Preliminary assessment of phytochemical contents and antioxidant properties of *Pistacia integerrima* fruit

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Abstract: Present study was focused on the evaluation of preliminary phytochemical screening and antioxidant properties in hydrophilic and lipophilic solvents extracts of *Pistacia integerrima* fruit, collected from Lesser Himalayas-Pakistan. Ethnobotanical data were collected through semi-structured interviews. Standard analytical methods were followed to estimate the proximate composition of nutrients, trace metals and phytochemical contents including phenolics, flavonoids, flavonols and ascorbic acid. The free radical scavenging activities of aqueous and acetone extracts were carried out against 2, 2-diphenyl-1-picrylhydrazyl, hydroxyl radical, hydrogen peroxide radical, ferrous ion chelation, ferric ion reduction, and phosphomolybdenium complex assay. Comparatively highest value of cultural importance index was determined for Margalla hills than other study sites. Crude fibers contents (33.65%) were found highest among nutrients, while in minerals utmost level was measured for K (947.3mg/kg, FW), followed by Ca, Mg, Na and Fe. Acetone extract exhibited highest total phenolics contents (113.7mg GAE/100g, FW) and antioxidant potential for ferric ion reduction (107.3µM GAE/100g, FW), phosphomolybdenum complex assay (99.32µM AAE/100g, FW) and DPPH radical scavenging (91.89%). Fruit of *P. integerrima* was found an excellent source of nutrients, minerals and antioxidants. Crude extracts exhibited noteworthy potential against free radicals and could be of immense significance in the prevention of different diseases related to free radicals. Present data could be an effectual tool for propagation programs intended to enhance the antioxidant and other phytochemical components in foods.

Keywords: Phytochemicals, antioxidant, wild fruit, *Pistacia integerrima,* Pakistan

INTRODUCTION

The pathogenesis of various health effects such as coronary artery disease, diabetes, stroke, alcoholic liver cirrhosis and cancer is caused by oxidative stress, which is initiated by reactive oxygen species (ROS) Hazra *et al.*, (2010), which are formed through usual physiological actions and the antioxidant system in organisms maintains a balance between the production and inactivation of ROS (Halliwell *et al.*, 1992). However, pathological conditions and inadequate endogenous antioxidant defenses result overproduction of ROS that leads to lipid per-oxidation, damage to proteins and DNA and causes oxidative stress (Manian *et al.*, 2008). Epidemiology shows contrary relation between the daily utilization of fruits and vegetables and the jeopardy of degenerative and unrelenting diseases, which have long been accredited to their antioxidant compounds Zibadi *et al.*, (2007), that own the ability to defend the body from harm caused by free radical induced oxidative strain. Polyphenols, carotenoids, anthocyanin, flavonols, vitamin C and E, in plants are powerful antioxidants for their roles in the maintenance and protection of human health against chronic degenerative diseases (Pellegrini *et al.*, 2000). Lately, there has been a worldwide tendency towards the eating of ordinary antioxidants (Abalaka *et al.*, 2011). These antioxidants are considered safer than synthetic ones Zheng & Wang, (2001), can defend the body from free radicals and slow down the development of many chronic diseases as well as lipid oxidative rancidity in foods (Lai *et al.*, 2001).

Pistacia integerrima (fig. 1) locally, known as Shnaie, Kangar or Kakar singhi belongs to family Anacardiaceae. This species is native to Indo-Pakistan and exotic in USA. Its flowering period is from March-May and fruiting from June-October. The fruits are globular, purplish or blue at maturity (Padulosi *et al.*, 2002). Leaves and bark of this plant has been used traditionally against rheumatic, fever, diarrhea, vomiting and jaundice (Ahmad *et al.*, 2008; Khan *et al.*, 2008).

Fig. 1: *Pistacia integerrima* (fruits)

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Although antimicrobial, phytotoxic, anti-diarrheal, antioxidant, immune-modulatory, analgesic, anti GIT motility, antidepressant, analgesic, hepato-protective and anti-diabetic effects of the leaves and bark of *P. integerrima* have been reported by different workers (Ahmad *et al.*, 2013; Ilahi *et al.*, 2012; Ismail *et al.*, 2012; Rahman *et al.*, 2011; Joshi & Mishra, 2010), but to our knowledge, there is no information available on the distribution of nutrients, minerals, phytochemical and antioxidant properties in the fruits, hence present work will give first appraisal of the characterization of chemical composition as well as antioxidant activity of *P. integerrima* fruit.

