SUMMARY

Medicinal plants are known to be a repository of bioactive compounds having a wide array of therapeutic properties. The World Health Organization (WHO) estimates that near about 80% of the world’s residents depends on the traditional medicines for their major health benefits (Farnsworth et al., 1985). The National Cancer Institute (NCI) collected approximately 35,000 plant samples from 20 countries and screened nearly 114,000 extracts for having their anticancer potential (Shoeb et al., 2005). The medicinal importance of plants is due to chemical substances i.e. phenolic compounds (flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins) present in them, having multiple biological effects that produce a specific physiological action on the human body (Edeoga et al., 2005). The immense therapeutic potential of medicinal plants includes antioxidative, anti-inflammatory, antiviral, antitumor, antimalarial, antimutagenic, anticancerous and analgesic. The in vitro studies on the antioxidant compounds present in plants showed that plants help to protect against oxidation damage caused by free radicals through inhibiting or quenching them (Ali et al., 2008). Thus, antioxidant compounds present in plants terminate the action of free radicals and hence protects the body from various diseases (Lai and Chou, 2001). Furthermore, the essential organic compounds present in plants are embroidered to diminish the toxicity caused due to chemotherapy. An ideal anticancer drug should kill or debilitate cancer cells without causing the unnecessary damage to normal cells. The ability of medicinal plants to inhibit the cancer cell proliferation is measured as a sign of anticancer potential. The reason behind their action is the balance of tumor cell proliferation over cell death which has been proposed to be one of the key factors in cancer initiation and progression. Keeping above in view, the present study was conducted to assess the antioxidant and anticancer potential of leaf extract/fractions of Acacia nilotica collected in summer and winter seasons.

Acacia nilotica (Mimosa nilotica) is a medium sized tree and locally named as “Babul” or “Kikar”. It belongs to the Fabaceae family. It is a native from Egypt to Mozambique and grows in tropical and subtropical countries and particularly quite
abundant in Pakistan (Baquar, 1989). The plant is used in folk medicine as the bark, leaves, pods and fruits are widely used to treat diarrhoea, fever, tuberculosis and menstrual problems (Ambasta, 1994 and Singh et al., 2008). The plant is also reported to show evidences of antiinflammatory (Dafallah and Mustafa, 1996) and antiplatelet actions (Shah et al., 1997).

Materials and Methods

The leaves of *Acacia nilotica* (L.) Willd Ex Del. were collected from trees growing in Guru Nanak Dev University Campus, Amritsar, Punjab in two seasons i.e. August-September (summer) and February-March (winter). Further, Botanical identification and authentication was done from the Herbarium of Department of Botanical and Environmental Sciences, GNDU, Amritsar and voucher specimens were submitted (Accession No. 7241). The extraction method involves grinding the leaves into fine powder after washing and drying. The finely powdered material was extracted by the method as described in materials and methods to get the methanol extract which was further extracted to get hexane, chloroform, ethyl acetate, n-Butanol and aqueous fractions. The supernatant was recovered by filtering through Whatman no. 1 sheet and the solvents were evaporated by rotary evaporator. The leaf extract/fractions (summer and winter) were then evaluated for the following parameters:

1. **Phytochemical analysis**

   ➢ Qualitative analysis of extract/fractions for the different phytochemicals (Lalitha and Jayanthi, 2012).

   ➢ Quantitative analysis for total phenolic content (Yu et al., 2002) and total flavonoid content (Kim et al., 2003).

   ➢ Quantification of polyphenolic compounds by ultra high pressure liquid chromatography (UHPLC) (Kumar et al., 2008).

2. **In vitro antioxidant studies**

   The leaf extract/fractions (summer and winter) of *A. nilotica* were assessed for their antioxidant properties by *in vitro* antioxidant assays.
Summary

- **Hydrogen and electron donating assays:** Molybdate ion reduction assay (Prieto *et al.*, 1999), CUPRAC assay (Cupric ion reducing antioxidant) (Apak *et al.*, 2007), ABTS radical cation decolorization assay (Re *et al.*, 1999) and β-carotene linoleic acid assay (Tepe *et al.*, 2005).

- **Radical scavenging assays:** Superoxide anion scavenging assay (Nishikimi *et al.*, 1972), lipid peroxidation assay (Ohkawa *et al.*, 1979) and pBR322 plasmid nicking assay (Lee *et al.*, 2002).

3. **In vitro antiproliferative studies**

The antiproliferative activity of leaf extract/fractions of both the seasons was studied by using *in vitro* 3-(4,5-Dimethylthiasol-2-yl)-2,5,-diphenyltetrazolium Bromide (MTT) assay in different human cancer cell lines (Twentyman and Luscombe, 1987).

