DISCUSSION

Medicinal plants have contributed a lot to the welfare of human beings because of their potential in the treatment of various diseases like immunological disorders, microbial infection and cancer (Newman and Cragg, 2007). In the present scenario, the interest to unlock the secrets of ancient herbal remedies has been enhanced (Izzo and Ernst, 2009). The herbs known as “Botanical medicines”, used for the treatment of cancer are increasingly used because of the unwanted side effects of synthetic drugs and their toxicity. These provide an abundant pharmacopoeia of products that have been prescribed for many diseases over many centuries. In recent times, the natural plant products mainly the traditional and indigenous medicines have gained increased scientific attention as they serve as therapeutic agents and an essential raw material for the makeup of traditional and modern medicines (FAO, 2004; Vickers, 2002; Han et al., 2002; El Shemy et al., 2007). Many studies have led to the discovery and development of new active ingredients from natural sources. The variety of plants contain different secondary metabolites with high biological value which can be used for the treatment of diverse diseases including cancer.

The plant derived antioxidants are used in nutritional or pharmaceutical fields for the prevention of free radical related diseases (Dai et al., 2009; Thambiraj et al., 2012). Plant extracts and their bioactive compounds like flavonoids, phenolic acid and tannins possess many activities viz. antiinflammatory, anticarcinogenic, antiatherosclerotic that is due to their antioxidant potential (Chung et al., 1998; Wong et al., 2006). The antioxidants play an assure activity in cancer therapy because of their palliative action and minimum painful side effects (Kennedy and Bruninga, 2001). Flavonoids and flavonols have important role in stabilizing the lipid oxidation that is associated directly with antioxidant activity as they are highly effective electron donors (Yen et al., 1993; Duh et al., 1999). Polyphenolic compounds have an inhibitory effect on mutagenesis and carcinogenesis in humans when they are ingested daily in diet rich in fruits and vegetables (Tanaka et al., 1998). It has been found that different polyphenolic compounds exhibit different functional properties as quercetin, rutin and catechin can scavenge the reactive oxygen species, $p$-coumaric acid inhibits the
generation of free radicals, chain breaking activity and also metal chelation (Liu et al., 2008; Van Acker et al., 1998; Laranjinha et al., 1995).

Over the last thirty years, the rapid progress made in cell and molecular biology played a significant role in understanding the functioning of cancer cells in order to develop more specific treatments. It has become possible to target more precisely the processes involved in the development, proliferation and survival of cancer cells. A better understanding of the characteristics of tumor cells has recently led to the development of more targeted treatments which are less toxic and include conventional cytotoxic molecules targeting non-specific molecules expressed on the surface of cancer cells and at less degree in normal proliferating cells (DNA, enzymes, microtubules) or of molecules directed against targets specific to cancer cells (oncogenes). The cytotoxicity in cancer cells is induced by impairing the cell cycle at certain stage and also by targeting molecular factors responsible for causing apoptosis. The antitumor effect of certain metabolites is observed in their ability to inhibit abnormally expressed growth factors like protein tyrosine kinase and thus inhibit the growth of cancer cells.

