CHAPTER 11

INVITRO ANTI-OXIDANT ACTIVITY

11.1 INTRODUCTION

In living systems, free radicals are generated as part of the body's normal metabolic process and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxidase activity, atmospheric pollutants and from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage [212].

Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders. Oxygen free radical can initiate per oxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long-term complication of diabetes [213-215]. Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias. Flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties [216-221].

Plants are potent biochemical factories and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds,etc i.e. any part of the plant may contain active components.The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to particular plant species or groups are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct [222]. Antioxidant based drugs or formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last three decades [223,224].This has attracted a great deal of research interest in natural antioxidants.

Subsequently, a worldwide trend towards the use of natural phytochemicals present in berry crops, tea, herbs, oilseeds, beans, fruits and vegetables have increased. Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chilli, pepper, ginger, and several Chinese medicinal plants extracts. The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins and isocatechins.In addition to the above compounds found in natural foods, vitamins C and E, beta carotene and tocopherol are also known to possess antioxidant potential [225].With this background and abundant source of unique active components harboured in plants, the present evaluation was taken up on the two compounds isolated from medicinal plant namely *Limonia acidissima*.

11.2 MATERIALS AND METHODS

Antioxidant Assay

The antioxidant activity of the compound was determined by *in vitro* method - the DPPH free radical scavenging assay. The assay was carried out in triplicate and average values were considered.

Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), Ascorbic acid, Folin-Ciocalteau reagent, Potassium ferricyanide, Ferric chloride ($FeCl_3$), Sodium bicarbonate, Trichloroacetic acid (TCA), Sodium carbonate, Hydrogen peroxide, Sodium nitroprusside, Phosphoric-acid, Napthylethylenediamine-dihydrochloride, Tri bromo acetic acid(TBA).

All chemicals used including solvents were of analytical grade.

DPPH Radical Scavenging Activity

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources [226]. There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants [227,228]

Principle

In order to measure antioxidant activity, DPPH free radical scavenging assay was used. This assay measures the free radical scavenging capacity of the investigated compounds. DPPH is a molecule containing a

stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple colour which is typical for free DPPH radical decays and the change in absorbance at 517 nm was followed spectrophotometrically. This test could provide information on the ability of a compound to donate a hydrogen atom, on the number of electrons a given molecule can donate and on the mechanism of antioxidant action.

The free radical scavenging capacity of the compounds (I and II) isolated from MELA was determined using DPPH. DPPH solution (0.004% w/v) was prepared in methanol. Compound I and II was mixed with methanol to prepare the stock solution (10 mg/100mL) individually. From stock solution 2ml, 4ml, 6ml, 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent, the final volume was made of each test tube up to 10 ml whose concentration was then 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml respectively. Freshly prepared DPPH solution (0.004% w/v) in methanol was added in each of these test tubes containing compounds I and II (20 µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml) and after 10 min, the absorbance was taken at 517 nm using a Lambda 25 UV/VIS (Perkin Elmer, USA) Spectrophotometer. The procedure was repeated thrice to get triplicate values.

Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10 mg/100ml or 100µg/ml) of the compounds. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank reagent.

Percentage (%) scavenging of the DPPH free radical was measured using the following equation(11.1)

$$\% \text{ scavenging} = \frac{\text{Absorbance of control - Absorbance of test Sample}}{\text{(Absorbance of control)}} \text{X} 100 \quad (11.1)$$

IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical.

11.3 *INVITRO* CYTOTOXIC ACTIVITY

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A rich heritage of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharva Veda, Charaka, and Sushruta etc. Over 50% of all modern clinical drugs are of natural product origin [229] and natural products play an important role in drug development programs in the pharmaceutical industry [230]. Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. In the continuation of this strategy of new drug discovery we have studied the compounds isolated from the fruit pulp of methanolic extract of *Limonia acidissima* for their cytotoxic activity.

11.3.1 Cytotoxicity Screening

Cell line used

A *HeLa* cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who

died from her cancer in 1951. Initially, the cell line was said to be named after a "Helen Lane" in order to preserve Lacks's anonymity.

11.3.2 Micro culture Tetrazolium (MTT) Assay

The MTT Cell Proliferation and Viability Assay is a safe, sensitive, in vitro assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability.

