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RESEARCH ARTICLE

Comparative analysis of the seasonal influence on polyphenolic content, antioxidant capacity, identification of bioactive constituents and hepatoprotective biomarkers by in silico docking analysis in *Premna integrifolia* L.

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Abstract The present study reports the effect of different seasons on polyphenol content and antioxidant potential of ethanolic, methanolic, ethyl acetate, and aqueous extracts of leaves, stems, and roots of *Premna integrifolia*. Ethyl acetate extract of leaves (EAEPI) collected in the rainy season showed potent antioxidant activity with highest total phenol (74.33 ± 2.26 µg/mg, gallic acid equivalent), and flavonoid (98.83 ± 0.26 µg/mg, rutin equivalent) content. Therefore, EAEPI extract was subjected to characterization by UHPLC-Q-TOF-MS/MS and GC–MS analysis for the identification of active constituents. UHPLC-Q-TOF-MS/MS analysis in + ve ion mode revealed the presence of eight polyphenolic compounds namely quercetin-3-D-xyloside, kaempferol-3,7-O-bis-alpha-L-rhamnoside, isorhamnetin-3-Oglucoside, luteolin-3′,7-di-O-glucoside, eriodictyol-7-O-glucoside, syringetin-3-O-galactoside, petunidin-3-O-beta-glucopyranoside and vitexin-2″-O-rhamnoside. GC–MS analysis confirmed the presence of 26 compounds with six major compounds viz; citronellol, phytol acetate, campesterol, squalene, stigmasterol, and hexadecanoic acid. These compounds are reported for the first time from *P. integrifolia* except phytol and stigmasterol. Our previous study validates the hepatoprotective potential of *P. integrifolia* but there was no idea about the bioactive compound responsible for the activity. So, in present work, the major compounds identified in spectrometry analysis were subjected to in silico docking against an important liver enzyme alanine amino transaminase to confirm its hepatoprotective properties. Docking analysis validates the presence of two hepatoprotective lead compounds stigmasterol, and campesterol, which satisfy the drug-likeness criteria with good absorption, distribution, metabolism, and toxicity properties. Thus, present work gives a clear insight about the influence of season on the total polyphenolic constituent in different plant parts of *P. integrifolia,* their antioxidant potential and preclinical evaluation of hepatoprotective lead compounds.

Keywords Antioxidants · Hepatoprotective · Lead compounds · Phenolics · Flavonoids

Abbreviations

EAEPI	Ethyl acetate extract of *Premna integrifolia*
DPPH	2,2-Diphenyl-1-picrylhydrazyl
NBT	Nitro blue tetrazolium
TBARS	Thiobarbituric acid-reactive species
TPC	Total phenolic content
TFC	Total flavonoid content
ALT	Alanine aminotransaminase
ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicity

Introduction

Changing environmental conditions (Fokum et al. 2017), living style (McKillop and Schrum 2005) and food habit (Shin et al. 2016; Singh et al. 2019) of people caused several health complications. Several phytoconstituents available in medicinal plants contribute major role in scavenging reactive oxygen species (ROS), which provides protection against damages caused by the xenobiotics.

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Several factors viz; genetical set up of the plant, physiological factors, environmental conditions, and geographical variations influence the phytochemical constituent of plant (Yosr et al. 2018). Seasonal variations have a major influence on the phytochemical composition of a species (Lopez-Lázaro 2009). *Premna integrifolia* L. commonly known as Agnimantha is a member of family *Lamiaceae* (Group 2009). Traditionally, it is used as anticoagulant (Gopal and Purushothaman 1984), cardioprotective (Bose et al. 2012), anti-inflammatory and anti-arthritic (Rajendran and Krishnakumar 2010), antidiabetic (Majumder et al. 2014), and anti-hyperlipidaemic (Patel and Patel 2012). The present study reports the comparative analysis of seasonal influence on polyphenolic constituent and antioxidant activity of different plant parts of *P. integrifolia.* Stems, roots and leaves of *P. integrifolia* were extracted in different solvents like ethanol, methanol, ethyl acetate and water in different season and subjected to analysis. Among different extracts, ethyl acetate extract of leaves was showing maximum antioxidant activity with high polyphenolic constituent. Therefore, this extract was characterized by UHPLC-Q-TOF-MS/MS and GC–MS analysis to know the active ingredient present in the extract. Previous reports validates the antioxidant property of *P. integrifolia* root (Gokani et al. 2011), stem bark (Majumder et al. 2014), wood (Muthukumaran et al. 2013; Rajendran et al. 2009) and leaves (Selvam et al. 2012), but there is no report of the comparative study of different plant parts influenced by season of collection for their total phenol, flavonoid content and antioxidant activity. In previous study, we reported the hepatoprotective activity of *P. integrifolia* (Singh et al. 2018) but we could not find the bioactive constituent responsible for hepatoprotective activity. Therefore, in present study we have performed in silico analysis of hepatoprotective property of major compounds of *P. integrifolia* identified by GC–MS and LC–MS as ligand by using an important liver enzyme alanine amino transaminase. This is the first report of the in-silico docking analysis in *P. integrifolia* for hepatoprotective biomarkers from UHPLC-Q-TOF–MS/MS and GC–MS identified compounds.

Materials and methods

Chemicals 0.1,1-Diphenyl,2-picryl hydrazyl (DPPH), riboflavin, nitroblue tetrazolium (NBT), L-methionin, thio barbutiric acid (TBA), ethylenediaminetetraacetic acid (EDTA) gallic acid, rutin, ascorbic acid, potassium ferricynide [$K_3Fe(CN)_6$], trichloro acetic acid (TCA),and ferric chloride ($FeCl_3$) were purchased from Hi- media Ltd. All reagents were of analytical grade.

Plant collection and extract preparation

Plant parts namely leaves, stem bark and root of *P. integrifolia* were collected from the ayurvedic garden of Banaras Hindu University, Varanasi during month of December for winter, May for summer and August for rainy season (Fig. 1). The plant was taxonomically identified by Prof. V.K. Joshi, Department of Dravyaguna, Faculty of Ayurveda, IMS, Banaras Hindu University, Varanasi, India. A voucher specimen BSI/CRC/2016–17 was deposited in the Botanical Survey of India. Collected plant sample leaves, stem bark and root were washed thoroughly under running tap water in order to avoid dust, and shade dried at room temperature and then powdered in a mechanical grinder. All extracts were prepared using protocol of Upadhyay et al. (2014).

DPPH radical scavenging activity

The free radical scavenging capacity of the extracts were estimated using modified protocol of Upadhyay et al. (2014). Different concentrations of plant extracts were mixed with 5 ml of a 0.005% methanolic solution of DPPH and incubated for 15 min at room temperature. Absorbance was recorded at 517 nm.

Lipid peroxidation assay

Lipid peroxidation was measured using modified protocol of Upadhyay et al. (2014). 50 µl of each extract and egg homogenate (250 µl, 10% in distilled water, v/v) were mixed in a flask, and the final volume was made up to 500 ml by adding distilled water. Finally, 20 µl $FeSO_4$ (0.07 M) was added to the above mixture and incubated for 40 min, to induce lipid peroxidation. This mixture was mixed with 750 µl of 20% acetic acid (pH 3.5) and 750 µl of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulphate) and 25 µl 20% TCA and then heated in a boiling water bath for 60 min. After cooling, 3.0 ml of 1-butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured against 3 ml butanol at 532 nm. Distilled water was used as blank in place of the extract.

Superoxide radical scavenging property

A modified protocol of Upadhyay et al. (2018) was used for superoxide radical scavenging activity. In this assay, reaction mixture of 5 ml was made by adding 0.01 M a phosphate buffer solution (PBS) (pH 7.8), 130 mM methionine, 60 µM riboflavin, 0.5 mM EDTA, 0.75 mM NBT and 0.5 ml of test sample solution. The reaction mixture was kept in front of fluorescent light for 5 min, and

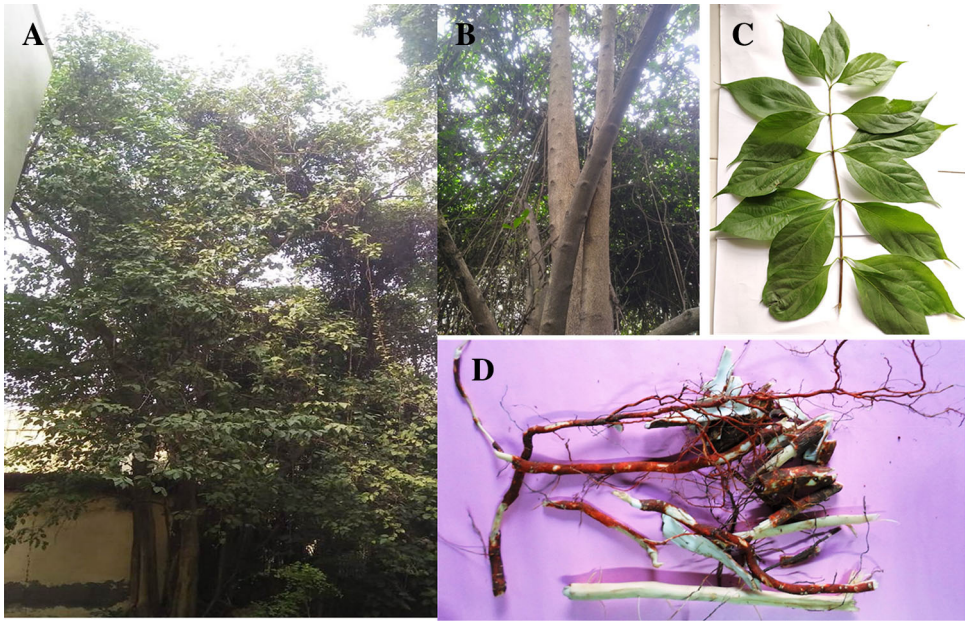


Fig. 1 *Premna integrifolia* **a** whole plant, **b** stem, **c** leaf, and **d** root

absorbance was recorded at 560 nm. For control, identical tubes containing the reaction mixture was kept in the dark. The percentage inhibition was measured by comparing the absorbance of the control and the test sample. The blank was 0.01 M PBS.

Reducing power assay (RP). The reducing power of extracts was determined by using modified protocol of Mishra et al. (2016). Different concentrations (50–1000 µg ml^{-1}) of extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)6$] (2.5 ml, 1%). The mixture was incubated at 37 °C for 15 min and after that 2.5 ml of trichloroacetic acid (TCA, 10%) was added to the mixture and centrifuged at 1000 rpm for 115 min. The upper organic layer of solution (2.5 ml) was taken and mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and then absorbance was recorded at 700 nm. High absorbance indicated high reducing power. Ascorbic acid was used as the standard in this assay.

Determination of total phenol content (TPC)

The TPC was determined by Folin-Ciocalteu method described by McDonald et al. (2001) with some modification. Briefly, 1 ml of distilled water, 0.1 ml of 1 mg ml^{-1} sample, and 0.3 ml of Folin-Ciocalteu reagent with distilled water (1:1) were mixed in a test tube, and kept for 5–8 min at room temperature. Then, 2 ml of 7% sodium carbonate solution was added, and reaction volume was maintained at 3 ml. Solutions were incubated for 1 h at room temperature, the absorbance was recorded at 750 nm. All determination was carried out in triplicate. Various concentrations of gallic acid used for the preparation of the standard curve and expressed as microgram per milligram of gallic acid equivalents (GAE).

Measurement of total flavonoid content (TFC)

Determination of TFC was done by $AlCl_3$ colorimetric method with slight modification in protocol (Chang et al. 2002). Different extracts (0.1 ml of 1 mg ml^{-1}) in ethanol were mixed with 0.1 ml of 2% $AlCl_3$, 0.1 ml of 1 M potassium acetate, and 2.7 ml of ethanol. The reaction mixture was kept at room temperature for 30 min and absorbance was recorded at 415 nm. TF content was calculated using rutin as the standard and expressed as micrograms per milligram of rutin equivalents (RE).

Profiling of bioactive compounds using UHPLC-Q-TOF-MS/MS

Ethyl acetate extract of leaves collected from rainy season (EAEPI) was characterized using modified protocol of (Singh et al. 2018). The separation of EAEPI was done on an Acquity UPLC system (Waters, Milford, MA, USA) equipped with a BEH C18 column (100 mm × 2.1 mm, 1.7 µm). The column temperature was set at 25 °C. The mobile phase consisted of acetonitrile (B) and methanol (C) acidified with 0.1% formic acid (A). The flow rate of the mobile phase was set at 300 µl/min while 5 µl injection volumes were used. Analytes were eluted by using a gradient elution programme as follows: The initial composition of B:C was 90:10% and increased to 80:20% in 2 min, 50:50% for 1–3 min, 30:70% for 3–6 min, then 10:90% for

1 min and finally, decreased quickly to 90:10% for 7–10 min. The sample was analyzed by mass spectroscopic method (positive and negative ion modes equipped with an electrospray ionization (ESI) source) for the presence of any bioactive compounds. The following mass spectroscopy parameters were applied: The cone and desolation gas flows were 52 l/h and 647 l/h, respectively; the source and desolation temperatures were 40 °C and 450 °C, respectively. The capillary and cone voltage were set at 2.72 kV and 40 eV, respectively. The Q-TOF mass spectrometer was conducted in MSE mode with a low collision energy set at 6 eV in the first function and a collision energy ramp from 20 to 40 eV in the second function. Centroid mode data was collected over the m/z range 100–1000 in both functions, and the scan time was 1 s with an interscan delay of 0.024 s. The accurate mass and molecular formula denomination were acquired with the Mass Lynx 4.1 software (Waters MS Technologies).