Epidemiological studies proved that the high intake of plant derived foods lead to lower rate of various cancers. The monophenolic and polyphenolic compounds from wide variety of plants that includes foods, spices and beverages have been shown to restrain the initiation, progression and spread of cancer in cells both in vitro and in vivo. The cellular process is modulated by phenolics to elicit the anticancer effects are comprehensive and include regulation of growth factor-receptor interactions and cell signaling cascades that include kinases and transcription factors. These determine the expression of genes involved in the cell cycle arrest, cell survival and apoptosis. A major focus has been the inhibitory effects of phenolics on the stress activated NF-κB and AP-1 signal cascades in cancer cells which are considered as major therapeutic targets. Phenolics can augment the body’s immune system to identify and demolish cancer cells as well as inhibiting the development of new blood vessels (angiogenesis) that is necessary for tumour development. They also attenuate adhesiveness and invasiveness of cancer cells thereby reducing their metastatic potential (Wahle et al., 2009).
The plant metabolites possessing different mechanisms of action exhibit targeted activities against the cancer cells. The anticancer metabolites are tested based on prior toxicological investigations to develop them as anticancer drugs and they can be subjected to clinical trials to account for their safety and effectiveness (Raina et al., 2014). Approximately, 25% of drugs in the world originate from plants and nearly 3000 species have been screened for their anticancer properties. Some plant extracts are cytotoxic in nature because of their ability to induce apoptosis in cancer cells and some of these plants are Andrographis paniculata, Centella asiatica, Newbouldia laevis, Nigella sativa, Panax ginseng, Plumbago zeylanica, Solanum incanum and Vismia laurentii (Rates, 2001; Graham et al., 2000). Hence, more attention is being paid to seek out for new anticancer agents from natural products.

Keeping above in mind, the present study is an effort to explore the antioxidative and anticancer potential of extract/fractions of A. nilotica leaves collected in summer and winter seasons. The leaf extract/fractions (summer and winter) were evaluated qualitatively as well as quantitatively. The leaf extract/fractions were evaluated qualitatively by different tests for the presence or absence of different classes of compounds. The quantitative analysis was done for total phenolic, total flavonoid as well as ultra high pressure liquid chromatographic analysis. The antioxidative activities were assessed by hydrogen and electron donating assays as well as radical scavenging assays viz. molybdate ion reduction assay, CUPRAC assay, ABTS radical cation decolorization assay, β-carotene linoleic acid assay, superoxide anion scavenging assay, lipid peroxidation assay and pBR322 plasmid nicking assay. The in vitro antiproliferative activity was done by using human cancer cell lines of different origin. As the ethyl acetate fraction (summer and winter) was found to be effective, therefore, it was further evaluated for the mechanistic studies using confocal and scanning electron microscopy, cell cycle analysis, generation of reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and colorometric analysis for the measurement of caspase-3 activity.
5.1 Phytochemical analysis of plant extracts

The presence of variety of phytochemical constituents indicate that the plant can be used in a multitude of ways which may be beneficiary to humans (Jayanthi et al., 2011). The natural plant products exhibit one or the other kind of biological activity as they are widely used in the human therapy, veterinary, agriculture, scientific research and in countless other areas (Kandukuri et al., 2009). The usefulness of plant is mainly due to the presence of bioactive constituents viz. alkaloids, tannins, flavonoids and phenolic compounds (Lata and Dubey, 2010). The qualitative analysis of extract/fractions of summer and winter seasons showed the presence of glycosides, phenols, flavonoids, proteins and carbohydrates. The presence of alkaloids was seen in all the extract/fractions except methanol and hexane fractions. The steroids and terpenoids were found to be absent in all the extract/fractions (summer and winter). In living systems, alkaloids play important role in metabolism and development. Flavonoids are known to inhibit the initiation, promotion and progression of tumours (Lata and Dubey, 2010). Both flavonoids and phenolic compounds in plants have been reported to have multiple biological effects including antioxidant, antiinflammatory and anticarcinogenic (Asha et al., 2011).