**Mechanistic studies on ethyl acetate fraction (summer and winter)**

- Morphological changes of PC-3 cancer cells by using confocal microscopy (Bhushan *et al.*, 2007) and scanning electron microscopy (Ye *et al.*, 2012).
- Cell cycle analysis by flow cytometer (Carneiro *et al.*, 2010).
- Measurement of reactive oxygen species generation (Shin *et al.*, 2009) and changes in mitochondrial membrane potential (Deng *et al.*, 2013) spectrofluorimetrically.
- Assessment of caspase-3 activity colorimetrically (Sigma Aldrich colorimetric kit).

**Results**

**Phytochemical analysis**

The leaves of summer and winter seasons were extracted in different solvents. 90g mother methanol extract of leaves (summer season) yielded hexane (12.03g), chloroform (13.73g), ethyl acetate (22.70g), n-Butanol (13.83g) and aqueous (14.76g) fractions. Similarly, 16.27g mother methanol extract of dried powdered leaves of winter season yielded maximum percentage of ethyl acetate fraction (2.93g) followed by
hexane (2.03g), chloroform (0.572g), n-Butanol (0.389g) and aqueous (0.355g) fractions.

The qualitative analysis of phytochemicals showed the presence of various phytoconstituents. Although, the alkaloids were present in the extract/fractions of both the seasons but the methanol and hexane extract/fraction did not show the occurrence of them. The glycosides, phenols, flavonoids, proteins and carbohydrates were found to be present in all the extract/fractions. The steroids and terpenes were not present in any of extract/fractions of both the seasons.

The total phenolic content (mg GAE/100mg dry wt. of extract or fraction) was calculated by Folin-Ciocalteu method. The results showed the presence of phenolic compounds in all the extract/fractions of summer season in varying amounts. The ethyl acetate fraction contained 93.30 mg GAE/100mg followed by chloroform (89.30), methanol (81.30), n-Butanol (67.60), hexane (51.30) and aqueous (5.30). However, the ethyl acetate fraction of winter season was found to contain more total phenolic content (94.32 mg GAE/100mg) as compared to same extract of summer season.

The amount of total flavonoid content was evaluated in terms of mg/g rutin. Among different extract/fractions of summer season, the ethyl acetate fraction was found to have maximum flavonoid content of 61.19 mg RE/100mg. Further, in case of extract/fractions of winter season, the ethyl acetate fraction has the highest flavonoid content (83.70) followed by hexane (69.70) > methanol (63.40) > chloroform (60.06) > n-Butanol (45.06) > aqueous (38.70) in mg RE/100mg.

The UHPLC analysis of extract/fractions showed the presence of different phenolic compounds. Among the different polyphenols studied in extract/fractions of summer season, it was observed that the methanol, hexane and chloroform extract/fractions showed the highest amount of umbelliferone and chlorogenic acid. The ethyl acetate fraction also showed the varying amounts of gallic acid, catechin, chlorogenic acid and umbelliferone. The n-Butanol and aqueous fractions showed very less number as well as amount of polyphenols.
It was further observed that the methanol, hexane and chloroform extract/fractions of winter season exhibited the maximum amount of umbelliferone. In ethyl acetate fraction chlorogenic acid, umbelliferone and rutin was found to be present in varying amounts. The n-Butanol and aqueous fractions also showed the presence of some of the polyphenolic compounds.

**In vitro antioxidant studies**

In vitro antioxidant studies were performed by Molybdate ion reduction assay, CUPRAC assay, ABTS radical cation decolorization, β-carotene linoleic acid, Superoxide anion scavenging, Lipid peroxidation and pBR322 plasmid nicking assays. The various concentrations of extract/fractions were used ranging from 20-200 µg/ml. In pBR322 plasmid nicking assay, 200-1000 µg/ml concentrations were used.

**Hydrogen and electron donating assays**

The molybdate ion reduction assay estimates the total antioxidant capacity in terms of mg ascorbic acid equivalents/100mg dry weight of extract or fraction. It was seen that ethyl acetate fraction of summer and winter season exhibited maximum antioxidant capacity of 93.25 and 97.73 respectively. The reduction potential of different extract/fractions (summer and winter) of *Acacia nilotica* was estimated by CUPRAC assay. It was observed that among extract/fractions of summer season, the ethyl acetate fraction was found to be most active with reduction potential of 99.06% at 200 µg/ml concentration. The ethyl acetate fraction of winter season was found to have more reduction potential of 99.23% at 200 µg/ml concentration which was slightly more than the same fraction of summer season. The free radical scavenging activities of extract/fractions was assessed by ABTS assay. The IC₅₀ value of 102.43 µg/ml was seen in summer ethyl acetate fraction. However, the extract/fractions of winter season showed the trend in terms of IC₅₀ values as: ethyl acetate (37.79 µg/ml) < methanol (81.68 µg/ml) < chloroform (131.10 µg/ml) < n-Butanol (178.39 µg/ml) < hexane (202.63 µg/ml) < aqueous (404.22 µg/ml) fractions, respectively.