Profiling of bioactive compounds using GC–MS analysis

The EAEPI was used for the GC–MS analysis using modified protocol of Tiwari et al. (2016). The bioactive constituents were identified by comparison of their retention indices (RI) relative to homologous alkane series (purchased from Sigma, St. Louis, USA) and by comparison of their mass spectral fragmentation patterns with those data provided in WILEY8.LIB and NIST11.LIB. Identification was assumed when a good match of mass spectrum and RI was achieved. EAEPI was subjected to GC–MS analysis on a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) system with headspace sampler (AOC-20 s) and auto-injector (AOC-20i), equipped with the mass selective detector, having ion source temperature of 230 °C, interface temperature of 270 °C, a solvent cut time of 3.50 min threshold of 1000 eV and mass range of 40 to 650 m/z. Compounds were separated using a Rtx 5 MS capillary column (Restek Company, Bellefonte, USA: cross bond 5% diphenyl/95% dimethyl polysiloxane) having dimensions 30 m (length) × 0.25 mm (diameter) × 0.25 µm (film thickness). The split mode at a ratio of 10:1 was used. The temperature of the injector was initialized to 260 °C, having a split injection mode. The temperature was programmed from 80 °C (2 min), then further increased to 250 °C at a ramp rate of 10 °C/min (5 min hold) and finely increased to 280 °C at a ramp rate of 15 °C/min (24 min hold). Helium (> 99.999%) was used as the carrier gas at a linear flow velocity of 40.5 cm/s. The debit of gas (helium) vector with a total flow of 1.21 mL/min was fixed to 16.3 mL/min. The volume of the injected sample was 2 µL of methanol extract. Total MS running time was 46 min.

Proteins and ligands for in silico docking

The major compounds identified in UPLC-Q-TOF-MS/MS and GCMS compounds and a standard hepatoprotective drug known as silibinin were used as ligand for molecular docking analysis. The three-dimensional (3d) structure of the compounds was downloaded from Pubchem database (https://pubchem.ncbi.nlm.nih.gov) in sdf file then converted into Pdb file through Open Babel Gui. The protein structure (3d) of the enzyme alanine aminotransaminase with pdb id 3IHJ was downloaded from protein data bank (https://www.rcsb.org/) in pdb format.

Molecular docking analysis

The molecular docking studies of all selected compounds and proteins were performed with Auto doc tools 1.5.6 (Morris 2009). The interaction between ligand and protein were assessed on the basis of binding energies.

Evaluation of drug likeness and ADMET properties

The drug likeness properties of the compounds were evaluated by Lipinski's rule of five (http://scfbio-iitd.res.in/software/drugdesign/lipinski.jsp#anchortag) (Lipinski et al. 1997). ADMET properties of compounds viz; adsorption, distribution, metabolism, excretion, and toxicity were evaluated using ADMETlab2 https://admetmesh.scbdd.com/service/evaluation/cal to know the pharmacologically potent hepatoprotective lead molecule (Xiong et al. 2021). For ADMET properties evaluation, different parameters viz; blood–brain absorption (BBB), human intestinal absorption (HIA), AMES toxicity, and carcinogenicity were used for scoring.

Statistical analysis

All experiments were performed in triplicate and data were expressed as means ± SE. Statistical comparisons were made by means of one-way ANOVA test followed by post hoc analysis with Dunnett's test by using SPSS (version 16). $P < 0.05$, $P < 0.01$ was considered a significant difference, while $P > 0.05$ was considered to be non-significant. EC_{50} values were calculated from linear regression analysis.

Results

DPPH radical scavenging assay

All three extracts of leaf, stem and root have significant free radical scavenging activity (Table 1). Lowest EC_{50}

Table 1 Comparative DPPH free radical scavenging activity of leaf, stem, and root extracts of *P. integrifolia* in different seasons

Concentration (µg/ml)	Ethanol extract			Methanol extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	1.50 ± 0.68**	1.01 ± 0.35**	0.93 ± 0.61**	3.30 ± 0.39**	2.85 ± 0.19**	2.67 ± 1.23**
100	12.83 ± 0.75**	12.53 ± 0.83**	11.56 ± 0.12**	12.07 ± 0.87**	11.54 ± 1.34**	11.23 ± 0.56**
200	22.10 ± 0.83**	21.23 ± 0.25**	21.63 ± 1.32**	19.59 ± 0.50**	19.01 ± 1.02**	18.67 ± 0.34**
300	27.31 ± 0.52**	26.12 ± 1.23**	26.10 ± 3.12**	28.22 ± 0.66**	27.12 ± 0.56**	26.65 ± 0.19**
400	39.53 ± 0.14**	38.35 ± 0.14**	37.15 ± 2.12**	34.07 ± 0.84**	32.83 ± 0.12**	31.83 ± 1.32**
500	51.82 ± 0.83**	50.65 ± 1.32**	49.48 ± 1.23**	47.46 ± 1.33**	46.45 ± 1.53**	45.32 ± 2.12**
600			59.23 ± 2.35**	53.43 ± 0.75**	51.67 ± 1.23**	51.26 ± 1.12**
700						
EC_{50}	**485.02 ± 1.32**	**498.14 ± 1.23**	**505.13 ± 1.26**	**578.34 ± 2.43**	**585.35 ± 2.12**	**588.59 ± 2.10**
Inhibition Percentage (Stem)						
50	11.50 ± 0.57*	10.91 ± 2.65*	10.34 ± 1.23**	2.84 ± 0.36*	1.94 ± 1.26**	1.35 ± 1.25*
100	17.88 ± 0.56**	16.89 ± 0.19**	16.43 ± 1.23 ns	5.05 ± 0.68*	4.85 ± 2.12**	3.98 ± 1.23**
200	23.24 ± 0.34*	23.01 ± 0.34*	22.01 ± 2.13*	19.30 ± 0.74*	18.21 ± 0.56*	17.21 ± 0.56*
300	31.56 ± 0.53**	30.65 ± 0.19**	29.83 ± 1.23**	27.40 ± 0.57**	26.15 ± 1.25**	24.25 ± 1.35*
400	41.65 ± 1.01*	41.12 ± 1.01**	38.96 ± 2.12 ns	34.56 ± 0.95*	32.85 ± 1.19**	30.83 ± 1.23*
500	53.31 ± 0.67*	52.67 ± 1.26**	50.12 ± 1.56**	42.36 ± 1.15**	39.12 ± 1.26*	36.95 ± 0.35**
600				52.19 ± 0.56**	48.56 ± 1.36**	46.35 ± 1.38**
700				63.32 ± 0.27**	58.45 ± 1.26**	56.84 ± 0.83**
800						
1000						
1200						
1400						
1600						
1800						
2000						
2200						
EC_{50}	**485.15 ± 0.75**	**496.26 ± 1.23**	**503.12 ± 2.13**	**615.12 ± 1.26**	**645.15 ± 1.26**	**668.12 ± 2.01**
Inhibition Percentage (root)						
50	5.16 ± 1.26**	5.05 ± 0.75*	4.98 ± 0.19**	11.48 ± 0.61*	10.86 ± 0.27*	10.87 ± 1.12**
100	14.01 ± 1.03**	13.67 ± 1.27**	14.03 ± 1.19*	12.53 ± 0.57*	12.21 ± 0.47**	13.01 ± 0.21*
200	33.12 ± 0.12 ns	32.18 ± 1.34 ns	33.12 ± 1.45*	16.53 ± 0.66*	14.45 ± 1.27**	14.26 ± 1.27*
300	45.12 ± 1.23**	44.72 ± 1.19*	44.74 ± 0.18**	20.12 ± 0.17*	19.87 ± 0.26*	18.76 ± 0.57*
400	57.36 ± 1.17*	57.12 ± 0.34**	57.19 ± 1.19 ns	25.66 ± 0.38*	23.23 ± 2.01**	24.27 ± 1.28**
500				28.1 ± 0.51 ns	28.41 ± 0.31*	28.18 ± 0.57 ns
600				31.17 ± 0.43*	29.96 ± 1.29*	29.65 ± 0.29*
800				43.16 ± 0.34*	43.12 ± 0.57**	43.17 ± 0.17*
1000				52.58 ± 0.51*	51.12 ± 1.24**	52.17 ± 0.56*
1200						
1400						
1600						
EC_{50}	**489.16 ± 0.29**	**490.18 ± 0.18**	**490.10 ± 1.67**	**998.12 ± 2.18**	**1001.19 ± 0.19**	**999.89 ± 1.34**
Concentration (µg/ml)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	22.00 ± 0.48**	20.56 ± 0.81**	19.31 ± 1.26**	0.70 ± 0.22**	0.60 ± 1.21**	0.52 ± 1.12**
100	24.86 ± 0.62**	24.86 ± 1.23**	23.12 ± 2.15**	4.25 ± 1.05**	3.65 ± 0.62**	3.12 ± 1.23**
200	40.14 ± 0.45**	39.12 ± 0.41**	37.12 ± 0.56**	10.17 ± 0.60**	9.64 ± 0.65**	8.64 ± 0.12**
300	50.25 ± 0.54**	48.35 ± 0.54**	46.95 ± 1.23**	20.65 ± 0.87**	19.46 ± 1.34**	18.12 ± 2.15**
400	61.34 ± 0.56**	57.32 ± 1.35**	54.12 ± 1.26**	27.62 ± 0.46**	25.12 ± 3.12**	25.00 ± 3.12**

Table 1 continued

Concentration (μg/ml)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
500				36.84 ± 0.75**	35.53 ± 1.23**	35.12 ± 1.23**
600				43.75 ± 0.98**	42.12 ± 1.12**	40.95 ± 1.12**
700				52.47 ± 0.69**	50.84 ± 1.26**	50.12 ± 1.26**
EC_{50}	**301.23 ± 1.25**	**345.46 ± 1.35**	**369.12 ± 1.23**	**715 ± 1.26**	**765 ± 0.83**	**777.05 ± 1.23**
Inhibition Percentage (Stem)						
50	17.93 ± 0.87**	16.38 ± 1.23**	15.12 ± 1.23**	0.47 ± 0.16**	0.46 ± 0.23**	0.43 ± 0.32**
100	23.00 ± 0.50*	22.56 ± 0.83**	20.18 ± 0.83**	1.02 ± 0.42*	1.21 ± 0.31**	1.31 ± 0.29**
200	33.80 ± 0.66*	31.45 ± 1.23**	29.83 ± 1.23**	2.99 ± 0.29**	2.801 ± 1.21*	2.58 ± 1.12**
300	43.86 ± 1.21 ^{ns}	40.95 ± 2.15 ^{ns}	38.35 ± 0.34**	4.21 ± 0.17 ^{ns}	4.51 ± 0.42**	3.83 ± 0.11**
400	53.49 ± 0.66*	49.85 ± 1.23*	46.35 ± 2.15**	5.07 ± 0.19*	4.95 ± 0.34 *	4.39 ± 0.38**
500	63.23 ± 0.39 ^{ns}	58.32 ± 0.86**	56.38 ± 1.23 ^{ns}	6.72 ± 0.45**	6.42 ± 0.51 ^{ns}	6.72 ± 0.39*
600				8.52 ± 0.25**	8.12 ± 0.21**	8.02 ± 0.35**
700				11.21 ± 0.29 ^{ns}	10.79 ± 0.42**	10.68 ± 0.39 ^{ns}
800				14.82 ± 0.44**	14.15 ± 0.23**	14.12 ± 0.35**
1000				19.57 ± 0.22*	18.95 ± 1.12*	18.05 ± 1.21**
1200				27.98 ± 0.42 ^{ns}	27.12 ± 1.12**	26.83 ± 1.12*
1400				32.65 ± 0.61**	31.95 ± 0.12**	30.98 ± 1.12**
1600				38.86 ± 0.34**	37.98 ± 1.25**	35.12 ± 0.53**
1800				43.72 ± 0.60*	43.12 ± 1.12*	42.12 ± 0.83*
2000				51.94 ± 1.00**	50.12 ± 1.21**	49.32 ± 1.25**
2200				61.61 ± 0.39**	58.61 ± 0.39**	56.23 ± 1.30**
EC_{50}	**398.12 ± 2.05**	**427.12 ± 1.35**	**445.23 ± 2.08**	**2015 ± 1.32**	**2041 ± 1.32**	**2105 ± 1.35**
Inhibition Percentage (root)						
50	8.81 ± 0.37*	9.18 ± 0.34**	9.20 ± 1.23**	6.34 ± 0.38*	6.32 ± 0.29**	6.15 ± 0.17**
100	12.54 ± 0.23**	12.63 ± 0.12*	12.67 ± 0.83**	8.29 ± 0.51 ^{ns}	7.94 ± 0.27*	8.21 ± 0.41**
200	24.66 ± 0.46*	23.15 ± 1.26*	23.25 ± 1.35 ^{ns}	10.84 ± 0.32**	10.89 ± 1.29**	9.98 ± 0.18*
300	31.40 ± 0.48**	30.56 ± 1.21*	30.12 ± 1.35**	14.21 ± 0.52 ^{ns}	13.21 ± 0.27 ^{ns}	13.14 ± 0.57 ^{ns}
400	44.77 ± 0.60 ^{ns}	44.85 ± 1.26*	44.01 ± 0.83 ^{ns}	17.55 ± 0.99*	17.49 ± 0.97**	17.50 ± 1.45**
500	51.78 ± 0.79*	50.12 ± 1.38*	50.12 ± 0.83*	18.21 ± 0.21 ^{ns}	17.44 ± 0.17 ^{ns}	18.10 ± 0.26*
600				20.33 ± 0.51**	19.76 ± 0.28**	19.67 ± 1.28**
800				26.58 ± 0.10*	26.87 ± 0.16*	26.29 ± 1.29**
1000				31.23 ± 0.51**	31.00 ± 0.32**	31.29 ± 1.21 ^{ns}
1200				35.94 ± 0.40*	35.87 ± 1.34*	34.76 ± 0.19**
1400				43.47 ± 0.61*	42.35 ± 0.83*	42.19 ± 0.29*
1600				51.38 ± 0.46**	51.19 ± 0.53*	51.19 ± 0.18**
EC_{50}	**502.56 ± 1.23**	**505.53 ± 0.17**	**507.29 ± 0.19**	**1603.28 ± 1.26**	**1605.16 ± 0.29**	**1608.19 ± 0.28**

