in a dose-dependent manner. At the concentration of 250 µg/mL, the norabogori extract is showing highest activity of 99% among the concentrations taken for analysis and it was close to the BHT standard with 99.46%. The IC_{50} value of the fruit extract and BHT are 7.455µg/mLand 8.545µg/mL respectively which are well within the concentrations taken for analysis.



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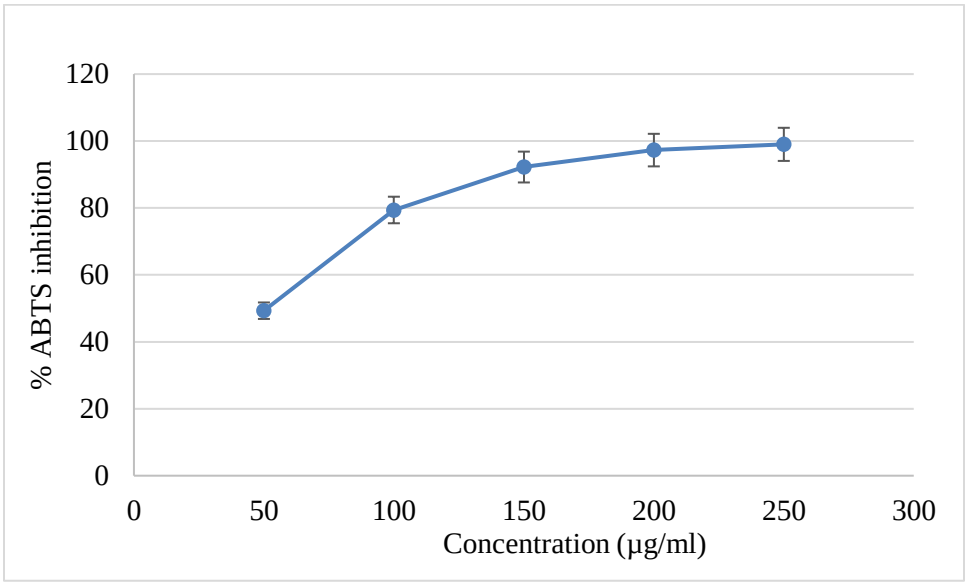

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Fig.3.2 ABTS radical cation decolorization assay of a) BHT standard, b) Norabogori fruit extract

3.3.2 Norabogori extract regulates expression of proinflammatory genes:

3.3.2.1 Cell viability assay

In order to determine whether extract from norabogori fruit could induce expression of inflammatory genes, THP-1 differentiated macrophages were treated with the extract. Treatment

with 25, 50, 100 and 125 µg/ml extract induced cell viability by 98.54%, 89.89%, 91.77% and 82.28% in 48 hr incubation, respectively. The results of MTT assay demonstrated that norabogori fruit extract was non-cytotoxic to THP-1 cells upto125 µg/ml as evidenced by 82.28% cell viability (Fig.3.3).

