Antioxidant, antibacterial and antiproliferative activities of pumpkin *(cucurbit)* peel and puree extracts - an *in vitro* study

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Abstract: Natural resources right from the beginning of the human civilization has paved the way to human being to combat different challenges. The big challenge was to safe the human being from diseases and shortage of food. Plants helped the man in both areas very efficiently. No doubt when plants are used as food actually we are also taking lot of compounds of medicinal values in an excellent combination which naturally reduce the risk of diseases. Extraction and purification of several medicinally important compounds also gave the way to develop pharmaceutical industry in addition to its own therapeutic effects against different lethal diseases. Pumpkin is one of the several medicinal important vegetables used in different way on the behalf of its admirable power to combat different diseases. Antioxidant and biological studies showed very important results. A good coherence was found among extraction yield (10.52 to 18.45%), total phenolics (1.13 to 6.78 mg GAE/100g), total flavonoids (0.23 to 0.72mg CE/100g) and antioxidant potential (≈70%). Antibacterial assays of peel and puree extracts advocated good potential to stop the growth and division of pathogenic bacteria. Further biological activity study was carried out using MDBK cancer cell line. The growth inhibitory effect on cancer cell line using MTT assay showed methanol extracts of peel and puree both remained efficient to inhibit growth (≈35%) and cell division of cancer cells. Our results showed that extracts of pumpkin puree and its waste, peel, may be utilize to prepare functional food against pathogenic born diseases and most active compounds may also be extracted, concentrated and converted into tablets or suspension form for therapeutic purposes.

Keywords: Antioxidants, Pumpkin fruit, MTT assay, Antibacterial potential, Plant extracts, TPC, TFC.

INTRODUCTION

Excess production of reactive oxygen species (ROS) in living body can hack or de-track key biochemical mechanisms of the body and could render them to stimulate signal transduction rout and consequently activation of key transcription factors. The activation of these transcription factors consequently may create disturbance in cell machinery, alteration in DNA, aging before time, infections or carcinogenic diseases susceptibility. Out of these susceptibilities, before time aging and infection are more susceptible as compared to cancer induction by ROS because the latter process required multistage, multistep processes (for example initiation, promotion, progression) to convert normal cells into neoplastic cells. However, programmed cancer induction by cancer cell lines injection into living body skips number of steps to convert healthy cells into carcinogenic cell as compare to genotoxicity. There are mainly two factors such as physical and chemicals which convert healthy cells to neoplastic cells. These two factors collectively are known as genotoxic factors or genotoxic agents. ROS are also one form of genotoxic agents include a wide range of chemicals such as nitric oxide, hydrogen peroxide, superoxide anion, hydroxyl radicals, peroxynitrite anions, alkylperoxyl and hydroperoxyl radicals. These chemicals are capable to oxidize biomolecules and initiate chronic diseases such as asthma, diabetes, cardiovascular and cancer through cell death and tissue damaging (Sugden *et al.,* 2001, Scarpato *et al.,* 2011, Sahar *et al.,* 2013, Naqvi *et al.,* 2013a,b).

Antioxidants have played very critical role in different ways to protect living body from possible chronic threat originated by genotoxic agents either by scavenging the free radicals produced in the body or by stopping the oxidation of biomolecules (Skandrani *et al.,* 2010). Living body possess a series of antioxidant defensive networks and repairing mechanisms against oxidative stress, however, the ability of these networks is limited and couldn't perform up to the mark of requirement especially when free radical production going very rapid. The practice of utilization of grinding material of different parts of plants was common to cure different ailments in long history of man. Over the period of last few decades, however, extensive experimental work was carried out to

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explore phytochemicals in plants, vegetables and fruits and their possible functions in living bodies. As a result of these experimental and epidemiological studies wide range of phytochemicals such as phenolics, flavonoids, isoflavone, flavones, carotenoids, catechin, isocatechin and anthocyanins was explored. Most of them have strong potential to prevent or slow down the oxidative stress induced cell/tissue damaging which leads to carcinogenesis by upsetting the molecular events in the initiation, promotion or progression stages (Gul *et al.,* 2013). On the basis of clinical studies and trails, it was proved that regular intake of vegetables and fruits could be helpful to reduce the risks of carcinogenic diseases especially infections and cancers. That's way today real consideration should be given to develop preventive medicines and natural antioxidants obtained from botanical sources, especially vegetables and herbal plants.