EC_{50} value of ascorbic acid is 54.23 ± 2.35; *Significant $P < 0.05$, **$P < 0.01$ and $P > 0.05$ non-significant (ns)

value was observed in leaf extract of ethyl acetate of rainy season (301.23 ± 1.25 μg/ml) compared to other extracts in their respective season. Antioxidant activity of rainy season ethyl acetate extract was higher compared to summer and winter extracts. Leaf sample of summer and winter season showed EC_{50} values 345.46 ± 1.35 and 369.12 ± 1.23 μg/ml respectively. EC_{50}value of leaf sample extracted in ethanol during rainy season was 485.04 ± 1.32 μg/ml, while for summer and winter season extracts EC_{50} values was 498.14 ± 1.23 and 505.13 ± 1.23 μg/ml respectively. In case of methanol and aqueous extract of leaf, EC_{50} value of different seasonal extracts was in this order rainy < summer < winter. If we compare the EC_{50}value of DPPH free radical scavenging activity of different extracts of stem, the trend was in this order ethyl acetate < ethanol < methanol < aqueous extract. On the basis of comparison of EC_{50} values of different root extracts the range was as follows: ethanol extract (EC_{50} = 489.16–490.16) < ethyl acetate (EC_{50} = 502.56–507.29) < methanol (EC_{50} = 998.12–999.89) < aqueous extract (EC_{50} = 1603.28–1608.19). Antioxidant activity of rainy season ethanol extract of root was higher

(EC_{50} = 489.16 ± 0.29) compared to summer (EC_{50} = 490.18 ± 0.18) and winter (EC_{50} = 490.10 ± 1.67) extracts.

Superoxide radical scavenging assay

Results revealed that leaf, stem and root exhibited potent scavenging activity for superoxide radicals in a concentration dependent manner (Table 2). Superoxide scavenging activity of leaf extract was higher compared to stem and root extracts as evident from EC_{50} values. Among leaf extracts, ethyl acetate extract has a lower range of EC_{50} values (349.12 ± 1.26–383.19 ± 1.67) compared to ethanol (477.15 ± 0.12–495.18 ± 0.12), methanol (677.02 ± 1.26–697.25 ± 0.27) and aqueous (1096.12 ± 2.10–1112.45 ± 0.67) extracts. In conclusion, among all extracts, ethyl acetate extract of leaf of rainy season exhibited (EC_{50} = 349.12 ± 1.26) highest potential for superoxide radical scavenging activity compared to stem (EC_{50} = 707.12 ± 0.27) and root (EC_{50} = 733.91 ± 1.21) extracts.

Lipid peroxidation assay

If we compare the EC_{50} values of lipid peroxidation of different extracts derived from different parts of plant, we can say that leaf extracts showed lower range of EC_{50} values (339.12 ± 0.23–599.19 ± 1.18) compared to stem (EC_{50} = 648.24 ± 0.38–1367.99 ± 0.54) and root (EC_{50} = 485.12 ± 0.67– 991.88 ± 0.65) extracts. It means leaf extract showed better inhibition of lipid peroxidation compared to stem and root extracts. Among all extracts, leaf ethyl acetate extracts exhibited lower range of EC_{50} value (339.12 ± 0.23–354.00 ± 0.13) compared to ethanol (503.15 ± 0.19–531.37 ± 1.12), methanol (579.56 ± 0.76–589.14 ± 0.56) and aqueous (585.23 ± 0.45–599.19 ± 1.18) extracts. EC_{50} values of ethyl acetate extracts obtained in different seasons are in the order rainy (339.12 ± 0.23) < summer (348.43 ± 0.25) < winter (354.00 ± 0.13). So, we can conclude that ethyl acetate extract of leaves derived during rainy season has highest lipid peroxidation inhibition (Table 3).

Total phenolic content (TPC)

The phenolic compounds may contribute directly to antioxidant action (Awika et al. 2003). Total phenolic content was determined spectrophotometrically by Folin-ciocalteu method and reported as GAE in reference to standard curve (y = 0.003x–0.041, R^2 = 0.945). In ethyl acetate extract of leaf, TPC was higher in rainy season which was 74.33 ± 2.26 mg g^{-1} followed by summer and winter having phenolic content 70.13 ± 0.26 mg g^{-1}, 65.10 ± 1.26 mg g^{-1}, respectively. In ethanolic extract of leaf the TPC was higher in rainy season which was 68.33 ± 0.56 mg g^{-1} while summer and winter had 64.3 ± 1.26 mg g^{-1}, 62.32 ± 0.23 mg g^{-1}, respectively. In methanol extract of leaf, TPC was higher in rainy season which was 64.43 ± 0.76 mg g^{-1} while by summer and winter had 60.3 ± 1.06 mg g^{-1}, 54.06 ± 0.23 mg g^{-1}, respectively. TPC in aqueous extract of leaf of rainy season was 49.0.35 ± 0.35 mg g^{-1} while by summer and winter season had 41.09 ± 0.20 mg g^{-1} and 39.00 ± 0.26 mg g^{-1}. TPC in ethyl acetate extract of stem bark of rainy season was 50.33 ± 1.23 mg g^{-1} while summer and winter season had 42.09 ± 0.25 mg g^{-1} and 39.00 ± 0.36 mg g^{-1}. TPC in ethanol extract of stem bark of rainy season was 48.66 ± 0.35 mg g^{-1} while summer and winter season had 39.09 ± 0.27 mg g^{-1} and 37.65 ± 0.44 mg g^{-1}, respectively. TPC in methanolic extract of stem bark of rainy season was 46.33 ± 1.73 mg g^{-1} while summer and winter season had 35.19 ± 0.20 mg g^{-1} and 32.01 ± 0.26 mg g^{-1}. TPC in aqueous extract of stem bark of rainy season was 41.66 ± 0.25 mg g^{-1} while summer and winter season had 31.29 ± 0.20 mg g^{-1} and 27.66 ± 0.46 mg g^{-1}. Root extract, TPC was higher in ethyl acetate extract of summer season which was 46.35 ± 2.12 mg g^{-1} while rainy and winter season had 46.33 ± 1.32 mg g^{-1} and 45.84 ± 0.81 mg g^{-1}, respectively, which indicates almost same value (Fig. 2).

Total flavonoid content (TFC)

Total flavonoid content was determined by $AlCl_3$ colorimetric method and rutin was used as standard for making curve (y = 0.006x–0.104, R^2 = 0.966). Flavonoid content in different extracts of different parts during change in the season was represented in Fig. 3. Flavonoid content was higher in ethyl acetate extract of leaf of rainy season (98.83 ± 0.26 µg/mg) compared to summer (86.43 ± 0.43 µg/mg) and winter season (76.33 ± 0.23 µg/mg). In ethanol extract of leaf, flavonoid content in rainy season was observed higher (68.5 ± 0.19 µg/mg) compared to summer (65.5 ± 1.23 µg/mg) and winter (51.81 ± 0.82 µg/mg). In methanol extract of leaves of rainy season, flavonoid content was higher (50.12 ± 2.13 µg/mg) compared to summer (45.23 ± 0.56 µg/mg) and winter (42.12 ± 1.23 µg/mg). The flavonoid content in aqueous extracts of different seasons was in this order: rainy (37.16 ± 0.82 µg/mg) > summer (30.12 ± 2.31 µg/mg) > winter (29.43 ± µg/mg). Flavonoid content in

Table 2 Comparative superoxide radical scavenging activity of leaf, stem, and root extracts of *P. integrifolia* in different seasons

Concentration (μg/mL)	Ethanol extract			Methanol extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	10.55 ± 1.00*	10.05 ± 0.23*	9.79 ± 0.15**	3.36 ± 0.48**	3.12 ± 1.28**	3.02 ± 1.16**
100	27.55 ± 0.84**	26.18 ± 0.45 ns	24.96 ± 0.29 ns	11.53 ± 0.48 ns	11.21 ± 1.67*	11.10 ± 1.27 ns
200	38.55 ± 1.35*	38.01 ± 1.23**	37.09 ± 0.19**	20.03 ± 0.55**	18.98 ± 0.17*	18.54 ± 0.28 ns
300	47.84 ± 2.15 ns	45.18 ± 0.34*	45.12 ± 0.29 ns	27.08 ± 0.73*	26.45 ± 1.12 ns	26.23 ± 0.38 ns
400	58.33 ± 2.00*	54.98 ± 0.13**	54.01 ± 0.67**	32.69 ± 0.96**	31.90 ± 1.28**	30.76 ± 0.86**
500				38.46 ± 1.26**	37.67 ± 1.67**	36.89 ± 2.19 ns
600				44.71 ± 0.10**	43.18 ± 1.89 ns	43.01 ± 0.16**
700				52.24 ± 0.99**	51.23 ± 1.28**	50.98 ± 0.45**
800						
900						
1000						
EC_{50}	**477.15 ± 0.12**	**487.35 ± 1.23**	**495.18 ± 0.12**	**677.02 ± 1.26**	**689.17 ± 0.18**	**697.25 ± 0.27**
Inhibition Percentage (Stem)						
50	2.52 ± 0.31**	2.19 ± 0.45**	2.10 ± 0.29*	3.79 ± 1.02 ns	3.17 ± 0.28*	3.04 ± 0.28**
100	7.20 ± 0.82*	6.89 ± 0.18*	6.36 ± 1.10*	9.39 ± 2.01**	9.28 ± 0.45 ns	8.97 ± 1.70**
200	12.97 ± 0.54*	12.45 ± 0.28**	12.10 ± 1.02*	13.75 ± 1.20*	13.34 ± 0.19**	13.02 ± 0.34 ns
300	21.44 ± 1.35*	20.86 ± 0.18 ns	20.18 ± 0.37 ns	16.54 ± 0.77**	15.98 ± 0.28*	15.65 ± 0.28*
400	30.26 ± 1.95**	30.18 ± 1.07**	29.67 ± 0.67*	19.46 ± 0.67 ns	18.97 ± 0.29**	18.54 ± 0.19**
500	38.19 ± 0.62**	37.89 ± 0.29 ns	37.84 ± 1.23**	23.71 ± 0.77*	23.65 ± 1.29 ns	22.97 ± 1.89 ns
600	45.07 ± 0.65*	44.89 ± 0.18**	44.18 ± 1.43*	25.35 ± 0.36**	25.19 ± 0.18**	25.19 ± 0.29**
700	50.62 ± 0.83**	50.56 ± 0.18*	49.19 ± 0.29**	31.31 ± 1.54**	30.97 ± 0.29**	30.78 ± 0.34*
800			58.08 ± 1.78*	36.46 ± 3.20**	35.34 ± 1.49 ns	35.12 ± 0.56 ns
900				44.63 ± 1.55*	44.42 ± 1.45*	44.12 ± 0.34**
1000				51.22 ± 1.93*	50.67 ± 0.28**	50.23 ± 1.23**
1200						
1400						
1600						
EC_{50}	**727.23 ± 0.26**	**738.18 ± 1.29**	**746.13 ± 1.91**	**1094.12 ± 1.23**	**1105.11 ± 0.18**	**1109.00 ± 0.10**
Inhibition Percentage (Root)						
50	3.82 ± 0.48**	3.56 ± 0.18*	3.45 ± 0.18*	3.36 ± 0.48**	3.31 ± 0.28*	3.29 ± 0.28**
100	14.36 ± 1.91 ns	14.15 ± 0.38**	13.98 ± 0.28**	11.53 ± 0.48**	11.20 ± 0.19**	11.10 ± 0.19*
200	21.36 ± 0.54*	20.89 ± 0.18**	20.29 ± 0.28**	20.03 ± 0.55*	19.89 ± 0.28**	19.28 ± 0.19**
300	33.17 ± 0.73*	32.67 ± 0.49*	32.78 ± 0.19*	27.08 ± 0.73*	26.98 ± 0.19 ns	26.34 ± 0.49**
400	41.62 ± 0.96 ns	41.07 ± 0.39**	40.19 ± 0.29**	32.69 ± 0.96**	32.65 ± 1.04*	31.19 ± 0.29 ns
500	52.26 ± 3.58*	51.67 ± 0.38**	51.29 ± 0.19*	38.46 ± 1.26**	37.19 ± 0.89*	37.19 ± 0.39**
600				44.71 ± 0.00*	44.13 ± 0.29*	43.96 ± 0.39**
700				52.24 ± 0.99**	51.01 ± 0.29*	50.98 ± 0.59 ns
800						
900						
1000						
1200						
EC_{50}	**485.12 ± 0.67**	**496.10 ± 0.19**	**499.10 ± 0.14'**	**677.02 ± 1.26**	**684.19 ± 0.29**	**686.02 ± 0.29**
Concentration (μg/mL)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	12.51 ± 2.58**	11.23 ± 0.34 ns	10.69 ± 0.19 ns	0.87 ± 0.31*	0.81 ± 1.26*	0.80 ± 1.10**
100	18.53 ± 1.09 ns	16.90 ± 1.13**	16.78 ± 0.14**	2.02 ± 0.21**	1.98 ± 0.45**	1.95 ± 0.38**
200	43.68 ± 0.68**	43.12 ± 0.67*	42.67 ± 1.17 ns	6.36 ± 0.33*	6.12 ± 1.34*	6.28 ± 1.20**
300	52.32 ± 1.87**	51.56 ± 2.13*	50.29 ± 2.01**	8.29 ± 0.27 ns	8.01 ± 0.28*	7.87 ± 0.18 ns