MATERIALS AND METHODS

Cultural significance

Well-versed approval semi-structured interviews have been conducted with key respondents of the five major sites of Lesser Himalayas-Pakistan, including Abbottabad, Haripur, Mansehra districts, Margalla Hills National Park Islamabad, Murree Hills and its allied areas to collect ethnobotanical information. Cultural significance of species incorporated cultural importance index (CI) and mean cultural importance index (mCI) was also calculated as Pardo-de-satayana *et al.*, (2007).

$$CI = \sum_{i=1}^{i=NU} \frac{URi}{N}$$

Where, N is the number of informants and UR is the use report in each use category.

Sampling

About, 2-3kg ripened fruits were collected. Plant specimen was recognized by Prof. Dr. Mir Ajab Khan, Department of Plant Science, Quaid-i-Azam University-Islamabad. Collected fruits were washed vigilantly with tap water followed by distilled water and, then placed in paper wrapper and dried at 55°C to 65°C for 24 hours in oven (Abuye *et al.*, 2003). The dried samples were ground with a pestle and mortar, fine powder was stored in polythene bottles and kept in desiccators until study.

Standards and reagents

Ascorbic acid; ethylenedi-aminetetraacetic acid (EDTA); aluminum chloride, ferric chloride, ferrous chloride, ferrous sulphate, Folin-Ciocalteu; 2,2-diphenyl-1-picrylhydrazyl (DPPH); sodium acetate, rutin, gallic acid, meta-phosphoric acid, 1,10-phenanthroline, ferrozine, dichloroindophenol, trichloroacetic acid (TCA) were purchased from Sigma Co. (St. Louis, MO, USA). Standards solutions of different metals were purchased from (Merck, Germany). Sulphuric acid; Sodium carbonate, sodium hydroxide, sodium nitrite, disodium hydrogen phosphate and hydrogen peroxide were obtained from Wako Co. (Osaka, Japan). Potassium ferricyanide, sodium dihydrogen phosphate, Nitric acid, perchloric acid, hydrochloric acid, potassium sulphate, sodium sulphate, copper sulphate, ammonium molybdate, selenium dioxide, boric acid, sodium carbonate, acetone, petroleum ether, methyl red/bromo-cresol green indicators used were of analytical grade and purchased from Merck Co. (Darmstadt, Germany).

Proximate nutritional and minerals analysis

Proximate estimation of ash contents, calorific values, carbohydrates, crude proteins, fats, fibers and moisture contents were carried out, following AOAC methods (AOAC, 1995). For minerals estimation, (~1.0g) of fruit sample was digested in a mixture of nitric acid and perchloric acid at 80-85°C until a clear solution was obtained (Arora *et al.*, 2008). A blank was also prepared in the same way. Trace metals such as Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Pb, Sr and Zn were measured through an atomic absorption spectrophotometer (AAS).

Phytochemical analysis

Extraction

A two-step hydrophilic (aqueous) and hydrophobic (acetone) extraction procedure was adopted as (Ji *et al.*, 2011). 1.000g of dried powered sample was blended with 10mL of distilled water and homogenate was centrifuged at 6000 rpm for 15 minutes, and supernatant was collected in a clean test tube. This procedure was repeated thrice and supernatants were pooled in a flask. The solid residue was then extracted 3 times in acetone (1:10 w/v) and supernatants were also collcted.