The phenolic compounds are known antioxidant agents as they act as free radical oxidation terminators (Shahidi and Wanasundara, 1992). They are known to show medicinal activities as well as exhibit various physiological functions (Sofowora, 1993). The total phenolic content in terms of mg GAE/100mg dry weight of extract or fraction of all the extract/fractions (summer and winter) was calculated and the presence of phenolic compounds was seen to occur in varying amounts. Among the different leaves extract/fractions (summer), the ethyl acetate fraction was found to contain maximum mg GAE/100mg viz. 93.30. In case of extract/fractions (winter), again the ethyl acetate fraction was found to have highest phenolic content (94.32 mg GAE/100mg). It is well known that phenolic compounds contribute to the quality as well as to nutritional value of food in terms of modifying color, taste, aroma and flavor. They also play a significant role in plant defense mechanisms to counteract reactive oxygen species in order to endure and prevent molecular damage and the damage caused by microorganisms, insects and herbivores (Vaya et al., 1997).
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The quantitative estimation of flavonoid content showed to have good amount of flavonoid content in terms of mg Rutin Equivalents/100mg dry weight of extract/fraction. The plant flavonoids act as highly effective free radical scavenging agents. They are used for the prevention and cure of many diseases that are related with free radicals (Havesteen, 1983; Deepa et al., 2009). It was seen that ethyl acetate fraction of both the seasons showed highest flavonoid content viz. 61.19 mg RE/100mg (summer) and 83.70 mg RE/100mg (winter). The results indicated the highest flavonoid content in terms of mg RE/100mg in all the extract/fractions of winter season as compared to summer season.

Herbal medicines contain hundreds of diverse phenolic compounds. Chromatography offers an extremely important and useful tool to assess the individual profiles of compounds present in samples. The UHPLC analysis of different extract/fractions (summer) showed that the methanol extract have the highest percentages of umbelliferone and chlorogenic acid. Also, the hexane fraction showed the maximum amount of umbelliferone. Similarly, the chloroform fraction was found to contain highest percentage of umbelliferone followed by chlorogenic acid, catechin and gallic acid. The major amount of polyphenols found in ethyl acetate fraction was viz. gallic acid, catechin, chlorogenic acid, epicatechin, umbelliferone and ellagic acid. While the n-Butanol fraction was found to contain highest percentage of umbelliferone only and the aqueous fraction also showed the presence of number of different polyphenols. Deconinck et al. (2012) studied the development and validation of an ultra high pressure liquid chromatographic method for the characterization of different natural products. In this study, a fully validated UHPLC-DAD method for the identification and quantification of pharmaceutical preparations that contains molecules frequently found in illegal slimming products was developed. The UHPLC method developed was also used for the analysis of polyphenols in the extract/fractions of winter season. The methanol extract was found to contain maximum percentage of umbelliferone, catechin, gallic acid and ellagic acid. Likewise, the hexane and chloroform fractions showed the presence of many polyphenols. The ethyl acetate fraction was found to show maximum percentage of gallic acid, catechin, chlorogenic
acid, epicatechin, umbelliferone and ellagic acid. Many other polyphenols were found to be present in n-Butanol and aqueous fractions respectively.

5.2 **In vitro antioxidant studies**

Reactive oxygen species (ROS) play an important role in numerous pathological conditions as the antioxidants present in plants are known to be of great help in modulating their effect (Agbor et al., 2007; Halliwell 1994). These free radicals have been implicated in over hundred diseases in humans (Bagchi et al., 2000; Nohl et al., 2005; Wang et al., 2007). The inherent defense in human body may not be enough for severe or continued oxidative stress. So, the exogenous antioxidants are constantly required to sustain an adequate level of antioxidants in order to balance the ROS. Medicinal plants are used as a source of phytochemicals to cure various illnesses such as urinary infections, skin infections, blood infections and gastrointestinal disorders (Meyer et al., 1996). The phytochemicals have been found to act as antioxidants by scavenging free radicals as they have therapeutic potential for free radical associated disorders (Lee et al., 2000). Therefore, it is important to assess antioxidant activity of the plants used in the herbal medicines either to elucidate the mechanism of their pharmacological action or to provide information on antioxidant activity of these herbal plants.