Table 2 continued

Concentration (μg/mL)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
400				16.59 ± 1.13 ns	15.98 ± 1.16 ns	15.85 ± 0.18**
500				25.24 ± 1.45**	25.21 ± 1.45**	25.12 ± 0.29 ns
600				27.98 ± 0.13**	25.95 ± 0.18**	25.07 ± 1.20*
700				36.40 ± 0.17 ns	34.38 ± 1.28**	34.30 ± 1.10*
800				44.34 ± 0.33**	43.18 ± 0.18**	42.17 ± 0.28**
900				47.89 ± 0.45*	45.98 ± 0.29 ns	44.29 ± 0.19**
1000				59.55 ± 0.31**	57.06 ± 1.17**	56.89 ± 0.29**
EC_{50}	**349.12 ± 1.26**	**376.45 ± 0.24**	**383.19 ± 1.67**	**1096.12 ± 2.10**	**1105.29 ± 1.34**	**1112.45 ± 0.67**
Inhibition Percentage (Stem)						
50	6.32 ± 1.40*	5.97 ± 0.56*	5.24 ± 0.56*	0.47 ± 0.64**	0.42 ± 0.17*	0.42 ± 0.6*
100	14.14 ± 1.16*	13.76 ± 1.23 ns	13.34 ± 0.56*	2.5 ± 0.34**	2.18 ± 0.45**	2.10 ± 1.76*
200	19.92 ± 0.85**	19.01 ± 0.56*	18.89 ± 0.56 ns	7.10 ± 0.12**	7.10 ± 0.28 ns	7.10 ± 0.28 ns
300	25.32 ± 0.85 ns	24.98 ± 0.34*	24.45 ± 1.79*	10.12 ± 0.31 ns	9.98 ± 1.45*	9.76 ± 0.17*
400	32.40 ± 0.56*	31.12 ± 0.34 ns	31.01 ± 0.45*	12.70 ± 0.55*	11.96 ± 1.29**	11.45 ± 0.13*
500	39.66 ± 1.48**	38.56 ± 2.01*	38.53 ± 0.34 ns	15.50 ± 0.50**	15.01 ± 0.29 ns	14.89 ± 0.28*
600	47.41 ± 0.46**	45.87 ± 1.03*	45.54 ± 1.24**	18.01 ± 0.19*	17.86 ± 1.67 ns	17.85 ± 0.56 ns
700	53.81 ± 0.31*	52.02 ± 0.34 ns	51.76 ± 0.78*	21.43 ± 0.11 ns	21.23 ± 0.54**	21.21 ± 0.87**
800				24.56 ± 0.34**	24.29 ± 0.18 ns	23.79 ± 0.56*
900				28.50 ± 0.38**	28.36 ± 0.28*	28.01 ± 0.11**
1000				33.09 ± 0.62**	32.19 ± 0.28*	32.12 ± 0.45*
1200				37.01 ± 0.55**	36.89 ± 0.24*	36.56 ± 0.23**
1400				42.57 ± 0.44**	42.10 ± 0.18*	41.23 ± 0.34**
1600				52.48 ± 0.47*	50.96 ± 0.29*	50.23 ± 0.22**
EC_{50}	**707.12 ± 0.27**	**719.32 ± 0.13**	**725.19 ± 0.17**	**1638.45 ± 0.18**	**1645.19 ± 1.27**	**1651.67 ± 0.23**
Inhibition Percentage (Root)						
50	3.95 ± 0.69**	3.85 ± 0.29*	3.80 ± 1.29**	2.86 ± 0.95*	2.78 ± 0.29**	2.67 ± 0.45*
100	8.82 ± 0.61**	8.76 ± 0.29*	8.67 ± 0.29*	7.96 ± 2.21**	7.94 ± 1.29**	7.70 ± 0.34*
200	15.21 ± 0.80 ns	15.10 ± 0.29**	15.00 ± 0.29 ns	13.07 ± 0.72**	12.78 ± 0.19*	12.70 ± 0.29*
300	23.43 ± 0.60 ns	23.21 ± 0.39 ns	23.01 ± 0.29 ns	16.90 ± 0.72 ns	16.87 ± 0.29*	16.82 ± 0.19*
400	29.98 ± 0.15**	29.39 ± 0.32*	29.19 ± 0.21**	22.32 ± 0.27**	22.13 ± 0.29**	22.10 ± 0.29 ns
500	36.52 ± 0.69*	36.32 ± 0.39**	36.29 ± 0.19*	28.06 ± 0.27 ns	27.45 ± 0.38	27.43 ± 0.45 ns
600	46.72 ± 0.94 ns	46.29 ± 1.23 ns	45.78 ± 0.29**	32.37 ± 0.73**	32.32 ± 0.34*	31.76 ± 0.45**

Table 2 continued

Concentration (μg/mL)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
700	56.31 ± 1.84**	54.43 ± 0.19**	54.89 ± 0.87*	36.04 ± 0.73 ns	35.01 ± 0.67*	35.00 ± 0.67 ns
800				42.34 ± 0.42 ns	42.02 ± 1.76*	41.89 ± 0.45**
900				48.32 ± 1.26*	48.23 ± 0.23 ns	48.29 ± 0.29*
1000				51.99 ± 0.27**	51.35 ± 0.45 ns	51.46 ± 0.29 ns
1200				55.65 ± 0.99**	55.12 ± 0.45*	54.87 ± 0.38**
EC_{50}	**733.91 ± 1.21**	**742.19 ± 0.38**	**743.19 ± 0.19**	**978.01 ± 0.62**	**987.12 ± 0.63**	**990.43 ± 0.19**

EC_{50} of copper sulphate is 109 ± 0.21; *Significance $P < 0.05$, **$P < 0.01$ and $P > 0.05$ non-significant (ns)

different extracts of stem during rainy season was as follows: ethyl acetate (70.12 ± 0.56 μg/mg) > ethanol (51.81 ± 2.13 μg/mg) > methanol (49.16 ± 0.32 μg/mg) > aqueous extract (35.00 ± 0.32 μg/mg). Flavonoid content in ethanol, methanol, ethyl acetate and aqueous extracts of stem in summer season was 45.12 ± 0.12 μg/mg, 45.83 ± 0.53 μg/mg, 62.12 ± 0.73 μg/mg and 31.32 ± 1.32 μg/mg, respectively. Flavonoid content in ethanol extracts of roots of all three season was comparable. In rainy season, it was 64.33 ± 0.86 μg/mg, while in summer and winter flavonoid content was 65.12 ± 0.82 and 65.09 ± 0.82 μg/mg, respectively. TFC in methanol extract of rainy, winter and summer season was 45.66 ± 0.82, 45.45 ± 0.82, and 45.83 ± 1.21 μg/mg, respectively. Aqueous extract of winter contained lower flavonoid content (32.12 ± 0.39 μg/mg) compared to rainy (37.16 ± 0. 56 μg/mg) and summer season (32.15 ± 1.32 μg/mg). There was no significant difference in TFC of summer (65.12 ± 1.2 μg/mg) and winter (65.09.12 ± 0.13 μg/mg) extracts, while in rainy season extract it was 64.33 ± 0.43 μg/mg. TFC in ethyl acetate extract of root in rainy season it was 53.66 ± 1.13 μg/mg, while in summer and winter season it was 54.12 ± 0.41 and 55.01 ± 0.13 μg/mg, respectively. There was no significant difference in flavonoid content in methanol extract of root in rainy (45.66 ± 1.42 μg/mg), summer (45.45 ± 1.02 μg/mg) and winter (45.83 ± 0.73 μg/mg) seasons. Similarly, flavonoid content in aqueous extract of roots of all three season was almost similar and it was 32.16 ± 0.41, 32.15 ± 0.19 and 32.12 ± 0.57 μg/mg in rainy, winter and summer season, respectively (Fig. 3).

Reducing power assay

The reducing power of all the three extracts of leaf, stem and root in three different seasons like rainy, winter and summer, increased in concentration dependent manner as depicted in Figs. 3 and 4. Ethanol extract of leaf showed higher reducing potential in rainy season compared to summer and winter (Fig. 5a–c). Methanol extract of leaves of rainy season exhibited higher reducing potential compared to summer and winter season (Fig. 6a–c). Ethyl acetate extract of leaves of rainy season showed higher reducing potential compared to stem and root. Absorbance of rainy season's leaves extract was highest (OD = 1.21) (Fig. 6a–c). Aqueous extract of leaf showed higher reducing potential in rainy season compared to winter and summer season (Fig. 7a–c). On the basis of above results, it was observed that ethyl acetate extract of leaves during rainy season exhibited highest reducing potential.

Correlation between total antioxidant activity and total polyphenol content

Ethyl acetate extracts of different parts of the plant during rainy season contained highest polyphenol content, so a correlation between the total phenol, flavonoid content and antioxidant activity in rainy season extracts was presented in Fig. 8. A positive correlation was observed between phenol and flavonoid content as well as total antioxidant activity in the ethyl acetate extract of leaf (R^2 value ranges from 0.975 to 0.999). Similarly, the strong positive correlation was observed between antioxidant activity and phenol and flavonoid content in ethyl acetate extract of stem (R^2 value ranges from 0.921 to 0.927) and root (R^2

Table 3 Comparative lipid peroxidation activity of leaf, stem, and root extracts of *P. integrifolia* in different seasons