Estimation of phytochemicals

The total phenolics content (TPC) was deliberated following Lin *et al.*, (2011) method. Aliquots of 1.0mL of water or acetone extracts were mixed with 5mL of 10 fold diluted Folin-ciocalteu reagent and 4mL of 7.5% sodium carbonate. The mixture was allowed to stand for 90 minutes at room temperature and the absorbance was measured at 760nm. Results were articulated as mg gallic acid equivalents in 100g fresh weight (mg GAE/100g, FW). Flavonoids content (TFC) was intended through modified colorimetric method described by Lin *et al.*, (2011). Briefly 5mL of water or acetone extract was mixed with 0.3mL of 5% sodium nitrite for 5 minutes in a test tube. Then 0.3mL of 10% aluminium chloride was added into it. After 6 min, 2mL sodium hydroxide was added to stop the reaction and mixture was further diluted with distilled water up to 10mL. The absorbance was instantly measured at 510nm and results were expressed as mg rutin equivalents in 100g fresh weight (mg Rt/100g, FW).

Total flavonols content (TFlC) was measured through the method of Kumaran & Karunakaran (2006). 2.0mL of 2% aluminium trichloride and 3mL sodium acetate (50g/L) solutions were added in 2.0mL of sample extract. The absorption was measured at 440nm after 2.5h at 20°C. Results were mentioned as mg rutin equivalents in 100g

fresh weight (mg Rt/100g, FW). Ascorbic acid content (AAC) was calculated following Klein & Perry (1982) scheme. Water and acetone extracts were re-extracted with meta-phosphoric acid (1%, 10mL) for 45 min at room temperature and filtered. The filtrate (1.0mL) was mixed with 9mL of 2, 6-dichloroindophenol (0.8g/1000 mL) and the absorbance was measured within 30 minutes at 515 nm. Ascorbic acid contents were estimated on the basis of calibration curve of L-ascorbic acid (0.006-0.1 mg/mL; y=3.006x + 0.007; R^2=0.999). Results were expressed as mg ascorbic acid equivalents in 100g fresh weight (mg AA/100g FW).

Antioxidant assays

DPPH free radical scavenging assay

DPPH scavenging activity was calculated following the method described previously Aoshima *et al.,* (2004). Briefly 2.0mL of the extracts or standards was added to 5 mL of DPPH solution (0.1mM in methanol) and vortexes vigorously and incubated in dark for 30 minutes at room temperature. The decolourization of DPPH was measured against blank at 517 nm. Percentage inhibition was calculated as

$$\%\,Inhibition = \frac{(A_{Blank} - A_{Sample})}{(A_{Blank})} \times 100$$

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity, based on Fenton reaction was estimated as reported earlier Yu *et al.,* (2004). 2.0mL of 0.2 M phosphate buffer (pH 7.2), 0.04mL ferrous sulphate (0.02 M), 2mL of extract and 1 mL of 1, 10-phenanthroline (0.04 M) were added in to the test tube. The Fenton reaction was initiated by the addition of 0.1mL of 7mM H_2O_2. Absorbance was measured at 560 nm after 5 minutes incubation at room temperature and expressed in (%) relative hydroxyl radical scavenging activity as,

$$Scavenging.Activity(\%) = \frac{(A_{Blank} - A_{Sample})}{(A_{Blank})} \times 100$$

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2) scavenging activity of the samples was assessed following Aeyigoro & Okoh, (2010) procedure. About 4mL of extract was mixed with 2.4mL of 4mM H_2O_2 solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 minutes at room temperature. The absorbance was measured at 230 nm against blank without H_2O_2, and percentage-scavenging activity was determined as,

$$Scavenging.Activity(\%) = \frac{(A_{Blank} - A_{Sample})}{(A_{Blank})} \times 100$$

Ferrous ion chelating activity

The ferrous ion (Fe^{2+}) chelating ability was calculated by the method of Dinis *et al.,* (1994). About 2.0mL of extract was added to the 2.0mL of ferrous sulphate (0.125mM) and the reaction was started by the addition of 2mL of 0.3125mM ferrozine. The mixture was shaken vigorously, and left at room temperature for 10 minutes and absorbance was measured at 562nm against blank prepared in the same way using ferrous chloride and water. EDTA (0.625-5.0mg) was used as positive control and sample without extract or EDTA served as negative control, final results were expressed in the percentage inhibition of ferrozine-Fe (II) complex as follow:

$$Chelating\ .Activity\,(\%) = \frac{(A_{Control} - A_{Sample})}{(A_{Control})} \times 100$$