Pumpkin belongs to family Cucurbitaceae, was first cultivated in America and later on its seeds was taken to Mexico, North America and Asia due to its taste and health benifits. In Asian countries, it is a well-known vegetable which is primarily famous due to its moderate to very big size. However, it is a treasure of biologically very active compounds such as tocopherols, tocotrienols, phenolics, flavonoids, flavones, etc in its different parts. Different studies had shown its medicinal importance in sense of antioxidant, slowing down the development of hypertension (Vasdev, 2005; Vucinic *et al.,* 2008; Schiffrin, 2010), decreasing gastric level, therapy of breast, lung, and colorectal cancers (Huang *et al.,* 2004).

The aim of this study was to investigate the antioxidant, antibacterial and anti-proliferative activities of 60%, 75% and 99.9% methanol extracts of pumpkin peel and pulp cultivated in lower areas of Punjab of Pakistan.

MATERIALS AND METHODS

Chemicals

The chemicals used in this project were of reagent grade and purchased from Sigma-Aldrich chemical Company. The bacterial strains and MDBK cancer cell line was obtained from the Department of Allied Health Sciences and Chemical Pathology, University of Health Sciences, Lahore, Pakistan. A Millipore type filter (pore size 0.22 µm and 0.45 µm) was obtained from Sartorius, USA. Absorption in UV-visible range of samples was measured using double beam U-2800 UV-visible spectrometer, HITACHI, Japan.

Collection of samples

Pumpkins of different sizes were collected from pumpkin fields of Faisalabad vicinity (coordinates; 31.4180° N, 73.0790° E) and washed properly with tap water followed by distilled water to remove earth clay and any other impurities attached to it. The voucher specimen was deposited in the herbarium of the Department of Botany, Government College University, Faisalabad. The peel and puree of the fruit separated very carefully and dried in air under shade. The dried parts of pumpkin fruit stored in polyethylene bag at 4ºC until extraction process (~4-8 days).

Preparation of extracts

Cleaned and dried sample material was extracted in methanol (MeOH). 60g each sample was soaked in 300 mL solvent and stirred at room temperature for 120h continuously on orbital shaker at 200 rpm. The mixture was separated out by using Whatman filter paper. Filtrate was concentrated and dried on vacuum rotary evaporator. Concentrated and dried filtrate was stored at 4ºC for further analysis. Extraction yields were calculated in terms of percentage using following expression.

$$\text{Percentage yield}\,(\%) = \frac{\text{Weight of solvent free extract (g)}}{\text{Dried extract weight (g)}} \times 100$$

Determination of phenolics

Phenolics and flavonoids present in pumpkin peel and puree extracts were determined by following the procedure described by Chaovanalikit and Wrolstad (2004) with slight modification. Briefly, 1mL of extract solution (0.05g/mL ethanol) was mixed with 200µL of Folin-Ciocalteu reagent and 3mL deionized water and the mixture was kept at room temperature for 10min. Then 0.75mL of 20% aqueous Na_2CO_3 (w/v) was added. The mixture was allowed to heat at 40ºC for 20min using water bath followed by ice cooling. Finally absorbance was taken at 755nm. Amount of total phenolic content was calculated from calibrated curve of gallic acid (10-130ppm). The results were expressed as gallic acid equivalents (GAE) g/100g dry extract matter. Sample was analyzed thrice and results were averaged.

Determination of total flavonoid contents (TFC):

Total flavonoid contents were determined according to the procedure described by Dewanto *et al.,* (2002). Briefly, 1 mL of extract solution containing 0.01g/1mL of dry matter was added in a 10mL volumetric flask followed by the addition of 5mL distilled water and 0.3mL of 5% $NaNO_2$ solution. After alternative 5 min incubation period added 0.6mL of 10% $AlCl_3$ and 2mL of 1M NaOH solution, respectively and volume was made up to the mark with distilled water. Absorbance was measured at 510nm. Total flavonoid contents were expressed as catechin equivalents (CE) g/100 g of dry matter. Sample was analyzed thrice and results were averaged.