Concentration (µg/ml)	Ethanol extract			Methanol extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	3.75 ± 1.35**	3.43 ± 0.76**	3.29 ± 1.01*	14.57 ± 2.15*	14.28 ± 0.29**	14.12 ± 1.56*
100	6.51 ± 1.21*	6.29 ± 0.29**	6.19 ± 0.12**	21.78 ± 0.68**	21.23 ± 0.18**	20.89 ± 0.37*
200	20.79 ± 0.21**	19.89 ± 0.23*	18.28 ± 1.29*	24.80 ± 1.40**	23.94 ± 0.18**	22.45 ± 0.28**
300	30.32 ± 1.92*	29.97 ± 0.29*	29.18 ± 2.19**	31.24 ± 0.56*	30.19 ± 0.17**	30.10 ± 0.29**
400	41.47 ± 2.06*	40.49 ± 0.28**	40.19 ± 1.28**	37.30 ± 0.65**	36.20 ± 0.53*	35.95 ± 0.39**
500	52.75 ± 1.89**	51.02 ± 1.26*	50.78 ± 0.18*	41.47 ± 1.50**	41.00 ± 0.65*	41.00 ± 0.29*
600				52.83 ± 1.13**	51.26 ± 0.18**	50.87 ± 0.27**
700						
EC_{50}	**503.15 ± 0.19**	**527.54 ± 0.26**	**531.37 ± 1.12**	**579.56 ± 0.76**	**587.19 ± 0.19**	**589.14 ± 0.56**
Inhibition Percentage (Stem)						
50	1.67 ± 0.33*	1.65 ± 1.02**	1.47 ± 0.67*	4.19 ± 0.17*	4.10 ± 1.20**	4.01 ± 0.36**
100	4.39 ± 0.38**	3.98 ± 0.28**	3.37 ± 0.48**	6.96 ± 0.19**	6.46 ± 0.29*	6.34 ± 0.28**
200	8.31 ± 0.39**	8.19 ± 0.17^{ns}	8.11 ± 0.34**	11.47 ± 0.50^{ns}	11.17 ± 0.25*	11.56 ± 0.45^{ns}
300	17.68 ± 0.57*	17.28 ± 1.11^{ns}	16.57 ± 0.49*	15.39 ± 0.57**	15.32 ± 0.29**	15.35 ± 0.56*
400	28.03 ± 0.44**	27.39 ± 0.26^{ns}	26.89 ± 0.45^{ns}	20.93 ± 0.43*	19.59 ± 0.92^{ns}	18.79 ± 0.88^{ns}
500	38.13 ± 0.18**	37.75 ± 0.38*	37.27 ± 0.28^{ns}	26.69 ± 0.51**	26.16 ± 1.27**	26.19 ± 0.27**
600	47.99 ± 0.59**	47.10 ± 1.45*	47.00 ± 2.01**	33.34 ± 1.18*	33.18 ± 0.22**	33.00 ± 0.84**
700	56.58 ± 1.67**	54.87 ± 0.28**	54.03 ± 1.56*	41.39 ± 0.93**	40.11 ± 0.33*	39.79 ± 0.19**
800				47.81 ± 0.71**	46.28 ± 0.28**	45.09 ± 0.98^{ns}
900				56.63 ± 1.64**	55.35 ± 0.56*	54.93 ± 0.29*
1000						
1200						
1400						
EC_{50}	**684.23 ± 0.12**	**698.57 ± 0.34**	**704.34 ± 0.57**	**856.19 ± 0.39**	**875.12 ± 0.33**	**882.18 ± 1.22**
Inhibition Percentage (Root)						
50	3.82 ± 0.48**	3.32 ± 0.33**	3.33 ± 0.98**	3.36 ± 0.48**	3.25 ± 0.19**	3.18 ± 0.46*
100	14.35 ± 1.91*	14.10 ± 0.18^{ns}	14.01 ± 1.11^{ns}	11.53 ± 0.48**	11.18 ± 0.57*	11.01 ± 0.36*
200	21.36 ± 0.54*	20.96 ± 0.29*	19.98 ± 0.28*	20.03 ± 0.55**	19.52 ± 0.19**	19.16 ± 0.33**
300	33.17 ± 0.73*	32.11 ± 0.19*	32.00 ± 0.55*	27.08 ± 0.73*	26.75 ± 0.15*	25.89 ± 0.45*
400	41.62 ± 0.96**	40.29 ± 0.24**	39.88 ± 0.19*	32.69 ± 0.96**	30.67 ± 0.16^{ns}	29.45 ± 0.67*
500	53.26 ± 3.58**	51.89 ± 1.28**	50.89 ± 0.19*	38.46 ± 1.23**	38.18 ± 0.28^{ns}	37.49 ± 0.29*
600				44.71 ± 0.10*	43.11 ± 0.11**	42.89 ± 1.19^{ns}
700				52.24 ± 0.99*	50.78 ± 0.66**	50.19 ± 0.88*
800						
900						
1000						
1100						
EC_{50}	**485.12 ± 0.67**	**499.67 ± 0.19**	**509.00 ± 0.35**	**677.02 ± 1.26**	**685.11 ± 0.33**	**692.11 ± 0.33**

Concentration (µg/ml)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
Inhibition Percentage (Leaf)						
50	1.61 ± 0.62**	1.54 ± 0.29**	1.38 ± 0.39**	3.58 ± 2.35**	3.34 ± 1.13**	3.21 ± 0.61**
100	10.20 ± 0.97*	9.95 ± 0.49**	9.85 ± 0.29**	7.99 ± 1.06*	7.67 ± 0.39^{ns}	7.65 ± 1.18^{ns}
200	24.97 ± 0.47**	24.85 ± 0.13**	24.28 ± 1.39*	15.99 ± 0.61*	15.75 ± 1.21**	15.57 ± 1.02**
300	40.30 ± 1.27**	40.10 ± 0.29*	40.10 ± 0.57*	27.59 ± 1.38^{ns}	26.89 ± 0.49**	26.82 ± 0.29**

Table 3 continued

Concentration (μg/ml)	Ethyl acetate extract			Aqueous extract		
	Rainy	Summer	Winter	Rainy	Summer	Winter
400	55.93 ± 1.61**	54.89 ± 0.29**	53.79 ± 0.48**	37.75 ± 0.71**	36.29 ± 0.23**	36.01 ± 0.19*
500				46.04 ± 1.38**	46.00 ± 0.54**	46.19 ± 0.25**
600				50.55 ± 0.63**	49.49 ± 0.53^{ns}	49.43 ± 0.28^{ns}
700				58.03 ± 0.34**	57.31 ± 0.28**	57.10 ± 0.29**
EC_{50}	**339.12 ± 0.23**	**348.43 ± 0.25**	**354.00 ± 0.13**	**585.23 ± 0.45**	**597.01 ± 0.32**	**599.19 ± 1.18**
Inhibition Percentage (Stem)						
50	8.03 ± 0.50*	8.03 ± 0.44**	7.76 ± 0.77**	1.24 ± 0.19**	1.12 ± 0.77**	1.11 ± 0.22^{ns}
100	13.64 ± 0.61*	13.13 ± 0.27*	13.10 ± 0.29**	2.31 ± 0.11*	2.21 ± 0.39**	2.19 ± 0.35**
200	20.17 ± 0.13**	20.17 ± 0.22**	19.98 ± 0.57**	6.63 ± 0.23**	6.54 ± 0.32*	6.47 ± 0.23*
300	28.18 ± 0.04**	27.87 ± 0.28**	27.57 ± 0.29**	10.36 ± 0.21**	10.29 ± 0.39**	9.97 ± 0.67^{ns}
400	35.48 ± 1.21*	34.78 ± 0.29**	32.90 ± 0.27*	14.23 ± 0.11**	14.28 ± 0.76*	14.11 ± 0.22*
500	43.09 ± 0.24*	42.05 ± 0.24*	41.00 ± 0.24**	18.32 ± 0.18**	18.10 ± 0.45**	17.99 ± 0.55**
600	53.54 ± 1.44**	53.02 ± 0.55*	52.19 ± 0.29*	22.08 ± 0.29*	21.89 ± 0.34^{ns}	21.08 ± 0.39**
700				27.30 ± 0.54*	27.01 ± 0.28*	26.87 ± 0.29**
800				30.45 ± 0.31*	30.18 ± 0.29**	30.19 ± 1.11**
900				34.44 ± 0.37**	34.28 ± 0.38**	33.29 ± 0.29*
1000				38.94 ± 0.40**	38.38 ± 0.29*	36.38 ± 0.39*
1200				47.66 ± 0.62*	47.10 ± 0.39**	46.56 ± 0.24*
1400				54.23 ± 0.87**	54.10 ± 0.29^{ns}	53.00 ± 0.11**
EC_{50}	**648.24 ± 0.38**	**653.11 ± 0.22**	**664.11 ± 0.83**	**1345.45 ± 0.18**	**1358.09 ± 0.87**	**1367.99 ± 0.54**
Inhibition Percentage (Root)						
50	3.95 ± 0.69*	3.90 ± 0.44*	3.54 ± 0.77**	2.86 ± 0.95**	2.32 ± 0.47*	2.16 ± 0.29**
100	8.82 ± 0.61**	7.78 ± 0.22**	7.23 ± 0.39*	7.97 ± 2.21**	7.28 ± 0.19*	7.17 ± 0.22*
200	15.21 ± 0.80**	15.92 ± 0.44*	15.88 ± 0.55*	13.07 ± 0.72**	12.49 ± 0.49*	12.17 ± 0.33*
300	23.43 ± 0.60*	23.43 ± 0.47**	23.42 ± 0.18*	16.90 ± 0.72	16.59 ± 0.39*	16.24 ± 0.44^{ns}
400	29.98 ± 0.15*	28.88 ± 0.55**	28.39 ± 1.66*	22.32 ± 0.27**	22.19 ± 0.29**	21.89 ± 0.55*
500	36.52 ± 0.69**	36.22 ± 0.44*	35.67 ± 0.59**	28.06 ± 0.27**	27.49 ± 0.42**	27..11 ± 0.22**
600	46.72 ± 0.94**	45.89 ± 0.44**	45.11 ± 1.89**	32.37 ± 0.73**	32.10 ± 0.29*	31.98 ± 0.22*
700	56.31 ± 1.84*	54.98 ± 1.22**	54.19 ± 1.66*	36.04 ± 0.73**	35.29 ± 0.39^{ns}	34.79 ± 0.27^{ns}
800				42.34 ± 0.42*	42.10 ± 0.29**	42.01 ± 0.44**
900				48.32 ± 1.26*	48.01 ± 1.01*	47.01 ± 0.19*
1000				51.99 ± 0.27**	50.77 ± 0.22*	50.56 ± 0.19**
1100				55.65 ± 0.99**	54.49 ± 0.49**	53.89 ± 0.22**
EC_{50}	**733.91 ± 1.21**	**751.44 ± 0.33**	**757.99 ± 0.89**	**978.05 ± 0.62**	**988.11 ± 0.25**	**991.88 ± 0.65**

EC_{50} value of ascorbic acid is 87.39 ± 0.61; *Significance $P < 0.05$, **$P < 0.01$ and $P > 0.05$ non-significant (ns)

value ranges from 0.981 to 0.947), which is clear from the correlation coefficient (Fig. 8). A significant positive correlation was observed between ferric reducing antioxidant power (FRAP) and total antioxidant activity ($R^2 = 0.975$) of rainy season leaf extract. Similarly, the ethyl acetate extract of stem ($R^2 = 0.948$) and root ($R^2 = 0.792$) of rainy season showed positive correlation with total antioxidant activity and ferric reducing power (Fig. 9). These results indicate that there was strong relationship between polyphenol content in leaf, stem and root with antioxidant activity. Therefore, in conclusion it was observed that presence of flavonoid and phenol in extracts contribute significant role for their antioxidant potential.

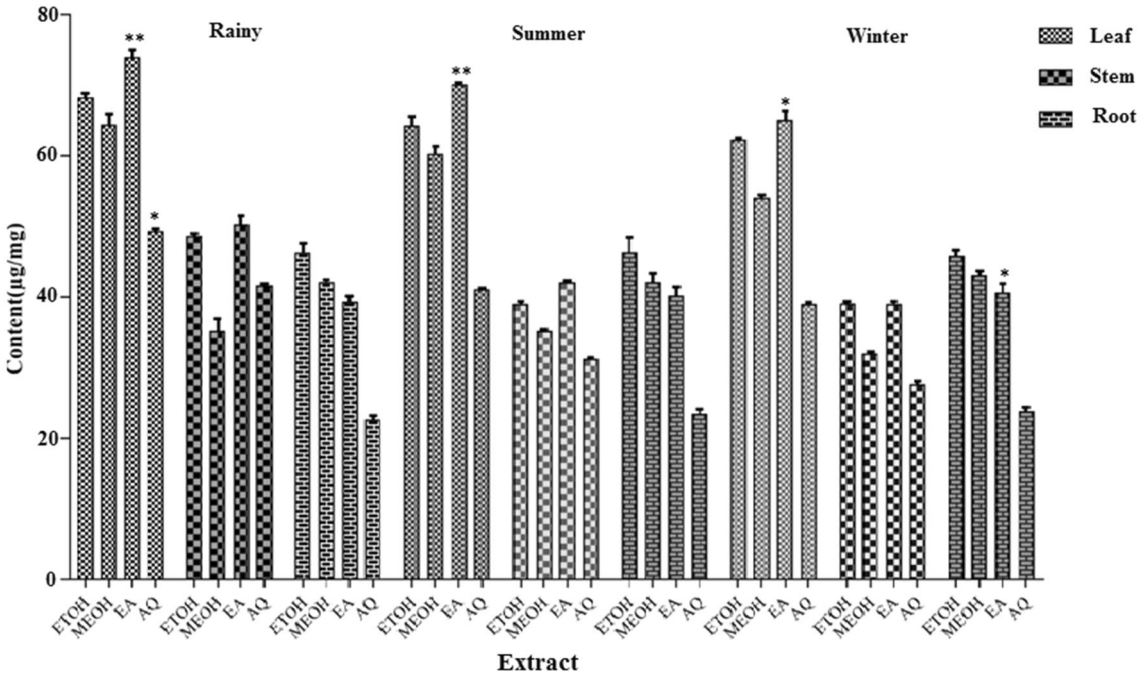


Fig. 2 Total phenol content in leaf, stem and root extracts of *P. integrifolia* in different seasons. Data are presented as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ significantly different from the control