The electron donating potential of extract/fractions was determined by molybdate ion reduction assay. It is a quantitative method which helps in analyzing the total antioxidant capacity of the leaves extract/fractions (summer and winter). The results revealed that, among the extract/fractions (summer), the ethyl acetate fraction has the highest capacity to reduce molybdate ions (93.25) in terms of mg AAE/100mg dry weight of extract or fraction. Likewise, among the different extract/fractions (winter) the tendency to donate electrons was highest in ethyl acetate fraction (97.73 mg AAE/100mg) followed by chloroform (93.23) > methanol (58.65) > n-Butanol (52.40) > hexane (51.65) > aqueous (15.57) mg AAE/100mg. Kanatt et al. (2005) observed the reducing ability of methanol extract of *Mentha* species. The results showed that the compounds present in methanol extract acts as reductones and promote the radical induced chain reaction by donating the electrons. Also, Prasad et al. (2009) found the
electron donating ability of different peels fractions of *Clausena lansium* (Lour.) Skeels. It was observed that the different fractions of *C. lansium* showed good antioxidant activity and ethyl acetate fraction (100µg/ml) was found to have antioxidant activity viz. 2.5±0.005, higher than BHT (1.3±0.005). The reducing potential of antioxidants was related to the presence of reductones that apply antioxidant action by breaking chain reaction which is the result of electron donation.

The antioxidant activity of different leaves extract/fractions (summer and winter) was also measured by CUPRAC assay. In general, the important feature of CUPRAC assay is its versatility, i.e., applicability to both hydrophilic and lipophilic antioxidants by a simple change of solvent (Apak *et al.*, 2004; Apak *et al.*, 2005). The results showed that ethyl acetate fractions (summer and winter) of *A. nilotica* were effective in converting Cu$^{2+}$ to Cu$^{+}$. The cupric ion (Cu$^{2+}$) reducing ability of seeds extracts of *Illicium griffithii* was studied by Kumar *et al.* (2013). The results showed the Cu$^{2+}$ reducing ability in concentration dependent manner. The trend follows the order as: BHT > methanol > ethyl acetate > hexane. Likewise, Li *et al.* (2015) investigated the antioxidant capacity by CUPRAC assay (with modifications) for the three different chinese medicinal plants viz. *Scutellaria baicalensis*, *Coptis chinensis* and *Sonchus oleraceus*. The results of the study were given in terms of mg gallic acid equivalents (GAE) per gram dried extract. The highest amount of antioxidant value was found in *S. baicalensis* viz. 82.4 mg GAE/g dried extract followed by *S. oleraceus* and *C. chinensis* viz. 76.5 mg GAE/g dried extract and 42.7 mg GAE/g dried extract respectively.

The ABTS radical cation decolorization method was used for the measurement of antioxidant activity of wide variety of carotenoids, phenolics and some plasma antioxidants by calculating the reduction potential of the radical cation (percentage inhibition) at 734 nm. The results showed that among extract/fractions (summer), the lowest IC$_{50}$ value was seen in ethyl acetate fraction (102.43 µg/ml). The ethyl acetate fraction (winter) also showed lowest IC$_{50}$ value (37.79 µg/ml) as compared to standard ascorbic acid viz. 95.84 µg/ml. Li *et al.* (2008) studied the effect of different powdered seeds extracts of *Vitis vinifera*. The results showed the highest ABTS scavenging activity for digestive enzymatic extracts viz. 1.4 to 10.8 fold, higher than the chemical
(solvent) extracts. The antioxidant potential of plant foods daily taken in the Spanish diet also revealed that the *in vitro* physiological method yielded a higher antioxidant capacity as compared to chemical procedure (Serrano et al., 2007). The scavenging activities of *Medicago polymorpha* metanolic extract showed a sequential increase with increasing concentration of plant extract (50 μg/ml < 100 μg/ml < 150 μg/ml < 200 μg/ml < 250 μg/ml < 500 μg/ml). Ascorbic acid used as standard also showed similar trend i.e. scavenging activities increased with increasing of ascorbic acid concentrations (50 μg/ml < 100 μg/ml < 150 μg/ml < 200 μg/ml < 250 μg/ml and 500μg/ml) (Khan et al., 2013).