Reducing power

The ferric ion Fe^{3+} reducing power (FRAP) value was calculated as Hazra *et al.,* (2008). 2.0mL of sample was mixed with the 2.0mL of phosphate buffer (0.2M, pH 6.6) and 2mL of 0.1% potassium ferricyanide, followed by incubation at 50°C in water bath for 20 minutes and then reaction was stopped by adding 2mL of trichloroacetic acid (10%). The upper portion of solution (2mL) was mixed with 2mL of distilled water and 2mL of 0.01% ferric chloride and left for 20 minutes at room temperature and absorbance was measured at 700nm against blank. Gallic acid was used as positive control. Final results were as the concentration of antioxidant having a ferric reducing ability in 100g fresh weight (µM GAE/100 g, FW).

Total antioxidant capacity

The total antioxidant capacity (TAC) was investigated by Phosomolybdenium complex assay as described by Prieto *et al*. (1999). About 2.0mL of sample solution in aqueous or acetone extract was added to the 6.6mL of reagent mixture (0.6mol/L sulphuric acid, 28mol/L sodium phosphate and 4 mol/L ammonium molybdate), capped and incubate at 95°C for 90 minutes. After cooling to room temperature, the absorbance was measured at 695 nm against blank. The fine results from three independent experiments are expressed as the mean of relative antioxidant activity (RAA) compared to ascorbic acid in 100g fresh weight (µM AAE/100 g, FW).

RESULTS

Ethno-medicinal uses and culture values

Local inhabitants of the study area use *P. integerrima* in different ways, such as its leaves are used as fodder for cattle; wood as fuel, timber, in carving, furniture, tools handles, musical instruments and thatch, while fresh ripened fruits are edible. Based on use reports (UR), cultural important (CI) and mean cultural important indexes (mCI) were intended within the five study sites of Lesser Himalayas, to guesstimate the cultural significance as mentioned in (Figure 2). It was observed that *P. integerrima* have highest cultural value in Margalla Hills (3.033), followed by Haripur (2.740) and Mansehra (2.367), while in Murree and its allied areas local communities are less familiar with ethno-medicinal

applications of *P. integerrima*. Mean cultural index of *P. integerrima* calculated for all study sites was 2.469.

Macronutrients and minerals

Measured values of nutritional contents are given in Figure 3, which revealed that fruit of *P. integerrima* contains highest crude fibers (33.65%), followed by carbohydrates (33.61%), crude fats (21.59%) and crude proteins (8.291%) contents. However, ash content was lowest, whereas energy value was determined about 361.9 Kcal/100g. Trace metal concentrations expressed as mg/kg on fresh weight basis are mentioned (Figure 4). In general, K metal shows the utmost level (947.3 mg/kg, FW), followed by Ca (120.7 mg/kg, FW), Mg (101.2 mg/kg, FW), Na (10.12 mg/kg, FW) and Fe (6.945 mg/kg, FW). However, comparatively lower levels were noted for Co, Sr, Mn, Cu, Cd, Pb, Cr and Li.

Phytochemical contents and antioxidant properties

Phytochemical contents measured in the fruit of *P. integerrima* are given in Figure 5. Total phenolic contents were more in acetone extract (113.7 mg GAE/100 g FW) than corresponding water extract (70.82 mg GAE/100 g FW). Similarly, total flvonoids were also high in the acetone extract (66.78 mg Rt/100 g FW) than aqueous extract (19.12 mg Rt/100 g FW). Measured value of flavonol contents were found more or less comparable (69.46 mg Rt/100 g FW) in both extracts. However, ascorbic acid content was was found fairly higher in aqueous extract (1.919 mg AA/100 g) compare to acetone extract (1.750 mg AA/100 g).

Result showing antioxidant potential of *P. integerrima* fruit are given in Figure 6, which indicates that relatively acetone extract shows more scavenging activity for DPPH (91.90%) than water extract. Hydroxyl radical scavenging activity was measured high in water extract (48.84%) compare to acetone extract (12.85%). Likewise, elevated levels for H_2O_2 radical scavenging activity and Fe^{+2} chelating activity were calculated in water extracts, 67.38% and 43.26% than corresponding acetone extracts. Ferric ion reducing power was found significantly higher in acetone extract (107.3 µM GAE/100 g, FW) than water extract. Similarly PMA value was also more in acetone extract (99.30 µM AAE/100 g, FW) than water extract.