DPPH radical scavenging activity

DPPH free radical scavenging activity of pulp and puree extracts were measured according to the method described earlier (Yen & Chen, 1995) with slight modification. To 1mL of 0.1mM ethanol solution of DPPH, 3mL of extracted sample of different concentrations (40, 80, 120, 160 and 200µg/mL) in separate test tubes, was added. The solution mixture was shaken well and left for 30 min at room temperature. Then

Table 1: Percentage yield, TPC and TFC values of peel and puree extracts of pumpkin

Extracts		%age yield	TPC (mgGAE/100g)	TFC (mg CE/100g)
Peel	99.9% MeOH	18.45±1.93	1.99±0.012	0.56±0.004
	80% MeOH	16.23±1.21	1.83±0.020	0.41±0.003
	65% MeOH	15.61±1.63	1.13±0.011	0.23±0.001
Puree	99.9% MeOH	14.10±1.03	6.78±0.06	0.72±0.004
	80% MeOH	12.41±1.77	5.13±0.034	0.65±0.002
	65% MeOH	10.52±1.45	4.31±0.023	0.51±0.001

Table 2: IC_{50} values of peel and puree extracts of pumpkin against MDBK cancer cell line

Extracts		IC_{50} for MDBK cancer cells (mg)
Peel	99.9% MeOH	7.12±0.24
	80% MeOH	16.11±1.07
	65% MeOH	34.94±2.03
Puree	99.9% MeOH	24.92±1.78
	80% MeOH	19.33±1.51
	65% MeOH	47.53±3.11

Each value is the mean ± SD of triplicate measurements.

absorbance was noted at 517 nm. BHT and blank (without extract) were used as positive and negative controls, respectively. Scavenging %age was calculated using following expression;

$$\text{Scavenging}\,(\%) = \times 100\left[\frac{A_{\text{Blank}} - A_{\text{Sample}}}{A_{\text{Blank}}}\right]$$

Antibacterial activity

Antibacterial activity of pumpkin peel and puree extracts were measured using disc diffusion method as described by Sahar *et al.,* (2013). Antibacterial activity was assessed against four bacterial strains *E. coli, P. multocida, S. aureus* and *B. subtilis*. Twenty mililitter media containing bacterial strain pour into nutrient agar petri plats and allowed it to set. After that, sterile filter paper discs (10 mm) placed on surface of the medium followed by loading 100 µL sample (10 mg/mL) dissolved in DMSO onto filter discs. The same concentration of rifampicin was also loaded as positive control. Petri plates were then incubated for 18-24 h at 37 ºC in an incubator. At the end of incubation period zone of inhibitions were measured by zone reader.

Antiproliferative assay

The antiproliferative assay was carried out as reported by Naqvi *et al.,* (2013a). MDBK cell line was used in this assay and extracts were diluted in five different concentrations i.e. 0.312mg/ml, 0.625mg/mL, 1.25mg/mL, 2.5mg/mL, 5mg/mL ethanol, in labeled wells while maintaining the cell number constant (2000 cells/well) in each well of 96-wells plate. GMEM medium was used for the growth of cells amended with 5% FBS and 5% CO_2 supply while incubating at 37ºC for 48 h. After incubation 20µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) stock solution of 5mg/mL concentration was added. The plate was again placed in 5% CO_2 incubator at 37ºC for 3h for the formation of crystals of purple formazan. Then Added 100µL of DMSO in each well to dissolve the crystals and optical density (OD) value was measured at 570nm with reference to control. Experiments were repeated thrice to obtain mean values.

RESULTS

Yields of extracts

All extractions were carried out in three different dilutions (99.9%, 80% and 65%) of MeOH solvent. Most of the components of the peel and puree of pumpkin was extracted in 99.9% MeOHl such as from peel in 99.9% methanol 18.45±1.93% and from puree 14.10±1.03% extract was obtained. Concentration dependent extraction was observed in all cases as shown in table 1.

Total phenolic and flavonoid contents

Total phenolic contents was measured using Folin-Ciocalteu reagent which is considered authentic protocol for calculating the phenolic contents in natural extracts. The 99.9% MeOH crude extract of *cucurbetea pepo* L. peel and puree showed the highest TPC, 1.99±0.012 and 6.78±0.06 mg GAE/100g extract, respectively. In case of flavonoids, similar behavior was investigated, however, very small concentration of TFC was found in peel and puree. Maximum quantity of flavonoids (0.72±0.004 mg CE/100g extract) was determined in 99.9% MeOH extract of puree and minimum (0.23±0.001mg CE/100g) was measured in 99.9% MeOH extract of peel. Other MeOH extracts also showed almost same results with slight difference as shown in table 1.