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria.The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. The MTT assay was tested for its validity in various cell lines [231]. More recent evidence suggests that reduction of MTT can also be mediated by NADH or NADPH within the cells and out of mitochondria [232].Further modification of the initial protocol described by Mossmann (1983) were proposed [233,234] in order to improve the repeatability and the sensitivity of the assay.

Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically.Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Cells are cultured in flat-bottomed, 96-well tissue culture plates. The cells are treated as per experimental design and incubation times are optimized for each cell type and system. The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is added to the wells and the cells are incubated. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. Detergent is then added to the wells, solubilising the crystals so the absorbance can be read using a spectrophotometer. Samples are read directly in the wells. The optimal wavelength for absorbance is 570 nm, but any filter that absorbs between 550 and 600 nm may be used. The data is analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakh cells/ml using medium containing 10% new born calf serum. To eachwell of 96 well micro titre plates, 0.1ml of diluted cell suspension was added.After 24 h, when the monolayer formed the supernatant was flicked off and 100 µl of different test compounds were added to the cells in micro titre plates and kept for incubation at 37ºC in 5 % CO_2 incubator for 72 h and cells were periodically checked for granularity, shrinkage, swelling. After 72 h, the sample solution in wells was flicked off and 50µl of MTT dyewas added to each well. The plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 570 nm.

The percentage growth inhibition was calculated using the formula below (11.2):

$$\% \text{ Cell inhibition} = 100 - \left\{ \frac{(\text{Absorbance of test} - \text{Absorbance of blank})}{(\text{Absorbance of control} - \text{Absorbance of blank})} \right\} X\,100 \quad (11.2)$$

Data Interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death,evidence of cell death may be inferred from morphological changes.

$$\% \text{ Cell survival} = \left\{ \frac{(\text{Absorbance of test} - \text{Absorbance of blank})}{(\text{Absorbance of control} - \text{Absorbance of blank})} \right\} X\,100 \quad (11.3)$$

$$\% \text{ cell inhibition} = 100 - \text{cell survival} \quad (11.4)$$

11.5 RESULT

***Invitro* Antioxidant Activity**

In the present study, both the compounds isolated from the Methanolic extract of *Limonia acidissima* showed a high effective free radical scavenging in the DPPH assay. These compounds exhibited a remarkable antioxidant effect at low concentrations. As the compounds from methanolic extract of *L.acidissima* exhibited at 10 µg ml^{-1} an extraordinary antioxidant effect (43% and 74% successively) whereas the ascorbic acid showed at this concentration an antioxidant effect of 45%. Out of the two compounds

isolated the compound-II started to exhibit a high effective free radical scavenging at 60 µg ml^{-1} (72, 57 and 68%, respectively). The free radical scavenging effect ranged between 10 and 38% at the highest concentration namely 100µg ml^{-1} (Table 11.1).

Table 11.1 Invitro antioxidant activity-DPPH method

Concentration (µg/ml)	% inhibition		
	Compound 1	Compound 2	Ascorbic Acid
20	32.5 ± 5.32	27.22 ± 2.15	35.8± 3.2
40	43.67 ± 4.85	34.57 ± 2.80	51.6±2.64
60	72.35 ± 2.57	57.97 ± 3.05	68.41±2.7
80	90.75 ± 4.26	68.75 ± 3.60	82.64±2.85
100	95.2 ± 2.86	83.56 ± 4.50	94.3±1.32