The antioxidant activity of different extract/fractions (summer and winter) was also assessed by β-carotene linoleic acid assay. In this assay, the standard ascorbic acid exhibited highest antioxidant activity with IC₅₀ value of 17.14 μg/ml as compared to all the extract/fractions of both the seasons. The highest antioxidant activity of 77.84% and 97.77% was found in ethyl acetate fractions (summer and winter), respectively. The antioxidant activity of the essential oils from the aerial parts of *Peucedanum longifolium* and *Peucedanum palimbioides* was studied by Tepe et al. (2011). It was observed that *P. palimbioides* have strong activity (90.58%) against linoleic acid oxidation (2.0 mg/mL). The synthetic antioxidants BHT and BHA were found to have 95.86% and 93.05% activity respectively. In another study, Umamaheshwari and Chatterjee, (2008) observed that the addition of hydromethanolic leaves fractions of *Coccinia grandis* reduced the discoloration of β-carotene. The petroleum ether, ethyl acetate and chloroform fractions showed strong inhibition on β-carotene bleaching and their IC₅₀ values were 0.068, 0.099 and 0.117 mg/ml respectively. These activities were significantly higher than that of α-tocopherol (0.14 mg/ml). The IC₅₀ value of the residual fraction of *C. grandis* was 0.145 mg/ml. In this assay, oxidation of linoleic acid, an unsaturated fatty acid occurs due to the production of reactive oxygen species formed from halogenated water. The reactive oxygen species initiate β-carotene oxidation leading to discoloration. All the fractions of *C. grandis* inhibited β-carotene oxidation, suggesting that the antioxidant activity could be related to high level of phenolic compounds (Gutierrez et al., 2006).
The assessment of superoxide anion radical scavenging activity was evaluated. The decrease in absorbance at 560 nm indicated the scavenging potential of the extracts. It was observed that among the extract/fractions, the ethyl acetate fraction of both the seasons was found to be most effective scavenger of superoxide anion radicals with lowest IC$_{50}$ value of 79.12 µg/ml (summer) and 43.37 µg/ml (winter). The gallic acid used as standard compound was found to have IC$_{50}$ value of 77.70 µg/ml. It was further observed that the extract/fractions other than ethyl acetate fractions also exhibited good activity. Kaur and Arora, (2011) evaluated the superoxide anion scavenging activity of bark and leaves of Cassia siamea and Cassia javanica. The results showed the inhibition of superoxide radicals in a dose dependent behaviour. Among all the bark extracts of C. siamea, the methanol extract showed the maximum inhibition of 60.5% at 800 µg/ml as well as the water extract also showed good antioxidant potential of 51.3% at 1000 µg/ml concentration respectively. The bark water extract of C. javanica showed antioxidant potential of 55% at 1000 µg/ml. The various leaf extracts of C. siamea showed moderate antioxidant potential of 25-50% (1000 µg/ml). Likewise, the methanol leaf extract of C. javanica showed antioxidant potential of 50.4% at 300 µg/ml concentration. In another study, Sundararajan and co-workers (2006) compared the O$_2^-$ scavenging activity of aqueous extract of Cystisus scoparius Link (leguminosae) with curcumin and results showed that the plant extract acted as more potent scavenger of O$_2^-$ with IC$_{50}$ value of 4.7µg/ml as compared to 5.84 µg/ml for curcumin. The highest activity of aqueous extract of Cystisus scoparius might be related to the synergistic effect exerted by the constituents like flavones, flavonols and isoflavones.