Antioxidant study

Antioxidant study of the extracts were carried out using DPPH free radical scavenging assay which revealed good

potential to scavenge the free radical results from the oxidation of different moieties. The antioxidant activity results were summarized in fig. 1. The activity at 0 min, if strictly speaking, actually measured at 20-30 seconds i.e. readily noted after addition of assay reagents. The 99.9%MeOH extract of peel and puree showed promising free radical scavenging potential. However, puree extract samples showed greater DPPH free radical scavenging potential as compared to peel extracts, 68.79±2.41 and 69.56±2.78, respectively. Less concentrated MeOH extracts (80 and 65%) showed comparatively low percentage inhibition of DPPH free radicals.

Antibacterial potential

Antibacterial activity of pumpkin peel and puree extracts were measured using disc diffusion method which showed satisfactory inhibition activity against four bacterial strains E.*coli*, P. *multocida,* S. *aureus* and B. *subtilis*. All extracts showed inhibition potential in the range of 10-15 mm while against three bacterial strains E.*coli*, S. *aureus* and B. *subtilis* while >15 mm against P. *multocida* as shown in fig. 2.

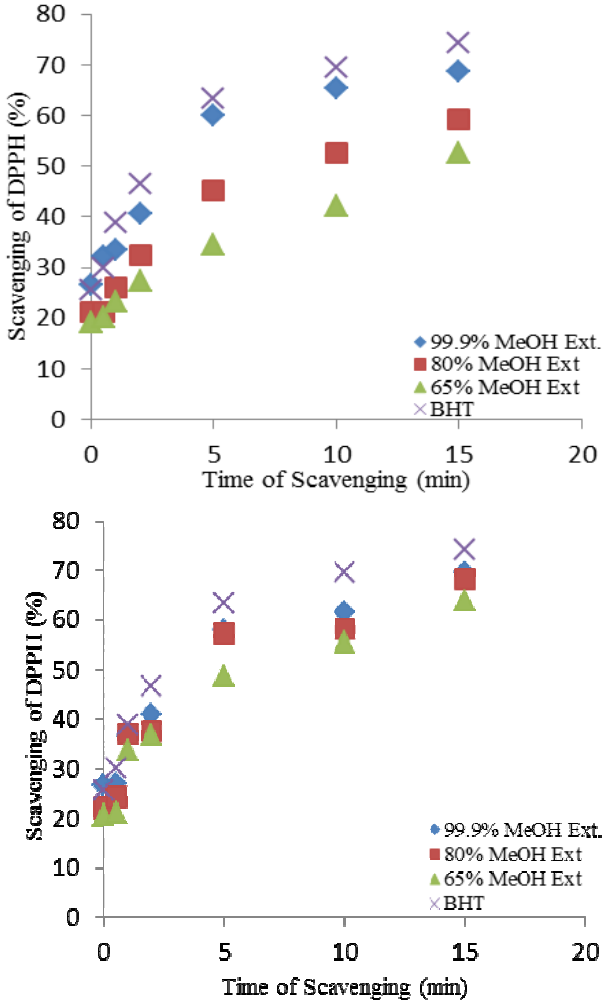

Fig. 1: DPPH free radical scavenging potential of methanol extract of Peel and Puree.