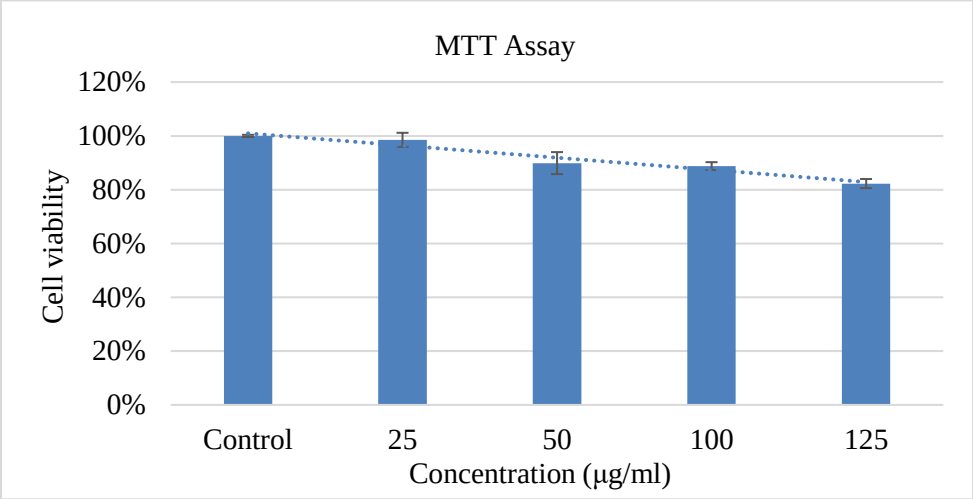
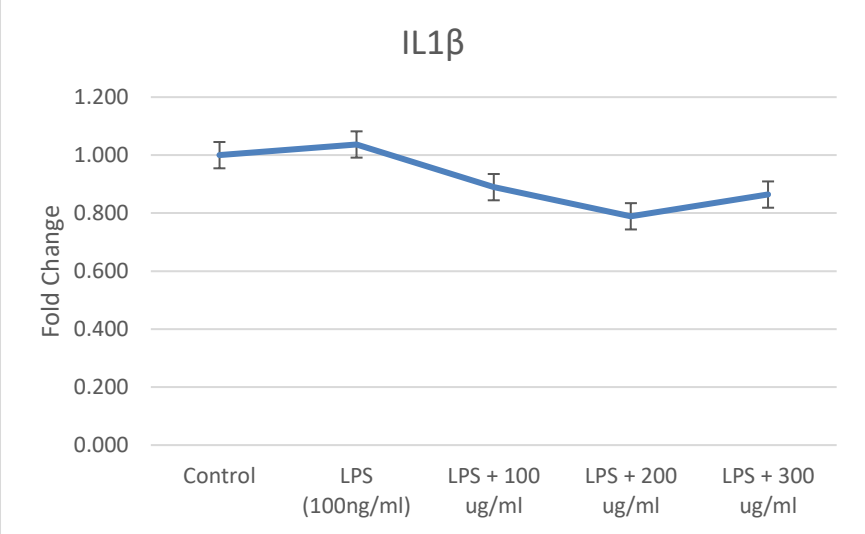


Fig.3.3 Cytotoxicity study of norabogori extract by MTT analysis

3.3.2.2 Effect of norabogori extract on pro-inflammatory genes

In the current study, the anti-inflammatory activity of norabogori fruit extract inlipopolysaccharides (LPS)-stimulated macrophages, a standard method for testing anti-inflammatory activity of drugs was performed. The human THP-1 cell line and THP- 1 derived macrophages are considered as effective cellular models for evaluation of anti-inflammatory drugs. In this study, THP-1 cells without any external stimuli was taken as control and100ng/ml LPS-stimulated THP-1 cells and norabogori extract treated LPS-stimulated THP-1 cells were compared against control. Three different doses (100, 200 and 300 µg/ml) of norabogori extract were used for treatment with cells. Inhibition with the extract demonstrated a significant decrease in the production of pro-inflammatory mediators in macrophages in concentration dependent manner (Fig. 3.4). Treatment with 100 and 200µg/ml of the extract resulted in gradual decrease to 80% fold change in IL1β expression. From 200 to 300 µg/ml, the fold change increased by a very small amount. In case of COX 2 and TNF-α, upon LPS-stimulation, the expression jumped to increase 2 folds and 3 folds respectively. Stimulation of THP-1 differentiated cells with 100 and 200 µg/ml of norabogori extract, significantly decreased the mRNA expressions of COX 2 and

TNF-α compared to control and LPS (Fig. 3.4). It shows that the phytochemical compounds present in norabogori extract successfully suppressed pro-inflammatory cytokine genes expression in lipopolysaccharides (LPS)-stimulated macrophages in human THP-1 cell line. But, in both cases, on going up in concentration of extract from 200 to 300 µg/ml, the expression levels of COX 2 and TNF-α genes increases significantly. This consistent occurrence in all three gene expressions might be because of slight toxicity at higher doses or concentration of the fruit extract [31]. In the preceding chapter [37], the presence of many phytochemical compound in the norabogori extract was explained. Among them, compounds like rutin, kaempferol, ellagic acid etc. are studied for their anti-inflammatory properties [37-41]. Combination of these phytochemicals like present in norabogori fruit may prove to be more effective than individual activity. In a previous study done by Seo et al., they studied the TNF-α, IL-1β and IL-6 mRNA expression levels in BV-2 microglial cell stimulated by the extract of *Prunus persica* aerial parts (leaves, fruits and twigs) [42]. They concluded that the extract exerts anti-inflammatory property and could suppress the expression of that the anti-inflammatory markers which was due to the presence of phenolics and flavonols.