The antioxidant activity of extract/fractions was also calculated by β-carotene linoleic acid assay. The results showed that the extract/fractions showed good antioxidant activity at all the concentrations used. The antioxidant activity of ethyl
acetate fraction (summer) was found to be maximum among all the extract/fractions viz. 77.84% at 200 µg/ml concentration. On the contrary, ethyl acetate fraction (winter) exhibited antioxidant activity of 97.77% at the same concentration followed by hexane (90.36%), chloroform (88.64%), n-Butanol (83.81%), aqueous (77.61%) and methanol (65.64%) fractions, respectively.

Radical scavenging assays

In superoxide anion scavenging assay, it was seen that among the extract/fractions of summer season, ethyl acetate fraction was most effective in comparison to other fractions with IC$_{50}$ of 79.12 µg/ml. Further, the winter ethyl acetate fraction showed inhibition with IC$_{50}$ value of 43.37 µg/ml. In lipid peroxidation assay, the order of peroxyl radical scavenging activity in terms of percentage inhibition for all the summer extract/fractions was: hexane (86.85%) > ethyl acetate (52.30%) > methanol (45.75%) > n-Butanol (28.57%) > chloroform (20.81%) at 200 µg/ml concentration. Similarly, the trend of percentage inhibition for winter extract/fractions followed the order viz. ethyl acetate (80.36%) > chloroform (78.94%) > aqueous (59.54%) > methanol (46.38%) > hexane (44.24%) and n-Butanol (22.00%) fractions.

The ability of extract/fractions (summer and winter) to protect the supercoiled plasmid DNA from the damage caused by hydroxyl radicals was estimated by using pBR322 plasmid nicking assay. It was observed that the amount of preserved supercoiled DNA was found to be 56.46% and 56.1% for methanol and hexane extract/fraction respectively. Similarly, the chloroform and ethyl acetate fractions were found to be protective with supercoiled DNA at 1000 µg/ml was 46.1% and 61.64% respectively. The n-Butanol and aqueous fractions showed recovery of supercoiled DNA by 48.93% and 49.60% respectively. Further, the extract/fractions of winter season were evaluated for their DNA protective activities. The amount of supercoiled DNA (%) for methanol, hexane, chloroform, ethyl acetate, n-Butanol and aqueous extract/fractions was 57.2%, 57.2%, 51.2%, 64.90%, 49.5% and 41.04% at 1000 µg/ml concentration respectively.
In vitro antiproliferative studies

The antiproliferative activity of different extract/fractions (summer and winter) was assessed on different human cancer cell lines like prostate (PC-3), breast (MCF-7), cervix (Hela), liver (Hep-G2), lung (A549) and brain (IMR 32) at different concentrations (10, 50, 100, 150, 200 µg/ml). It was observed that the extract/fractions (summer) showed moderate inhibitory effect against all the cancer cell lines. The methanol and hexane extract/fraction showed IC_{50} of 188.79 and 180.20 µg/ml respectively for Hep-G2 cell line at maximum concentration used. The chloroform and ethyl acetate fractions showed IC_{50} of 132.56 and 107.84 µg/ml against IMR-32 and PC-3 cell lines respectively. The n-Butanol and aqueous fractions exhibited IC_{50} of 216.4 µg/ml and 113.62 µg/ml for MCF-7 cell line respectively.

The cytotoxic activity of extract/fractions for winter season was also assessed. It was seen that IC_{50} value of 76.17, 112.85 and 95.62 µg/ml was found for PC-3 cells with methanol, hexane and chloroform extract/fractions respectively. The ethyl acetate fraction exhibited effective IC_{50} (µg/ml) of 22.01 for PC-3 cell line. The n-Butanol and aqueous fractions showed inhibitory effect with IC_{50} (µg/ml) of 164.34 for PC-3 cell line and 151.75 for Hep-G2 cell line.

Mechanistic studies

On the basis of results obtained in in vitro antiproliferative study against different human cancer cell lines, the ethyl acetate fraction of both the seasons was found to be effective, therefore it was evaluated for mechanistic studies on PC-3 cell line at IC_{50} and IC_{70} values.