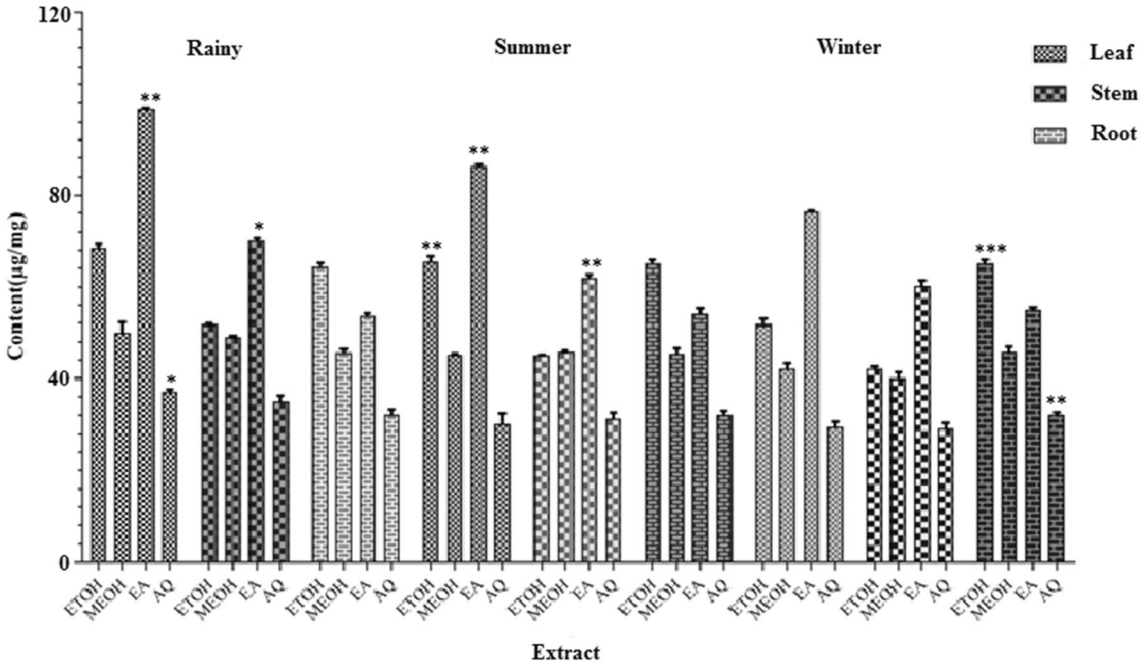


Fig. 3 Total flavonoid content in leaf, stem and root extracts of *P. integrifolia* in different seasons. Data are presented as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$ significantly different from the control

GC–MS analysis of EAEPI

The result obtained from GC–MS analysis of rainy season ethyl acetate extract of leaves of *P.integrifolia* showed the presence of important phytoconstituents. The GC–MS chromatogram (Fig. 10) showed the presence of several peaks. It indicates about presence of bioactive compounds. The major compounds identified based on their retention time (RT), molecular formula, molecular weight, peak area percentage and bio activities as presented in Table 4.

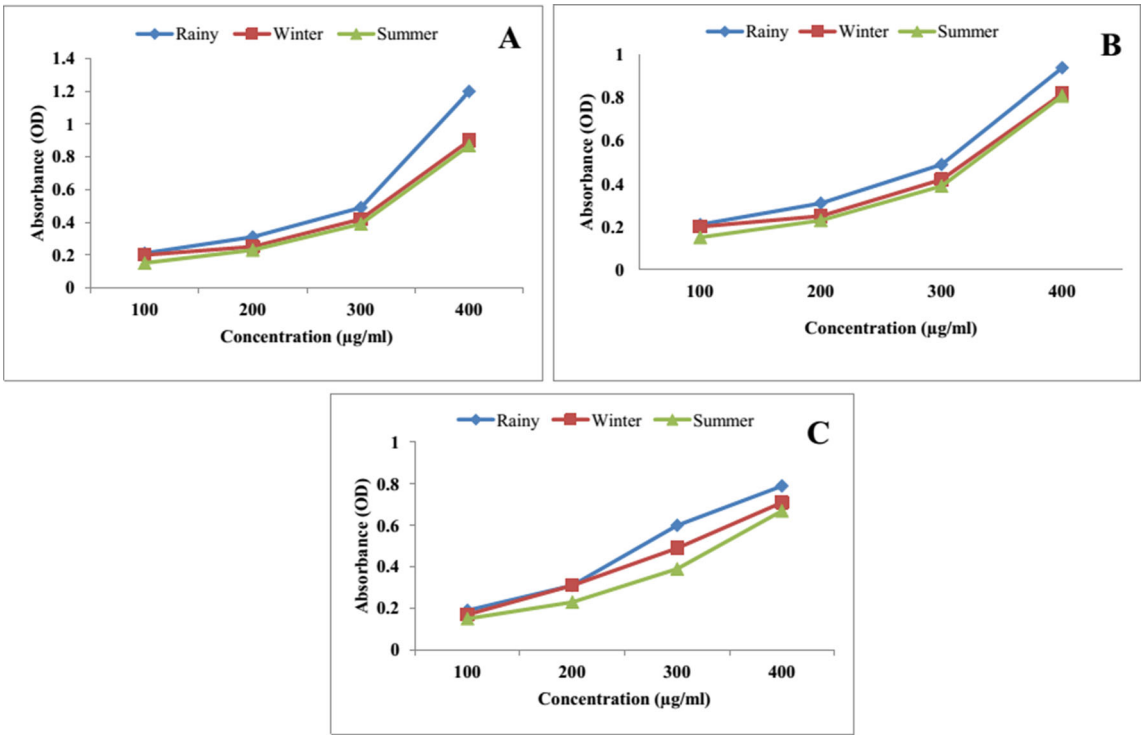


Fig. 4 Reducing power of ethyl acetate extract of **a** leaf, **b** stem and **c** root of *P. integrifolia* in different season

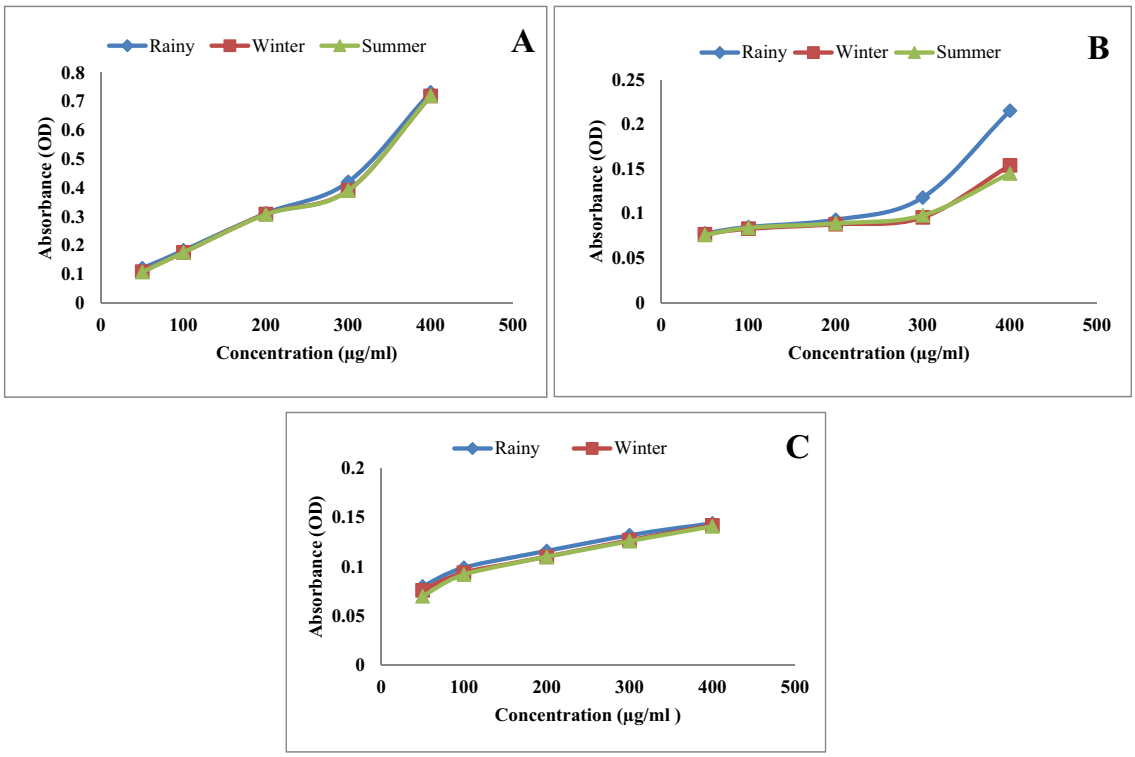


Fig. 5 Reducing power of ethanol extract **a** leaf, **b** stem and **c** root of *P. integrifolia* in different season

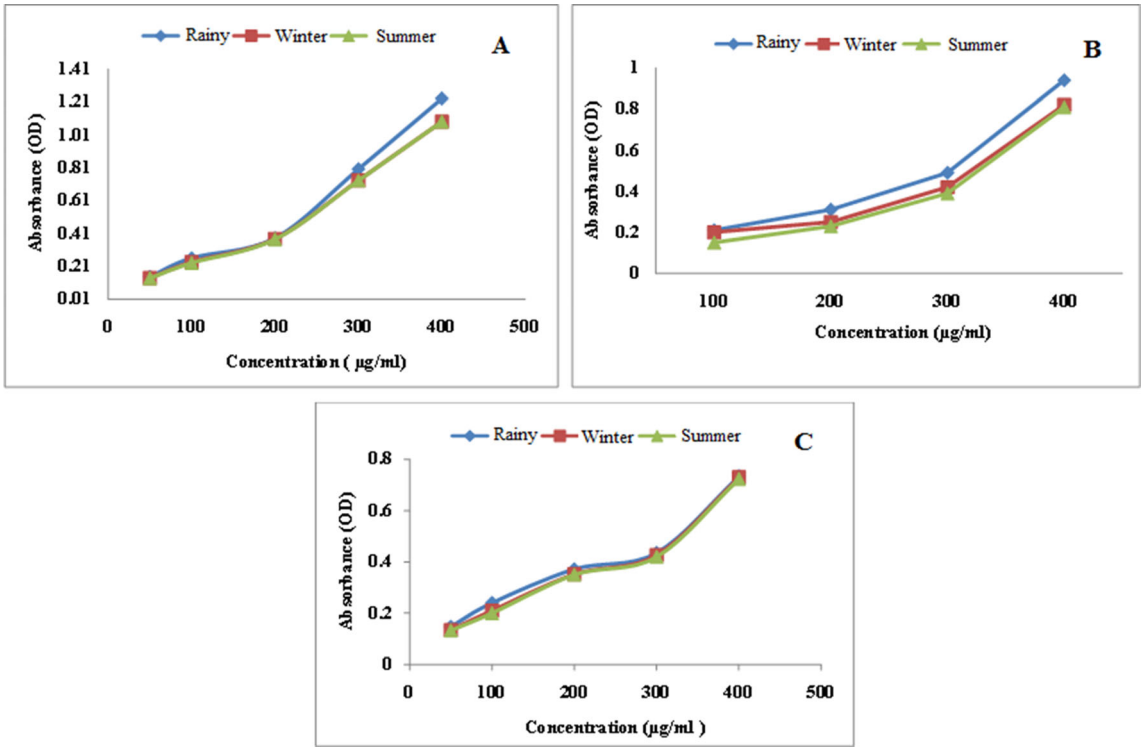


Fig. 6 Reducing power of methanol extract of **a** leaf, **b** stem and **c** root of *P. integrifolia* in different season

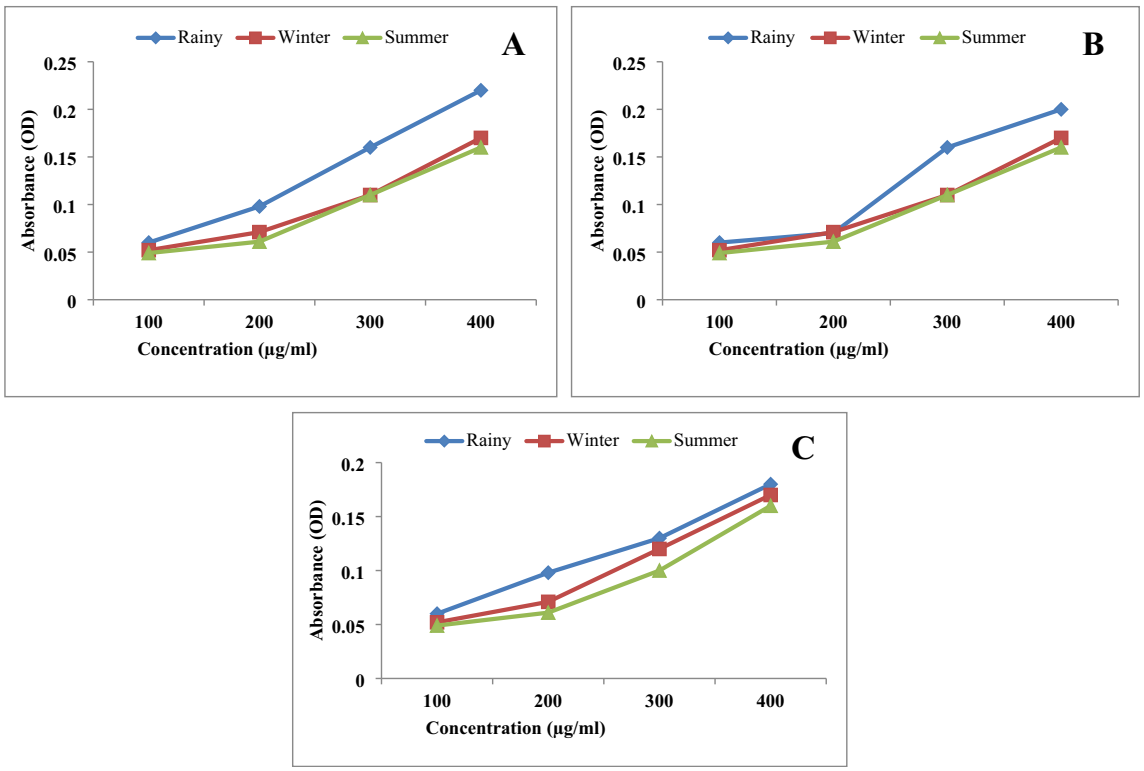


Fig. 7 Reducing power of aqueous extract of **a** leaf, **b** stem and **c** root of *P. integrifolia* in different season