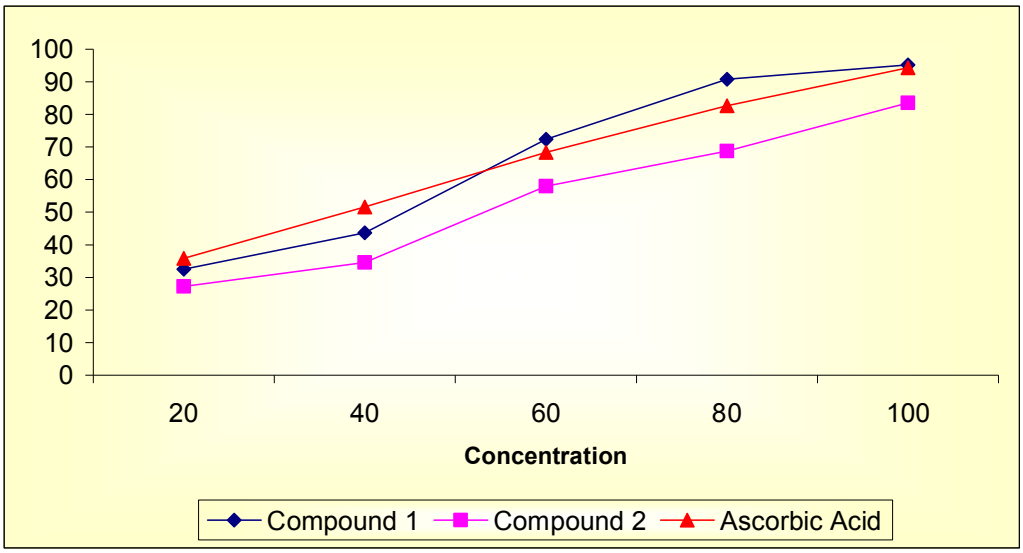

Figure 11.1 *Invitro* antioxidant activity-DPPH method

Table 11.2 Percentage cell viability and characterization of cell line

Cell line	% Viability	Live cell count	Total cell count	pH
HeLa	70-72%	1.728 X 10^5	2.40 X 10^5	6.9

Table 11.3 *Invitro* Cytotoxocity -MTT Assay

Cell line	Compound 1 (µg/ml)			Compound 2 (µg/ml)			Doxorubicin (µg/ml)		
	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50
HeLa	22.9	57.8	>100	32.7	73.2	>100	40.09	90.79	>100

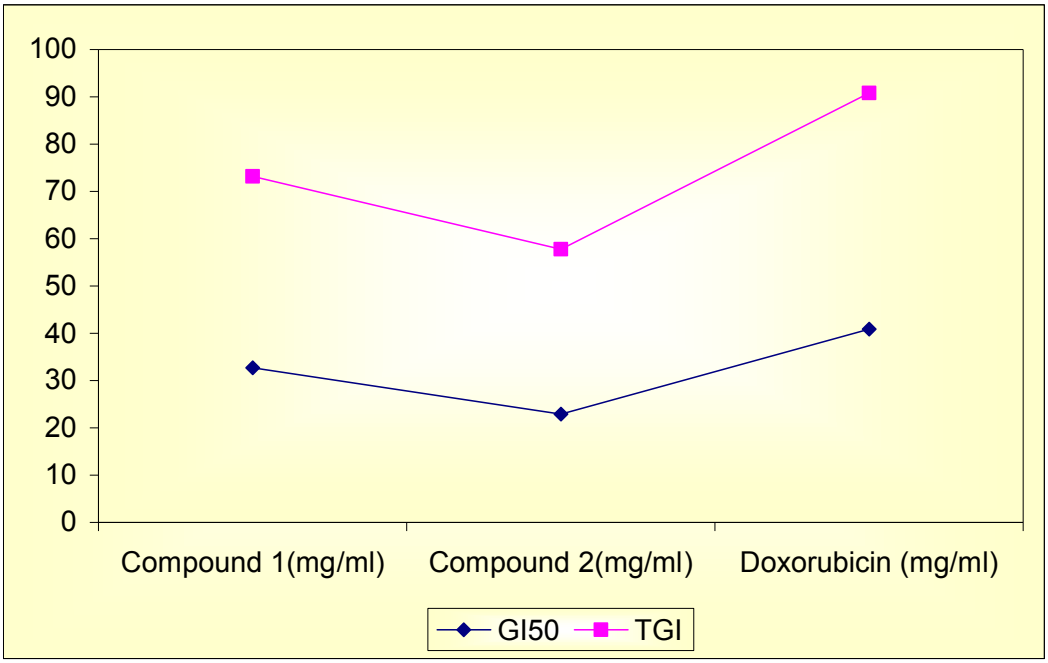

Figure 11.2 *Invitro* Cytotoxocity - MTT Assay

Invitro Cytotoxicity

Invitro confirmation of the toxicity on *HeLa* cell line

Percentage of viable cells can be obtained by performing trypan blue dye exclusion technique. The cytotoxicity activity was carried out by MTT assay.

Viability and characterization of cell lines

Cell line derived from NCCS, Pune were free from bacterial and fungal contamination.Percentage cell viability of cell lines were carried out by using Trypan blue dye exclusion technique. From the Table 11.2 it showed the percentage viability of *HeLa* cell line is 70-72 which is most suitable to perform cytoxicity studies.