The confocal microscopy showed the distorted morphology of cells as observed by presence of apoptotic bodies that indicated the induction of apoptosis with extract/fractions. SEM studies also confirmed the loss of surface projections, cell shrinkage and surface blebbing, the features characteristics of apoptosis. The PC-3 cells stained with PI (propidium iodide) confirmed the cells arrest at sub G_{0} phase of cell cycle. The PC-3 cells treated with IC_{50} and IC_{70} concentrations of ethyl acetate fraction (summer) showed 33.5% and 50.3% of cells arrested at sub G_{0} phase of cell cycle.
respectively. Whereas, cells arrest of 51.7% and 55.3% was found for PC-3 cells treated with IC\textsubscript{50} and IC\textsubscript{70} concentrations of ethyl acetate fraction (winter) respectively.

For measuring the hydrogen peroxides, hydroxyl radicals and reactive oxygen species generated in PC-3 cells, 2’,7’-dichlorofluorescein diacetate (DCFH-DA) was used. The results showed that ethyl acetate fraction (summer) treatment to PC-3 cells induced 1.29 and 1.52 fold increase in ROS production at IC\textsubscript{50} and IC\textsubscript{70} concentrations respectively as compared to untreated PC-3 cells. Similarly, 1.41 and 1.64 fold increase in ROS production was observed in PC-3 cells treated with ethyl acetate fraction (winter) at the same concentrations.

The Rhodamine 123 fluorescence was used to assess the shift in mitochondrial transmembrane potential in PC-3 cells treated with ethyl acetate fractions. The results revealed that ethyl acetate fraction (summer) exhibited loss of mitochondrial membrane potential of 96.15\% at IC\textsubscript{50} and 83.33\% at IC\textsubscript{70} concentrations. Likewise, ethyl acetate fraction (winter) at same concentration was found to be more effective and showed loss of mitochondrial membrane potential of 92.59\% and 80.64\% respectively as compared to cells treated with camptothecin (80\%). Further, the PC-3 cells were studied for the effect of ethyl acetate fractions (summer and winter) on caspase-3 activity. The results showed concentration dependent increase in caspase-3 activity of 162.96\%, 177.77\% for ethyl acetate fraction (summer) at IC\textsubscript{50} and IC\textsubscript{70} concentrations respectively. Similarly, an increase in caspase-3 activity of 174.07\% and 181.48\% was seen in ethyl acetate fraction (winter) at the same concentrations.

The highlights of the results obtained in the present study are as below:

- Among all the extract/fractions, the yield of ethyl acetate fractions was found to be maximum in both the seasons and also the qualitative analysis showed the presence of different phytochemicals in extract/fractions.
- The ethyl acetate fraction of winter season exhibited highest total phenolic and total flavonoid content as well as the maximum percentages of polyphenolic compounds as detected by UHPLC analysis.
The antioxidant activity of ethyl acetate fraction of winter season was found to be maximum among all the extract/fractions as measured by molybdate ion reduction, CUPRAC, ABTS radical cation, β-carotene linoleic acid and superoxide anion scavenging assays.

The hexane fraction of summer season showed maximum percentage inhibition of peroxyl radical scavenging activity.

Among all the extract/fractions, the ethyl acetate fraction of winter exhibited maximum hydroxyl radical potential in protecting Form I DNA at all the concentrations used.

Among all the extract/fractions, ethyl acetate fraction of winter season showed maximum cytotoxic activity in MTT assay.

Ethyl acetate fraction (winter) induced maximum morphological alterations in PC-3 cells as confirmed by confocal and scanning electron microscopy.

The maximum percentage of cells arrested at sub G₀ phase of cell cycle was found for the PC-3 cells treated with ethyl acetate fraction of winter season.

Ethyl acetate fraction (winter) was found to induce apoptosis in PC-3 cells by inducing ROS overproduction which in turn damaged mitochondria as measured by mitochondrial membrane potential.

The ethyl acetate fraction (winter) was found to have maximum caspase-3 activity in concentration dependent manner.

It can be concluded from the present study that, although all the extract/fractions of both the seasons showed good antioxidant and antiproliferative activities but the ethyl acetate fraction of winter season was found to be comparatively more active in almost all the assays. It was further studied that ethyl acetate fraction (winter) induced apoptosis in PC-3 cells as confirmed by different mechanistic studies. So, the results of present study suggest that the leaf extract/fractions of A. nilotica may have future prospects as an antioxidant and anticancer agent.