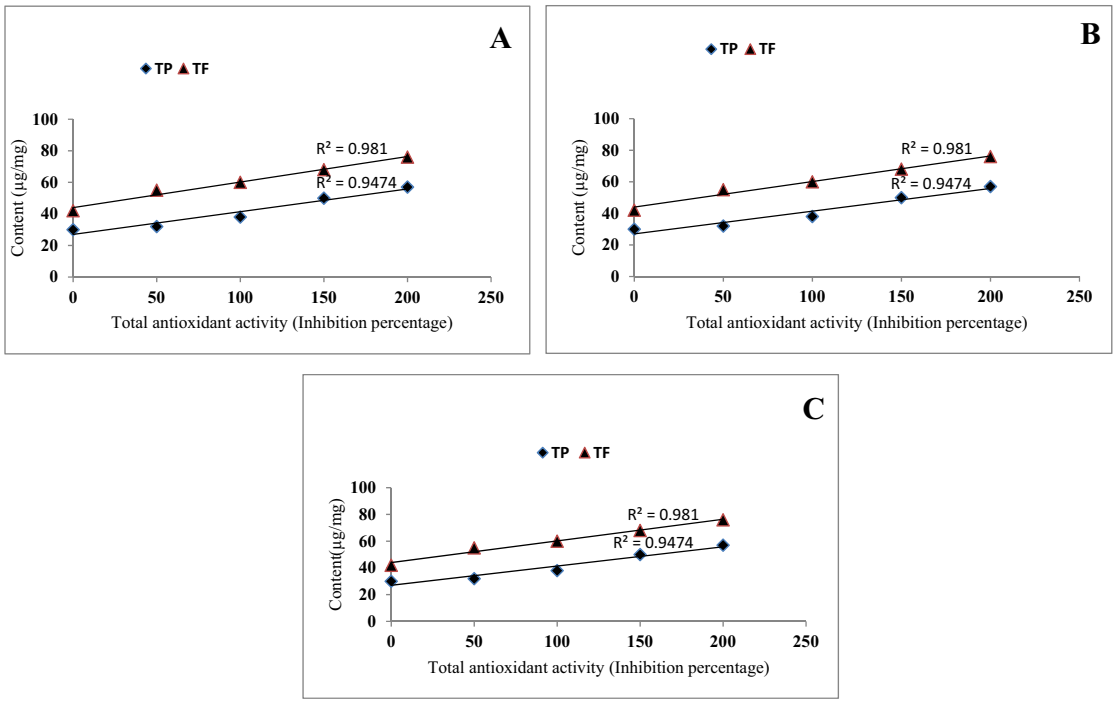


Fig. 8 Correlation between total antioxidant activity and polyphenol content of rainy season ethyl acetate extract of leaf (**a**), stem (**b**) and root (**c**)

Among these identified compounds some major compounds with their percentage pick area (%) and retention times (min) were as follows: citronellol (29.1%, 30.67 min), phytol acetate (16%, 32.48 min), campesterol (12.5%, 50.83 min), phytol acetate (5.83%, 59.74 min), squalene (4.78%, 41.77%), stigmasterol (4.76%, 49.16 min) and hexadecanoic acid (4.52%, 28.12 min).

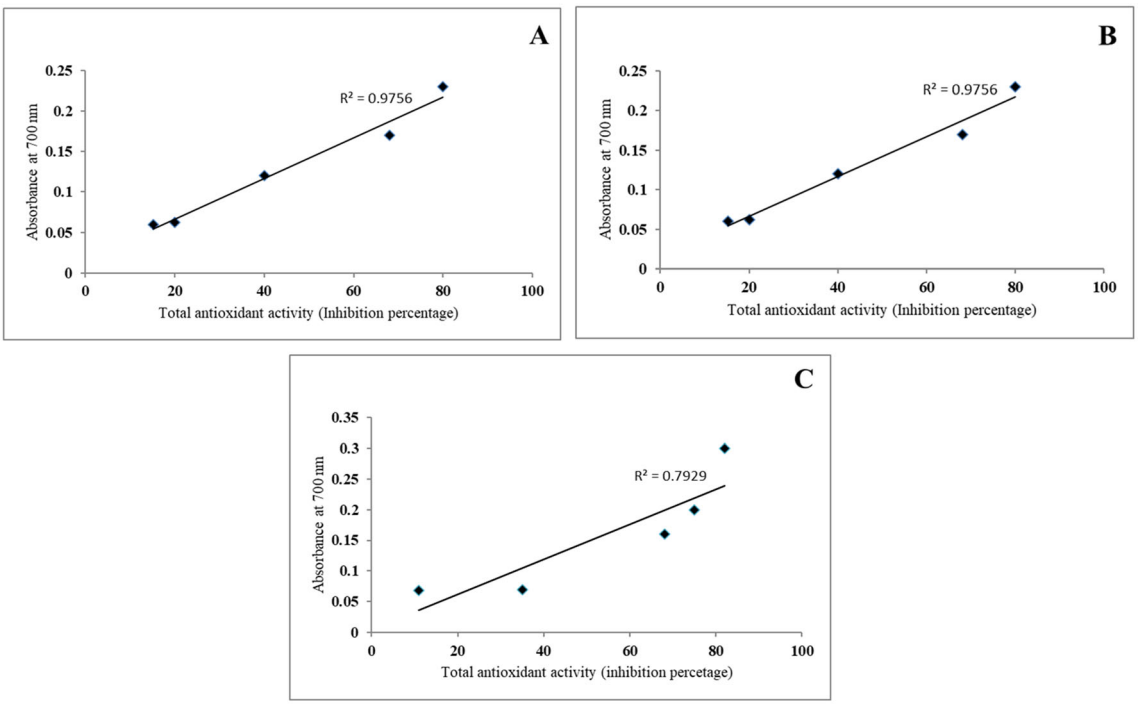


Fig. 9 Correlation between total antioxidant activities and reducing power of rainy season ethyl acetate extract of leaf (**a**), stem (**b**) and root (**c**)

UPLC-Q-TOF–MS/MS analysis of EAEPI

EAEPI was profiled for the presence of bioactive compounds by their mass through UPLC–Q-TOF–MS/MS (Fig. 10). The identified compounds with their biological activities were listed in Table 5. These compounds were tentatively identified based on their retention time, ESI [M-H-], MS/MS and m/z base ions. The compounds were identified by using the combination of mass spectrometry data and established database (http://spectra.psc.riken.jp/). Data revealed that EAEPI contained 8 polyphenolic compounds. These compounds with their m/z values were as follows: quercetin-3-Dxyloside (256), kaempferol-3,7-O-bis-alphaL-rhamnoside (284), isorhamnetin-3-O-glucoside (317), luteolin-3′,7-di-O-glucoside (149), eriodictyol-7-O-glucoside (872), syringetin-3-O-galactoside (359), petunidin-3-O-beta- glucopyranoside (329) and vitexin-2″-O-rhamnoside (425). These compounds possess different pharmacological activities like antioxidant,

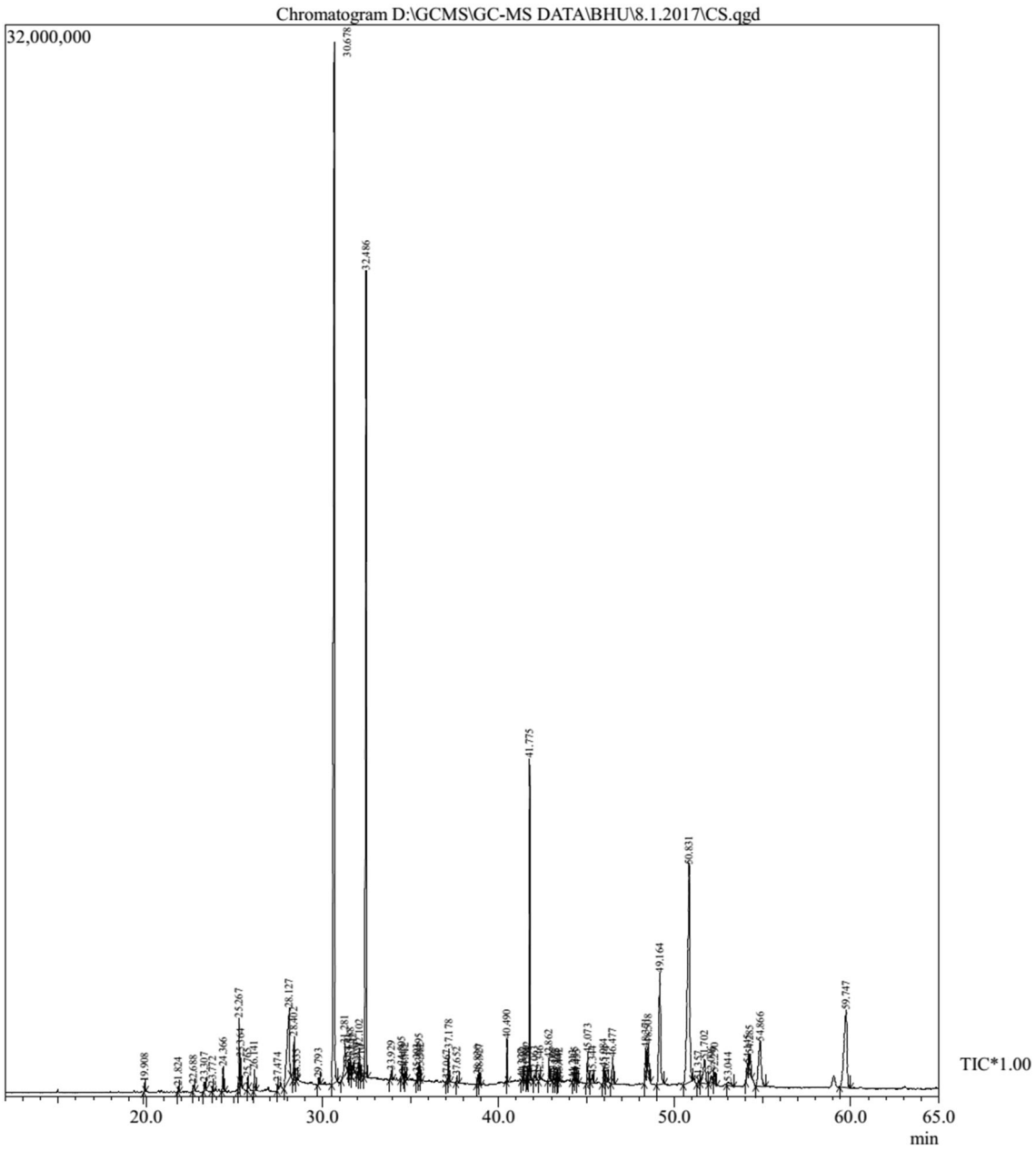


Fig. 10 GC–MS total ion chromatogram of rainy season ethyl acetate extract of leaves of *P. integrifolia*

Table 4 Bioactive compounds identified in ethyl acetate extract of leaves of *P.integrifolia* by GC–MS