DISCUSSION

P. integerrima is a well known plant species of the Lesser Himalayas, Pakistan. Traditionally local communities of the study areas use leaves and bark of *P. integerrima* to cure cough, asthma and jaundice which are in agreement with (Ahmad *et al.*, 2013; Ilahi *et al.*, 2012; Ismail *et al.*, 2012; Rahman *et al.*, 2011). This plant species also have miscellaneous ethnobotaical uses ad fodder; fuel, timber, furniture and thatch, while fresh ripened fruits are edible. Based on use reports (UR), cultural important (CI) and mean cultural important indexes (mCI) were intended within the five study sites of Lesser Himalayas, to guesstimate the cultural significance as mentioned in Figure 2, which revealed that this species is ethno-medicinally more familiar in Margalla Hills followed by Haripur and Mansehra, while in Murree and its allied areas local communities are less familiar with ethno-medicinal applications of *P. integerrima*. It might be due to less availability, community interaction with natural flora or lack of awareness about species consequence.

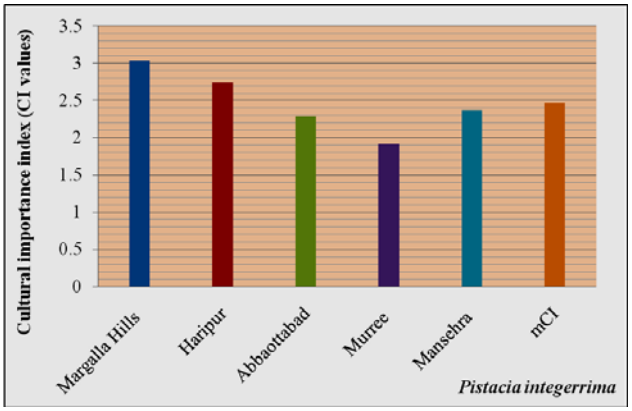

Fig. 2: Cultural importance index (CI) and mean cultural index (mCI) of *Pistacia integerrima*

Proximate nutritional assessment indicates that *P. integerrima* fruit is an excellent source of dietary fibers, which are essential to lower the cholesterol level, risk of heart disease, hypertension, constipation, diabetes, colon and breast cancer (Ishida *et al.*, 2000). Measured values of crude fibers, carbohydrates, fats, proteins and ash contents in the fruits of *P. integerrima*, were found in agreement to the reported levels in *Pisticai vera*, *Pisticia lentiscus*, *Pisticia atlantica*, *Pisticia khijuk* and Cashew tree (Amoo *et al.*, 2011; Ologunde *et al.*, 2011; Saffarzadeh *et al.*, 1999).

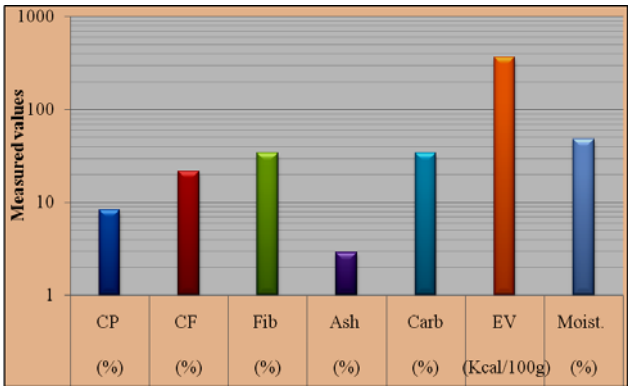

Fig. 3: Proximate nutritional composition of *Pistacia integerrima* fruit.