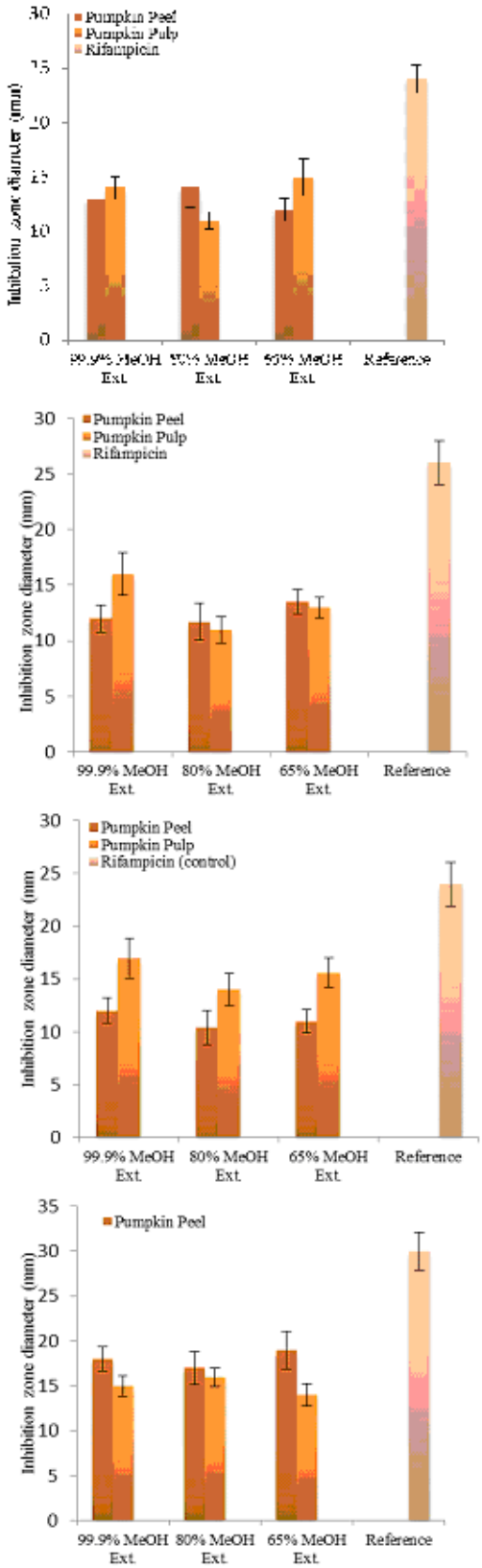

Fig. 2: Antibacterial activity of pumpkin peel and pulp extracts against A) *E.coli*, B) *P. multocida, C) S. aureus* and D) *B. subtilis.*

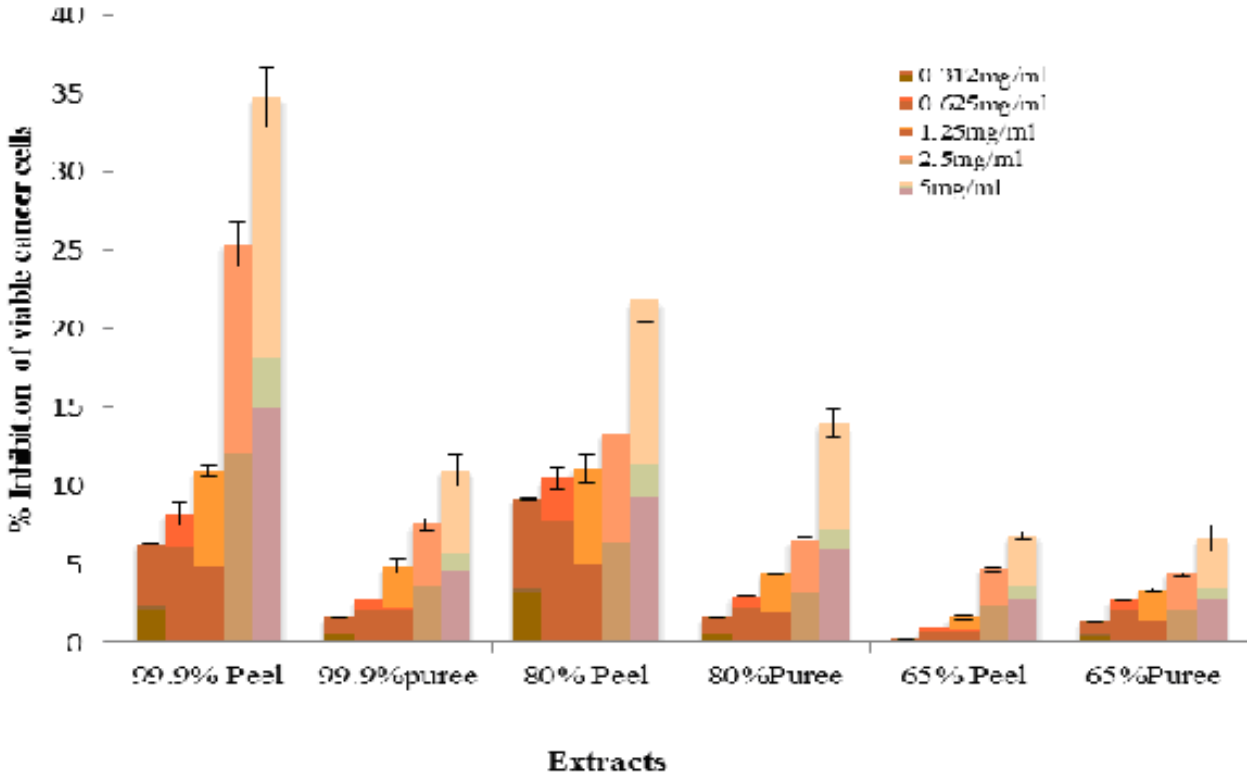

Fig. 3: Percent inhibition of viable MDBK cancer cell line against different concentrations of pumpkin peel and puree extracts