The effect of compound-I and II on the growth of HeLa cells were tested under *in vitro* conditions using different concentrations (5, 15, 25 and 50 µg/ml) and the cell survival after 48 hours was given in the Table 11.3. The IC_{50} value was calculated to be 32.7 µg/ml for compound-I and 22.9 µg/ml for Compound-II. Compound-II (50 µg/ml) exhibited significant cytotoxic activity (73%) on HeLa cells, whereas compound-I exhibited only 53% cytotoxic activity. The inhibition was found to be due to its triterpenoid concentration and time dependent with greater inhibition at highest concentration at 48 hours.The cytotoxic activity was compared with the effect of Doxorubicin and it was found to be equivalent to that of compound-I. These compounds were not toxic to the growth of normal cells tested under *in vitro* conditions.

11.6 DISCUSSION

Many human diseases are accompanied by activation of free radicals, considered a mechanism of biological membrane destruction. Free radicals induction occurs during inflammatory processes, cardiovascular problems, cancer, allergic reactions, among other diseases. The endogenous antioxidant system protects cells and tissues against free radicals. However, these endogenous antioxidants cannot totally prevent the development of oxidative stress. Therefore, it is of interest to search for some exogenous substances with antioxidant properties that might be used as prophylactics or therapy of free radicals activated diseases [235].

Polysaccharides and their conjugates were used for a long time in the food and medicine industries and have attracted much attention due to their biological activities. It has been reported that polysaccharides in general have strong antioxidant activities and can be explored as novel potential antioxidants [236]. Due to their antioxidant activity, polysaccharides extracted from fungal, bacterial and plant sources have been proposed as therapeutic agents [237]. It has been recently reported that pectins and pectic acids possess antioxidant activity, which has been related to the reduction power and free radical scavenging activity of these molecules [238,239].

The antiradical performance of Compound-1 and II with respect to DPPH radicals was measured. DPPH radicals have been widely used as a model system to study the scavenging activity of different natural compounds. The colour of the system change from purple to yellow when the absorbance at 517 nm decreases as a result of the formation of DPPH-H through donation of hydrogen by antioxidants [240].

The scavenging activity of Compound-1 and II against DPPH was equal to that reported for Ascorbic acid (60%) under the same concentration. It has been suggested that ascorbic acid interacts directly with oxidants and free radicals [241]. The higher range of antioxidant activity in Compound- II could be related to the galacturonic acid moiety. It has been reported that a relatively low molecular weight and a high uronic acid content in polysaccharides appeared to increase the antioxidant activity.

Another fact is that, the effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. Though the DPPH radical scavenging abilities of the extracts were less than those of Ascorbic acid at 100 µg/ ml, the study showed that the compound-II have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Previous literature reviews about *Limonia acidissima* also confirm the presence of terpenoids group. Many naturally occurring triterpinoids exhibited a good anti-inflammatory activity have been isolated from various plants [242,243].Tricyclic triterpenoids have a wide spectrum of biological activities and some of them may be useful in medicine. There is growing interest in natural triterpenoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities, they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, antiinflammatory, anti-cancer and antiallergic [244].These qualities would have attributed directly to the antioxidant activity of the compound I by DPPH method.

Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. In many instances, the actual compound isolated from the plant may not serve as the drug, but leads to the development of potential novel agents. The ability to attach agents to carrier

molecules directed to specific tumors holds promise for the effective targeting of highly cytotoxic natural products to the tumors while avoiding their toxic side effects on normal healthy tissues.

Plant derived polysaccharides and triterpenoids have been already reported to possess a wide range of biological activities, which includes the antioxidant, anticancer or cytotoxic activity. Several reports are available on the anticancer activity of triterpenoids isolated from other plants. Based on the results of this study, it can be concluded that the compounds isolated from MELA like Digalacturonic acid and Tetranortriterpene has a significant cytotoxic effect on HeLa cells.

11.7 CONCLUSION

It was concluded that compounds from methanolic extract of *Limonia acidissima*, contains polysaccharides and triterpenoids in enormous amount which exhibits high antioxidant thus free radical scavenging and cytotoxic activities. These *in vitro* assays indicate that both the compounds are a significant source of natural antioxidant, than its cytotoxic effect which might be helpful in preventing the progression of various oxidative stresses.