Results of mineral composition exposed that fruits of studied sample are rich source essential metals including K, Ca, Mg, Na, and Fe. Measured values of these metals were found in agreement to the reported levels in the leaf

galls of *P. integerrima* (Ibrar *et al.,* 2013), and fruits of *Pisticia vera, Pisticia itlantaca, Pisticia khijuk, Pisticia lentiscus, Rhus coiaria* and *Rhus typhina* (Saffari, 2012; Amoo *et al.,* 2011; Kossah *et al.,* 2009; Saffarzadeh *et al.,* 1999). Relatively lower levels were noted for Co, Sr, Mn, Cu, Cd, Pb, Cr and Li. In general, acetone extracts exhibited elevated levels of phenolics, flavonols and flavonoids contents compare to aqueous extracts, while AA contents were found higher in aqueous extract than acetone. Phenolic compounds are natural free radical scavengers and most significant antioxidants (Soobrattee *et al.,* 2005). TPC expressed as Gallic acid equivalent fresh weight (FW) were found higher in acetone extract compare to water extract, which was in agreement to the reported levels in the leaf galls of *P. integerrima* and fruits of *Pisticia lentiscus* (Hasan *et al.,* 2011; Sakong *et al.,* 2011). Total flavonol contents (TFlC) articulated in mg rutin equivalent/100 g fresh weight, were found more or less comparable in both extracts. Flavonoids have been found powerful scavengers of singlet oxygen and various other free radicals, related to DNA damage and cancer (Marchand, 2002). Acetone extract showed significantly higher TFC expressed as mg rutin equivalent than aqueous extract. TFC concentration in *P. integerrima* fruits was higher than reported levels in the fruits of *Lanna micrpcarpa* and *Sclerocarya birrea* of family anacardiaceae found in Burkina Faso (Meda *et al.,* 2008).

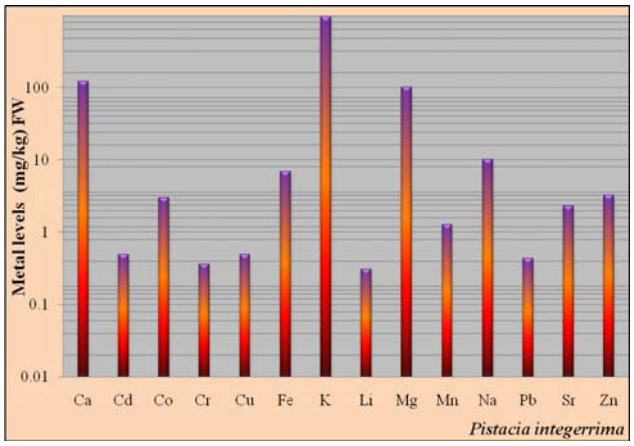

Fig. 4: Trace metal levels in the fruits of *Pistacia integerrima*.

Comparative level of ascorbic acid contents (AAC) expressed as mg ascorbic acid equivalent/100 fresh weight was found fairly higher in aqueous extract compare to acetone extract. Sakong *et al.,* (2011) reported12.68 mg GAE/100g dry weight ascorbic acid in *Spandias pinnata* fruit belonging to anacardiaceae family from Northeast Thailand, but it cannot be comparable as based on dry weight. Variations in nutritional, minerals and phytochemical contents *P. integerrima* fruits to reported values for other fruits, were conceivably due to ecological circumstances, growth conditions, verities, collection season and analytical methods used (Haciseferogullari *et al.,* 2005).

Preliminary antioxidant activity of different plant extracts is mostly assessed by 2, 2-diphenyl-1-picrylhydrazyl (Wong *et al.,* 2006), which is based on the ability of DPPH to react with proton donors such as phenols (Roginsky & Lissi, 2005). In the fruit of *P. integerrima* acetone extract revealed relatively higher values for extract, which is in agreement with (Ahmad *et al.,* 2013; Meda *et al.,* 2008). Hydroxyl radical scavenging is a significant antioxidant activity because OH is tremendously reactive and most injurious species in free cells (Yasuda *et al.,* 2000). OH^- scavenging activity was radical pathology, which causes damage to sugars, amino acids, lipids and nucleotides molecules found in living found to be significantly higher in the water extract of the studied sample, which is comparable to the reported value in *Zanthoxylum alatum* fruits (Batool *et al.,* 2010). Hydrogen peroxide can cross cell membranes quickly results the formation of hydroxyl radical and inactivation of a few enzymes directly (Miller *et al.,* 1993). H_2O_2 radical scavenging activity and ferrous ions chelating activity were determined for the first time and measured values were comparatively high in water extracts than corresponding acetone extracts.