Antiproliferative activity

The antiproliferative study results were summarized in fig. 3. The assay was carried out using MDBK cancer cell line. The antiproliferative activity appeared concentration dependent showing inhibition range from ≈5% to 35% of viable cancer cells by 65% to 99.9% MeOH extract sample, respectively.

DISCUSSION

The extraction of natural compounds from peel and puree of pumpkin fruit were carried out using most friendly and effectively working solvents i.e. methanol in different concentrations. Pure solvent extracted more efficiently as compared to the diluted solvent, might be due to the less polarity of the natural compounds as compared to water, which reduces the solvent-solute interaction and vice versa in case of MeOH.

Phenolics and flavonoids

It has been reported that phenolics and flavonoids are well recognized components of vascular plants and each class of compounds carry over 8000 compounds which are responsible for color, taste, flavor and medicinal properties of natural products. Since, pumpkin fruit is slightly tasteless, non-flavored and light yellow in color which attributed to low TPC and TFC as compared to some medicinal fruits or vegetables (Hasan *et al.,* 2012). The extraction was performed using different dilutions of MeOH. Since MeOH was suggested more effective solvent for the extraction of phenolic components. Although aqueous solvent can extract similar quantity of the phenolic components as MeOH but phenolic compounds extracted in MeOH showed more effective biological activities (Yildirim *et al.,* 2001, 2003; Arabshahi *et al.,* 2007). TPC values were measured in gallic acid equivalent due to its acid stability and good response to Folin-Ciocalteu reagent. The 99.9% MeOH crude extract of *cucurbetea pepo* L. peel and puree showed the highest TPC, 1.99±0.012 and 6.78±0.06 mg GAE/100 g extract, respectively. Other MeOH extracts also showed almost same results with slight difference as shown in table 1. This difference might be due to difference in polarity of the solvent mixtures. However, puree of the pumpkin was found reasonably rich in TPC as compared to MeOH extracts of peel. This could be attributed to the fact that basic place for metabolism and production of secondary plant metabolites is puree of the fruits. Phenolic compounds are considered to play very critical defensive role against tissue damaging either by free radical scavenging or probably by activation or repression of specific genes (Fardet *et al.,* 2008). The later mechanism is not fully elucidated, however, recent reports have elucidated that these compounds also act on gene expression via transcription factors, activating the Antioxidant Response Element (ARE) (Min *et al.,* 2003; Na & Surh, 2006). ARE on activation triggers the actions of antioxidant compounds (Myhrstad *et al.,* 2002). In case of flavonoids, similar behavior was investigated, however,

very small concentration of TFC was found in peel and puree. Maximum quantity of flavonoids (0.72±0.004 mg CE/100g extract) was determined in 99.9% methanol extract of puree and minimum (0.23±0.001 mg CE/100 g) was measured in 99.9% methanol extract of peel. However, even small concentration (µg quantity/100g) of flavonoids has strong ability to stop or reduce the oxidation and damaging action of bacteria.

Antioxidant study

Many studies have investigated the medicinal importance of plants, its different parts (roots, stems, bark and leaves) and its products (flowers and fruits) especially as an antioxidant and anti-infection. Oxidants produced in human body in the form of hydroxyl radical (OH•), superoxide anion (O2•-) etc., are predominantly involve in cellular and DNA damaging. Production of greater quantity of these moities may paralyse the defensive system of the body that lead to oxidative stress diseases such as cardiovascular diseases and hypertension in common (Uttara *et al.,* 2009; Rahal *et al*., 2014). The utility of the natural products which are considered significantly rich in those compounds which can combat to oxidative stress and its allied mechanisms, now a day are in common practice. Fig. 1 showed the antioxidant properties of extracted samples of peel and puree of pumpkin fruit. All samples were found in good position to stop oxidation process by accepting an electron and quenching DPPH free radicals in an *in vitro* antioxidant assay. The scavenging reaction was appeared time and concentration dependent in all cases and maximum scavenging was noted at maximum incubation time (15 min). The antioxidant activity at 0 min if strictly speaking at 20-30 seconds i.e. readily noted after addition of assay reagents. The 99.9% MeOH extract in all samples of peel and puree was appeared to carry higher quantity of free radical scavenging compounds. However, in contrast to pumpkin peel extract samples puree extract samples showed greater DPPH free radical scavenging potential, 68.79±2.41 and 69.56±2.78, respectively. Less concentrated MeOH extracts (80 and 65%) showed comparatively low percentage inhibition of DPPH free radicals.