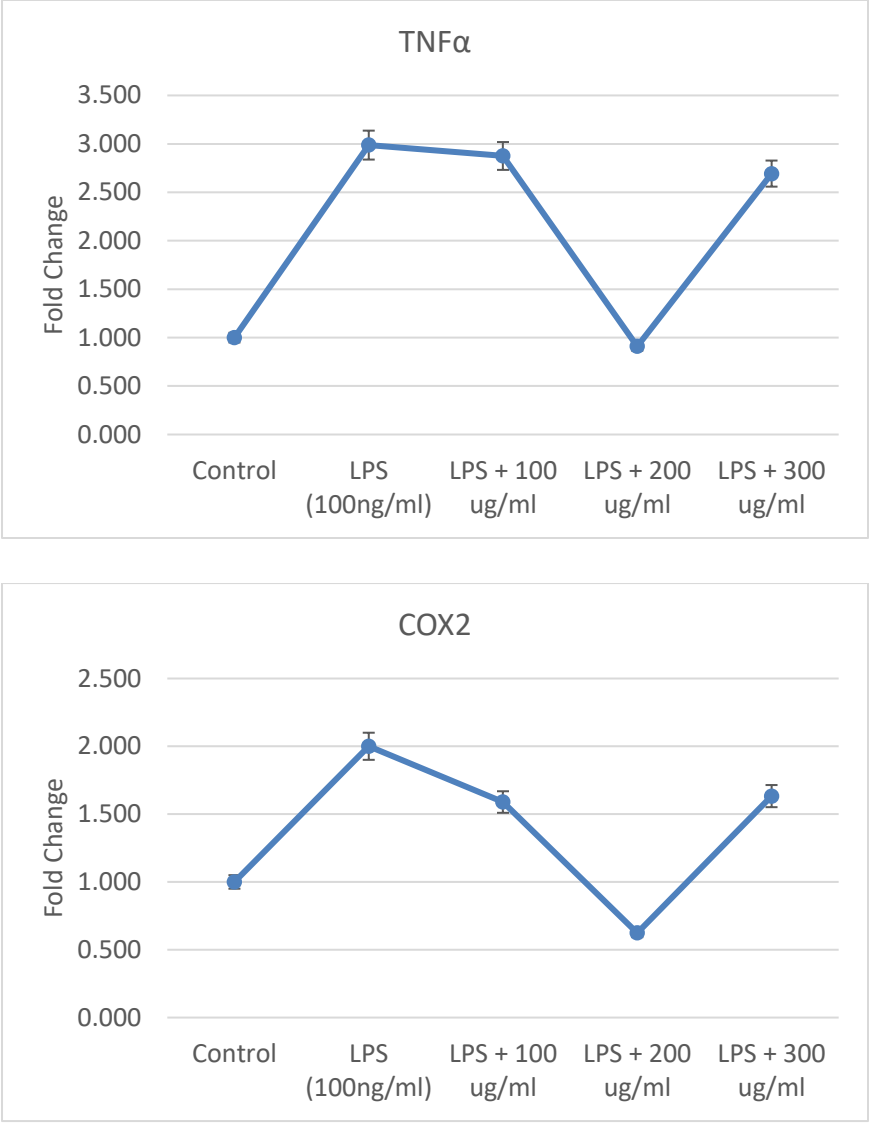


Fig.3.4 Effect of norabogori extract on pro-inflammatory genes

3.3.3 Anti-cancer activity of Norabogori extract by MTT assay

The most frequent gynecological malignancy that is deadly is ovarian carcinoma. Each year, ovarian cancer affects 230,000 women worldwide, and 150,000 of them pass away. As a result, it is urgently necessary to enhance the prognosis and treatment of ovarian cancer patients. [43]. As a result, it is urgently necessary to enhance the prognosis and treatment of ovarian cancer patients. To investigate the anti-cancer activity of norabogori fruit extract in EOC cells, we examined the effect of the extract on cell viability in the ovarian cancer cell line, SK-OV-3. The cells were treated with different concentrations (0, 50, 100 and 200 µg/ml) of norabogori extract

and the cell viability was estimated by using the MTT assay. The viability percentage came out to be drastically dropping from 0 to 50 µg/ml of the extract concentration. It might be meaning a highly effective anti-cancer activity exerted by norabogori fruit extract. Also, the other concentration variances showed that the norabogori extract could reduce cell viability and thus inhibited SK-OV-3 ovarian cancer cell proliferation in a dose-dependent manner, as shown in Fig.3.5. The IC50 value of the extract came out to be 2.06 µg/ml. A no. of previous study demonstrated the anticancer activity of different parts of peach on different cancer cell-lines.Cassiem and kock worked with peach kernel on human colon cancer [44]. Noratto and collaborators