Lipid peroxidation (LPO) has been broadly defined as the oxidative deterioration of polyunsaturated lipids (Kappus, 1991). Lipid peroxidation is a complex system where generation of the initiator molecule is followed by chain initiation, propagation and termination reactions (Catala, 2006; Schnitzer et al., 2007; Fukuzawa, 2008). LPO occurs mainly in biomembranes, where the content of unsaturated fatty acids is relatively high. The scavenging of peroxyl radicals occur due to the hydrogen donating property. Fe$^{2+}$ induced lipid peroxidation is also a good system for assessing antioxidant action of different extracts. One of the degradation products of lipid
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Peroxidation is malondialdehyde (MDA) that causes cell damage and form a pink colour chromogen with thiobarbituric acid (TBA). The antioxidant compounds present in the extract scavenged the hydroxyl radicals generated in the Fenton reaction in the liver homogenate. In lipid peroxidation assay, the potential of hexane fraction (summer) was found to be highest i.e. 86.85% at 200 μg/ml of concentration. In this assay, as the concentration of the extract/fraction was increased, the inhibition of lipid peroxidation was also increased. The broad range of antioxidant activity of the extracts indicates the potential of the plant as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits. Among the leaves extract/fractions (winter), the ethyl acetate fraction exhibited the highest peroxyl radical scavenging activity of 80.36% at the same concentration. The antioxidant activity in terms of percentage inhibition of lipid peroxidation for various seed extracts (hexane, ethyl acetate and methanol) of *Illicium griffithii* was observed by Kumar *et al.* (2013). The results were found in the following order: hexane (20%) > ethyl acetate (30%) > methanol (38%). The potential of different seed extracts of *Vitis venifera* to inhibit lipid peroxidation was also reported by Li *et al.* (2008). It was observed that all the extracts exhibited 55.10% to 81.80% inhibition of peroxidation at different concentrations used. The inhibition of peroxidation of all extracts of *V. venifera* showed the following order: methanol (80%) > dialysate (75%) > retentate (68%) > acetone: water (60%) > ethanol: water (60%) > water (53%).

DNA nicking assay is a sensitive method for the detection of potent oxidants. In this assay, OH radicals generated in Fenton’s reagent mixture convert the cesium chloride purified plasmid DNA (Form I) to open circular DNA (Form II) through single strand breaks whereas double strand breaks resulted in the formation of double strand nicked and linear form of DNA (Form III) (Zhang and Omaye, 2001). The results indicated that the addition of different leaves extract/fractions to pBR322 DNA containing Fenton’s reaction minimizes the formation of Form II and Form III DNA and thus maintained the Form I DNA viz. supercoiled DNA, by a hydrogen abstraction mechanism (Russo *et al*., 2000). The extract/fractions rich in phenols protect the DNA from hydroxyl radical mediated damage by scavenging these radicals (Lee *et al.*, 2002). It was observed that different leaves extract/fractions (summer and winter) of *Acacia*
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*nilotica* were active scavenger of hydroxyl radicals as DNA nicking caused by Fenton’s reagent was prevented by the addition of different leaves extract/fractions at different concentrations. Amongst all the leaves extract/fractions (summer and winter), the ethyl acetate fraction of both the seasons was found to be highly effective in preserving the supercoiled DNA i.e. 61.64% (summer) and 64.90% (winter) at highest concentration (1000 µg/ml). The study done by Lee *et al.* (2002) investigated the efficacy of *Opuntia ficus-indica* var. *saboten* (ethanol extract) to protect plasmid DNA against the strand breakage caused by hydroxyl radicals generated in Fenton’s reaction mixture. The addition of extract in nicking reaction mixture having pBR322 plasmid DNA resulted in the increase of Form I (supercoiled) formation and also reduced Form II DNA as compared to reaction mixture without the extract. In another study by Kadam *et al.* (2008) suggested that sugarcane juice have a remarkable potential to prevent the conversion of supercoiled DNA to open circular form of plasmid DNA due to single strand breaks or formation of linear form as a result of double strand breaks against radiation induced DNA damage in pBR plasmid DNA and *Escherichia coli* cultures.