Antibacterial potential

Extracted components of pumpkin peel and puree was found bacteriostatic as shown in fig. 2. Four different infection causing bacterial strains were used to test the bacteriostatic activities of MeOH extracts. The components extracted from peel were appeared slightly less active than extracts of puree. On the other hand, very small difference was noted in bacteriostatic activities from 99.9% MeOH extracts to 65% MeOH extracts. Diluted MeOH extracts showed lesser potential than concentrated MeOH extracts. This might be due to the increased ratio of water in MeOH as Wong and Kitts (2006) reported that aqueous extracts were found less likely to inhibit bacterial growth than organic solvent extracts despite of the fact that both MeOH and aqueous extract carry similar range of phenolic contents. Lesser activity of aqueous extracts against bacterial growth could be attributed to strong Van Der Waal's interaction with polar functionalities of phenolic compounds for water. Phenolic compounds are strongly associated with antibacterial activities because it was suggested in previous reports that polar isopropyl functionality of phenolic components may involve in bacteriostatic activities (Farag *et al.,* 1989). Before this report Raccach (1984) had been published his findings that phenolic compounds present in natural extracts have strong ability to react with cellular membrane components resulting in leakage of nucleotide (Degré & Sylvestre, 1983) and protienaceous material into extracellular areas (Davidson & Branen, 1980). In present study, it was noted that only in case of B. *subtilis* MeOH peel extract appeared more active and against other three strains such as *E. coli*, S. *aureus* and P. *multocida* pulp extracts were more active with almost similar effect. In past different studies were conducted to conclude the effect of extraction solvents especially for antibacterial activity (Yildirim *et al.,* 2001, 2003; Kumar *et al.,* 2012). In these studies it was reported that phytochemicals extracted in MeOH or ethanol did well as compared to aqueous solvent. Our results has clearly showed that all MeOH extracts of peel and puree were found in good position to inhibit the growth of bacteria either by cell membrane rupturing or by disruption of active transport of nutrients at the cytoplasm membrane (Cerruti & Alzamora, 1996).

Antiproliferative activity

Since cancer is most leading cause of mortality throughout the world beyond the discrimination of developing and developed countries. Oncologists commonly use chemotherapy or radiotherapy way of treatment but first of all preference is given to chemotherapy to keep the body away from exposure to radiations. Cancer chemotherapeutic mechanism mainly involves the targeting the infected cells either to destroy or to stop the proliferation of cells by interfering into one or more different cell mechanisms (Kumar *et al.,* 2007). MDBK cancer cell line was used to quantify the anticancer/anti-proliferative activity of the extracts of pumpkin peel and puree. The anti-proliferative assay was carried out with five different concentrations as shown in fig. 3. *In vitro* study modeling results showed concentration dependent behavior of all extracts as was observed in anti-bacterial study. However, 99.9% MeOH peel extract showed significant depletion of the cancer cells followed by the 80% MeOH peel extract with IC_{50} values 7.12±0.24 and 16.11±1.07 mg/L respectively, (table 2). However, notable decrease in activity was seen in case of 65% MeOH extracts. This might be due to the shielding/solvation of hydroxyl functionalities of phenolic compounds by water molecules due to reasonable dilution of peel and pulp extracts. But the story not ends here, in

addition to antioxidants which play its role to stop chronic mechanism some other classes of compounds are also involved to stop or slow down DNA damaging/mutation or abnormal proliferation of cells. Approximately >50 % drugs which are being used to treat cancer or in clinical trials were obtained from natural sources or modified them to enhance the anti-infection and anti-proliferation potential (Munari *et al.,* 2014).

CONCLUSION

From this study it could be concluded that the pumpkin fruit cultivated in lower Punjab areas of Pakistan is rich in TPC and TFC. The strong antioxidant, antibacterial and antiproliferative potential suggested pumpkin fruit peel and puree extracts could be a good candidate for the development of antioxidant rich functional food.

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