used peach fruit extract on xenograft models in female mice to show inhibition of tumor growth [37], Vizzotto and colleagues used peach extract to show its effect on two cancerous breast cell-lines [38]. All these studies proved positive results for peach to inhibit cancerous growth in different cell-lines. Cyaniding-3-glucoside, flavonoids, anthocyanins, hydroxyciinnamic acid etc. are known to be anti-cancerous from a large no. of studies with various samples in different concentrations. Compounds like chlorogenic acid, rutin, catechin, gallic acid etc. present in norabogori fruit has shown effective results in these experiment [45-47].

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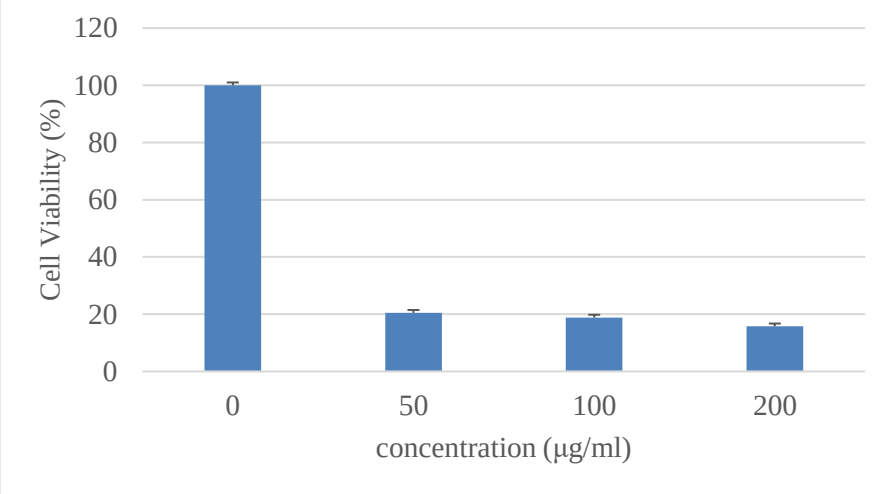

Fig.3.5 Anti-cancer activity of norabogori fruit extract

3.4 Conclusions

The antioxidant capacity of fruits varies in relation to antioxidant moieties present in the different species, although variations can occur among cultivars within a single species [48-50]. A concentration-effect relationship was found for the norabogori fruit in both ABTS and DPPH assay. The therapeutic activities like anti-oxidant, anti-inflammatory and anti-cancer activity of the fruit are the attributes for its phytochemical constituents. The presence of kaempferol, rutin, gallic acid, coumaric acid, catechin like polyphenols, flavonoids etc. phytoconstituents are the primary contributors.This aspect of the study with respect to the anti-inflammatory activity will be further discussed in next chapter.

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