5.3 **In vitro antiproliferative studies**

The conventional therapeutic and surgical modalities have been responsible for the marked improvement in the survival and enhanced quality of life of patients suffering with different types of cancer. There is a need to attenuate the increasing incidence rate of various cancers in western and industrialised populations. The intake of diet rich in phytochemicals appears to be a strong lifestyle change that meets its requirement (Doll and Peto, 1981; Mesina *et al.*, 1994; Duthie, 2007). Phytochemicals (phenols) play a significant role in suppressing all three stages (tumour initiation, promotion and progression) of tumour formation and metastasis. The positive epidemiological evidence of phytochemical intake prevents cancer. A great deal of research has identified a variety of cellular signaling mechanisms that support this hypothesis and shows that numerous phytochemicals can modulate distinct cell receptor and signal transduction pathways to suppress the carcinogenic process *in vitro* and *in vivo* and to a lesser extent in humans (Wahle *et al.*, 2009). The present study aimed at determining the *in vitro* cytotoxicity study of different leaves extract/fractions (summer
and winter) against different human cancer cell lines and study the mechanism of action on most effective ethyl acetate fraction of both the seasons.

MTT assay is a tetrazolium based colorimetric assay. MTT is reduced by succinate dehydrogenases and reducing agents present in metabolically active cells to form water insoluble formazan crystals (violet-blue) which are dissolved in organic solvents (Van Meerloo et al., 2011; Stockert et al., 2012). The results showed that among the different extract/fractions (summer), the methanol extract showed highest growth inhibition against Hep-G2 cell line whereas hexane fraction showed IC$_{50}$ value of 180.20 µg/ml. The chloroform and ethyl acetate fractions showed IC$_{50}$ values of 132.56 µg/ml (IMR-32) and 107.84 µg/ml (PC-3) respectively. Other fractions viz. n-Butanol and aqueous, have highest inhibition percentages (200µg/ml) i.e. 49%, 53% for MCF-7 cell line respectively. Among the extract/fractions of winter, the methanol extract exhibited the highest growth inhibition for PC-3 cells. The lowest IC$_{50}$ values was found for hexane and chloroform fractions against PC-3 cell line. The ethyl acetate fraction was found to have highest growth inhibition (80%) for PC-3 cell line at maximum concentration. Likewise, the n-Butanol and aqueous fractions have lowest IC$_{50}$ values against PC-3 and Hep-G2 cell lines, respectively. The antiproliferative activity with human embryonic kidney cells (HEK 293) was studied by Ravi et al. (2012) on ethanolic aerial extract of Pupalia lappacea (EAPL). The results showed the dose dependent decline in the growth of cells with increasing concentrations of EAPL. The IC$_{50}$ value of EAPL for K562 cells was found to be 40 µg/ml. In another study, Kumar et al. (2012) assessed the cytotoxicity of hexane, acetone, iso-propyl alcohol (IPA) and crude protein fruit extracts of Helicteres isora (Malvaceae) against human lung cancer cells (NCI-H460). The results showed cytotoxicity of acetone, iso-propyl alcohol, crude protein and hexane extract viz. 93.7% (10 µg/mL), 96.2% (40 µg/mL), 90.3% (40 µg/mL) and 62.5% (40 µg/mL), respectively.

The morphological changes in the cells are regarded as the gold standard for apoptosis detection in vitro as apoptotic cells are characterized by membrane blebbing, cell shrinking, nuclear condensation, chromatin aggregation, degradation of chromosomal DNA and formation of apoptotic bodies (Pulido and Parrish, 2003; Cummings et al., 2004). In the present study, PC-3 cells were treated with ethyl acetate
fractions (summer and winter) that exhibited typical morphological features of apoptosis in confocal microscopy using DAPI stain and scanning electron microscopy (SEM).