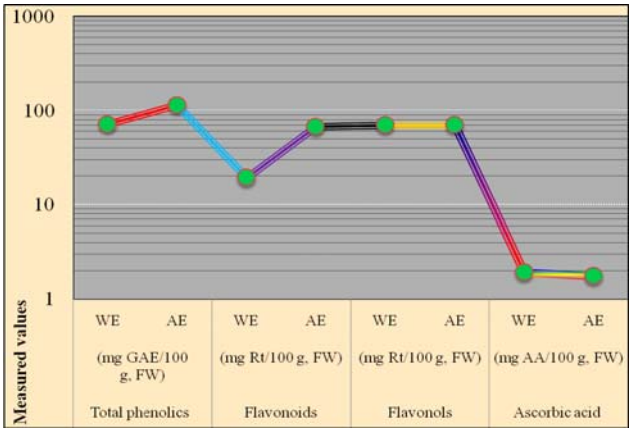

Fig. 5: Phytochemical contents in *Pistacia integerrima* fruits.

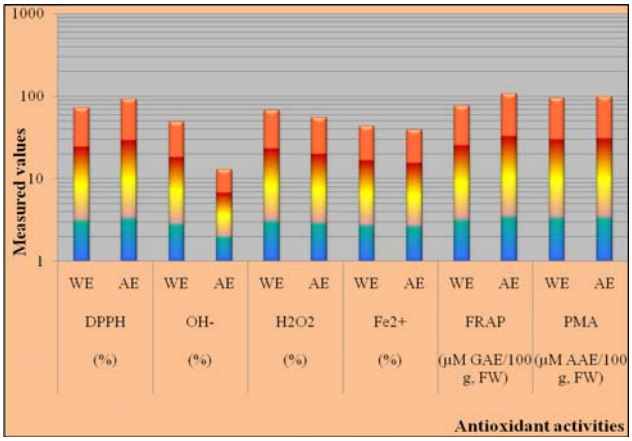

Fig. 6: Antioxidant activities in water and acetone extracts of *Pistacia integerrima* fruits.

FRAP assay is the only assay that directly measures the reducing ability of antioxidants that react with ferric tripyridyltriazine (Fe^{3+} – TPTZ) complex and produce a colored ferrous tripyridyltriazine (Fe^{2+}–TPTZ) (Benzie and Strain, 1996), while Phosphomolybdenum complex assay (PMA), that generally helps in the detection of ascorbic acid, phenolics, tocopherols and carotenoids is used for the determination of total antioxidant capacity (TAC) of the food samples (Miladi & Damak, 2008). Results of FRAP essay expressed in µM Gallic acid equivalent/100 g and that of Phosphomolybdenum complex assay µM ascorbic acid equivalent/100 g fresh weight, revealed that acetone extract showed higher antioxidant potential in terms of ferric ions reducing antioxidant power and Phosphomolybdenum complex assay. Nevertheless, water extract also showed significant value for Phosphomolybdenum complex assay whereas, relatively lower values were noted for FRAP in the water extract of studied sample. Ferric ion reducing antioxidant power of *P. integerrima* fruits was found in agreement to the reported values in the fruits of *Spondias pinnata*.

CONCLUSION

Current investigation deals with cultural importance index, proximate nutritional contents, minerals composition, phytochemical contents and antioxidant activities of *P. integerrima* fruits, which revealed that these wild edible fruits are an excellent source of nutrients, minerals and natural antioxidants. Crude extracts of studied sample exhibited significant potential as free radicals scavenger and could be of great importance in the prevention of different diseases associated with them. This work is an important addition to our ongoing effort in developing a comprehensive database of nutrition and health related secondary metabolites in the wild edible fruits germplasm for their improvement. Moreover researchers can also use the data as effectual tool for propagation programs intended to enhance the antioxidant and other phytochemical components in foods.

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