To further confirm the apoptotic events, the cell cycle analysis by flow cytometry was done in PC-3 cells treated with ethyl acetate fractions (summer and winter), after staining with propidium iodide. The structural and biochemical events occurring during cell death at cellular level were analysed by flow cytometry. Along with the observed changes in cell volume and granularity, it also helps to quantify apoptosis (Schmid et al., 1992; Lecoeur et al., 2002). Flow cytometric analysis can be employed for precise evaluation of cellular DNA content and subsequent identification of hypodiploid cells, which generally appear in the sub G_0/G1 phase. The analysis is based on the ability to stain the cellular DNA with a fluorochrome in a stoichiometric manner and the proportion of hypodiploid cells in total cell population represents the intensity of apoptosis inducing activity of the extract/fractions. The cell cycle analysis was performed by staining the treated cells by using propidium iodide (PI), a fluorescent dye that intercalates with cellular DNA (Li et al., 2005; Riccardi and Nicoletti, 2006; Agrawal et al., 2011). The results showed that ethyl acetate fractions of both the seasons exhibited concentration dependent increase in the apoptotic sub G_0 fraction that indicate that the extract/fractions induced cell cycle arrest at the G_0/G1 phase. Among the ethyl acetate fractions of both the seasons, ethyl acetate fraction of winter season was found to be most effective with 51.7% and 55.3% cells at hypodiploid stage at IC_{50} and IC_{70} values respectively.

The role of mitochondria, as a key executioner of intrinsic pathway of apoptosis is well known as this pathway for apoptosis is activated by non-receptor mediated intracellular signals that are oncogenes, hypoxia, radiation, ROS overproduction or direct DNA damage. These stimuli induce changes in the inner mitochondrial membrane that results in the loss of mitochondrial transmembrane potential causing the release of apoptogenic factors from the intermembrane space into the cytoplasm (Yang et al., 1997; Elmore, 2007; Ashkenazi et al., 2008). Reactive oxygen species are known as triggers of the intrinsic apoptotic cascade via interactions with proteins of the mitochondrial permeability transition complex, thus resulting in cytochrome c release from the intermembrane space. The significant mitochondrial loss of cytochrome c will
lead to further ROS increase due to a disrupted electron transport chain (Chen and Lesnefsky, 2006; Tsujimoto and Shimizu, 2007; Circu and Aw, 2010). In addition, 2’,7’-dichlorofluorescein diacetate (DCFH-DA) was used to measure reactive oxygen species generated in PC-3 cells treated with ethyl acetate fractions of both the seasons. The ROS generation ability of ethyl acetate fraction (winter) showed them to be effective generators of ROS intermediates leading to activation of redox sensitive transcription factors by 1.41 and 1.64 (fold) at IC$_{50}$ and IC$_{70}$ values respectively. The ethyl acetate fraction of summer was observed to be less elicitors of intracellular ROS.

The integrity of mitochondrial membranes in PC-3 cells following treatment with ethyl acetate fractions (summer and winter) was examined by measuring their ability to retain Rh-123, a fluorescent dye that gets integrated in the membranes of intact mitochondria (Kim et al., 2007). The results revealed that ethyl acetate fraction (summer) exhibited loss of mitochondrial membrane depolarization by 96.15% and 83.33% at IC$_{50}$ and IC$_{70}$ values respectively. Likewise, the ethyl acetate fraction (winter) was found to be more effective and showed loss of mitochondrial potential of 92.59% and 80.64% at IC$_{50}$ and IC$_{70}$ values respectively. The effect on activation of caspase-3 showed an increase in caspase-3 activity of 162.96% (IC$_{50}$) and 177.77% (IC$_{70}$) for ethyl acetate fraction of summer season and also, an increase in caspase-3 activity of 174.07% (IC$_{50}$) and 181.48% (IC$_{70}$) was seen in ethyl acetate fraction of winter season.

From the above studies, it is clear that the different leaves extract/fractions of _Acacia nilotica_ (summer and winter), the ethyl acetate fraction of winter season exhibited most protective effects with respect to their antioxidative and anticancer potential. The most active ethyl acetate fraction of both the seasons caused cell death via apoptosis. The results of the present study have shown that all the leaves extract/fractions are potentially good source of free radical scavengers. They contain high phenolic and flavonoid content and hence exhibits good antioxidant and anticancer activities.