Peak	RT (min)	Name of the compounds	Area %	Chemical formula	Nature of the compounds	Bioactivity	References
1	19.90	3-Octadecene, (E)-	0.13	$C_{18}H_{36}$	Essential oil	Free radical scavenging activity	Adeosun et al. (2013)
2	22.68	1-Tetradecanamine, N,N-dimethyl	0.18	$C_{16}H_{35}N$	Amine	Antibacterial activity	Idan et al. (2015)
3	23.30	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-6-H	0.20	$C_{11}H_{16}O_3$	Furanone	Antimutagenic activity	Ganaie et al. (2016)
4	23.77	Myristic acid	0.09	$C_{14}H_{28}O_2$	Fatty acid	Antioxidant and cyclo oxygenase activity	Henry et al. (2002)
5	24.36	1-Nonadecene	0.47	$C_{19}H_{38}$	Long chain fatty acid	Anti-fungal activity	Poongulali and Sundararaman (2016)
6	25.26	2,6,10-trimethyl,14-ethylene-14-pentadecne	1.15	$C_{20}H_{38}$	Pentadecene	Antimutagenic activity	Ganaie et al. (2016)
7	25.36	2-Pentadecanone, 6,10,14-trimethyl-	0.41	$C_{18}H_{36}O$	Pentadecanone	Anti-asthmatic	Ogunlesi et al. (2009)
8	25.76	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R	0.18	$C_{20}H_{40}O$	Hexadecen-1-ol	Antimicrobial	Jananie et al. (2011)
9	27.47	1-Hexadecen-3-ol, 3,5,11,15-tetramethyl-	0.11	$C_{20}H_{40}O$	Isophytol	Antimalarial and antifungal	Agnihotri et al. (2008)
10	28.12	Hexadecanoic acid	4.52	$C_{16}H_{32}O_2$	Palmitic acid	Antioxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic, Hemolytic 5-Alpha reductase inhibitor	Sermakkani and Thangapandian (2012)
11	28.40	Palmitic acid	0.87	$C_{24}H_{48}O_2$	Fatty acid	Antitumor activity	Harada et al. (2002)
12	30.67	Citronellol	29.1	$C_{20}H_{40}O$	acyclic monoterpenoid	Anticancer, anti-inflammatory, promoting wound healing	Zhuang et al. (2009)
13	31.28	9,12 Lienoleic acid	1.30	$C_{18}H_{32}O_2$	Fatty acid	Antioxidant	Leung and Liu (2000)
14	31.74	Stearic acid	0.31	$C_{18}H_{36}O_2$	Fatty acid	Control cholesterol	Bonanome and Grundy (1988)
15	32.48	Phytol, acetate	16.4	$C_{22}H_{42}O_2$	Phytol	Antimycobacterial	Rajab et al. (1998)
16	40.49	Celidoniol, deoxy-	0.63	$C_{29}H_{60}$	straight-chain hydrocarbon	Antioxidant	Yulia et al. (2016)
17	41.77	Squalene	4.78	$C_{30}H_{50}$	Triterpene	Hepatoprotective, Antibacterial, Antioxidant, Pesticide, Antitumor, Cancer preventive, Immunostimulant, Chemo preventive, Lipoxygenase-inhibitor	Sivakrishnan and Muthu (2014); Ko et al. (2002); Desai et al. (1996)
18	45.07	γ -Tocopherol	0.73	$C_{28}H_{48}O_2$	Vitamin	Traps mutagenic electrophiles	Leonetti et al. (2003)
19	46.47	dl-.alpha.-Tocopherol	0.75	$C_{29}H_{50}O_2$	Vitamine	Antioxidant, anti haemorrhage	Chiswick et al. (1983); Bottje et al. (1997)
20	48.50	Campestrol	0.94	$C_{28}H_{48}O$	Phytosterol	Antioxidant	Yoshida and Niki (2003); Safarpour et al. (2012)
21	49.16	Stigmasterol	4.76	$C_{29}H_{48}O$	Phytosterol	Antioxidant, Antitumor activity	Yoshida and Niki (2003); Ghosh et al. (2011)
22	50.83	Campesterol	12.5	$C_{28}H_{48}O$	Phytosterol	Prevention of cancer by antiangiogenic activity	Pandey (2016); Choi et al. (2007)

Table 4 continued

Peak	RT (min)	Name of the compounds	Area %	Chemical formula	Nature of the compounds	Bioactivity	References
23	51.70	α Amyrin	1.50	$C_{30}H_{50}O$	Pentacyclic triterpenol	Antibacterial	Lajubutu et al. (1995); Saeed and Sabir (2001)
24	52.09	5H-3,5a-Epoxynaphth [2,1-c]oxepin, dodecahydro-3,8,8,11a-	0.27	$C_{18}H_{30}O_2$		Antibacterial	Wang et al. (2011)
25	52.29	Stigmasta-5, 22-dien-3-ol, acetate, (3.beta.)-	0.40	$C_{31}H_{50}O_2$	Ester	Antibacterial	Okeleye et al. (2013)
26	59.74	Phytol acetate	5.83	$C_{22}H_{42}O_2$	Ester	Antimycobacterial	Rajab et al. (1998)

hepatoprotective, antitumor, anticancer and antidiabetic, which is summarized in Table 5.

In silico docking analysis

During in-silico docking analysis, three compounds stigmasterol, campesterol, and eriodictyol-7-O-glucoside were showing best binding potential with their binding energies − 8.98, − 8.67 and − 5.99 respectively (Table 6) (Fig. 11a and b). Their binding affinities were even higher than silibinin, the standard hepatoprotective compound.

Drug likeness and ADMET Properties All the compounds showing best binding score with enzyme were also satistfying drug likeness criteria according to Lipinski's rule of five except eriodictyol-7-O-glucoside (Table 7). The Lipinski's rule differentiates between drug-like and non drug-like properties of compounds by compliance with 2 or more than two of five different parameters viz; molecular mass less than 500 Dalton, Log *P* value (indicator of lipophilic property) less than 5, hydrogen bond donor less than 5, hydrogen bond acceptor less than 10 and molar refractivity between 40 and 130. Among three compounds stigmasterol, campesterol, and eriodictyol-7-O-glucoside only two compounds stigmasterol, and campesterol were satisfying Lipinski's rule of five while eriodictyol-7-O-glucoside was rejected by above criteria (Table 7). Besides drug likeness, any drug should fulfill ADMET parameters viz. absorption, distribution, metabolism, excretion and toxicity. It is evident from Table 8 that both stigmasterol, campesterol were showing best intestinal absorption and distribution. These two compounds were also non-ames toxic and non-carcinogenic. While the third compound eriodictyol-7-O-glucoside was toxic and carcinogenic with poor intestinal absorption (Table 8). Thus, it could not be recommended in pharmaceutical formulations.

Discussion

Medicinal Plants are the rich sources of polyphenolic compounds. These polyphenolics act as antioxidants by scavenging free radicals and chelation of metal ions (Halliwell et al. 1995). Plant accumulates phenolic compounds under various stress conditions such as drought, heat, ultraviolet light exposure, air pollution and pathogens attack (Pasqualini et al. 2003). Changes in climatic conditions also influence the biosynthesis of polyphenol (Christie et al. 1994). The stresses enhance the production of reactive oxygen and nitrogen species in the body of the host plant. Host defends itself against ROS by escalating the biosynthesis of phenolic compounds (do Nascimento and Fett-Neto 2010). Temperature fluctuation from optimal level also cause alteration in physiology and biochemistry of several plants (Malhi et al. 1998). In present study, significant variation in total phenol and flavonoid content in different plant part extracts collected in different season have been recorded (Figs. 1 and 2). The polyphenol (TPC and TFC) content in all extracts derived from different plant parts varied with the seasonal variation. Phenolic content was highest in ethyl acetate extract leaf collected in rainy season, while it was lowest in winter season leaf extract. Since the microbial load enhances during the rainy season, so the biotic stress during rainy season might be responsible for higher polyphenol production in rainy season in *P. integrifolia*. Seasonal variation also affected the phenolic content in leaf exudates in *Citrus* species (Chaves et al. 1997). Plant parts extracted in different

Table 5 Major bioactive compounds identified in ethyl acetate extract of rainy season leaves of *P. integrifolia* using UPLC-MS/MS in + ve ion mode

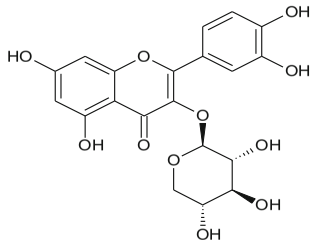
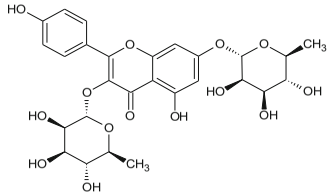
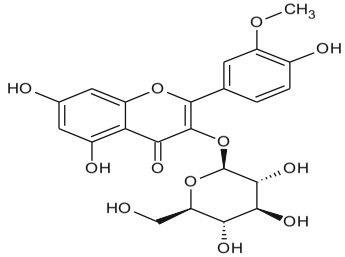
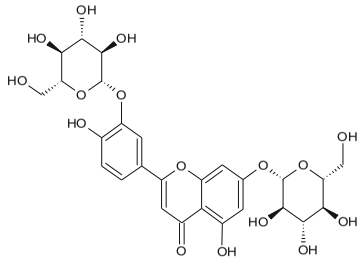
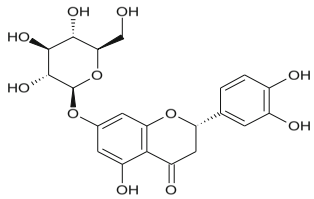
Peak No.	RT(min)	Tentative identification	Element Composition	M.W. (Da)	Ion (m/z)	Structure	Class	Reported activity	References
1	12.9	Quercetin-3-D-xyloside	$C_{20}H_{18}$	434.35	256		Flavonoid	Hepatoprotective	Choi et al. (2011)
2	15.6	Kaempferol-3,7-O-bis-alpha-L-rhamnoside	$C_{27}H_{30}O_{14}$	578.51	284		Flavonoid	Hepatoprotective	Wang et al. (2015)
3	1.33	Isorhamnetin-3-O-glucoside	$C_{22}H_{22}$	478.40	317		Flavonoid	Hepatoprotective, antiadipogenesis	Igarashi et al. (2008)
4	4.34	Luteolin-3',7-di-O-glucoside	$C_{27}H_{30}$	610.51	149		Flavonoid	Anticancer	López-Lázaro (2009)
5	10.05	Eriodictyol-7-O-glucoside	$C_{21}H_{22}$	450.39	872		Flavonoid	Hepatoprotective and anticytotoxic	Hu et al. (2012)

Table 5 continued

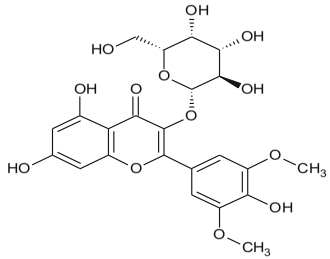
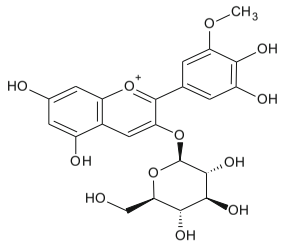
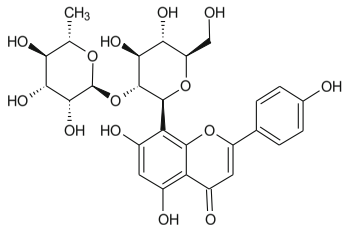
Peak No.	RT(min)	Tentative identification	Element Composition	M.W. (Da)	Ion (m/z)	Structure	Class	Reported activity	References
6	13.20	Syringetin-3-O-galactoside	$C_{23}H_{24}$	508.42	359		Flavonoid	Antiproliferative	Gómez-Alonso et al. (2012; Wu et al. (2012)
7	15.89	Petunidin-3-O-beta-glucopyranoside	$C_{22}H_{23}$	479.41	329		Flavonoid	Antioxidant	Kähkönen and Heinonen (2003)
8	16.37	Vitexin-2′′-O-rhamnoside	$C_{27}H_{30}$	578.51	425		Flavonoid	Hepatoprotective,antiadipogenesis and antitumor	Igarashi et al. (2008)

Table 6 Docking of Alanine amino transferase with major compounds identified in UPLC-MS/MS and GC–MS analysis

S. No.	Comound name	ID	Binding energy	No. of hydrogen bonds involved in binding	Amino acids involved in bonding
1	Silibinin	CID31553	− 5.63	3	Glu 407 Asp 304 Ser 308
2	Quercetin-3-D-xyloside	CID5320863	− 4.36	4	Tyr302, Ser340, Asp94, Tyr302
3	Kaempferol-3,7-O-bis-alpha-L-rhamnoside	CID 5,323,562	− 4.87	4	Gln215, Tyr62, Ser220, Tyr62
4	Isorhamnetin-3-O-glucoside	CID5318645	− 4.10	Nil	Nil
5	Eriodictyol-7-O-glucoside	CID13254473	− 5.99	6	Gly342, Asn412
6	Vitexin-2''-O-rhamnoside	CID5282151	− 3.96	4	Gly345, Asn305, Asn94
7	Citronellol	CID8842	− 5.24	2	Arg312, Arg370
8	Phytol acetate	6,428,538	− 4.88	1	Asn412
9	Campestrol	173,183	− 8.67	2	Ser 308, Cys311
10	Squalene	638,072	− 5.69	Nil	Nil
11	Stigmasterol	5,280,794	− 8.98	2	Val 409, Asn412
12	Hexadecanoic acid		− 3.25	2	Asn412, Lys416

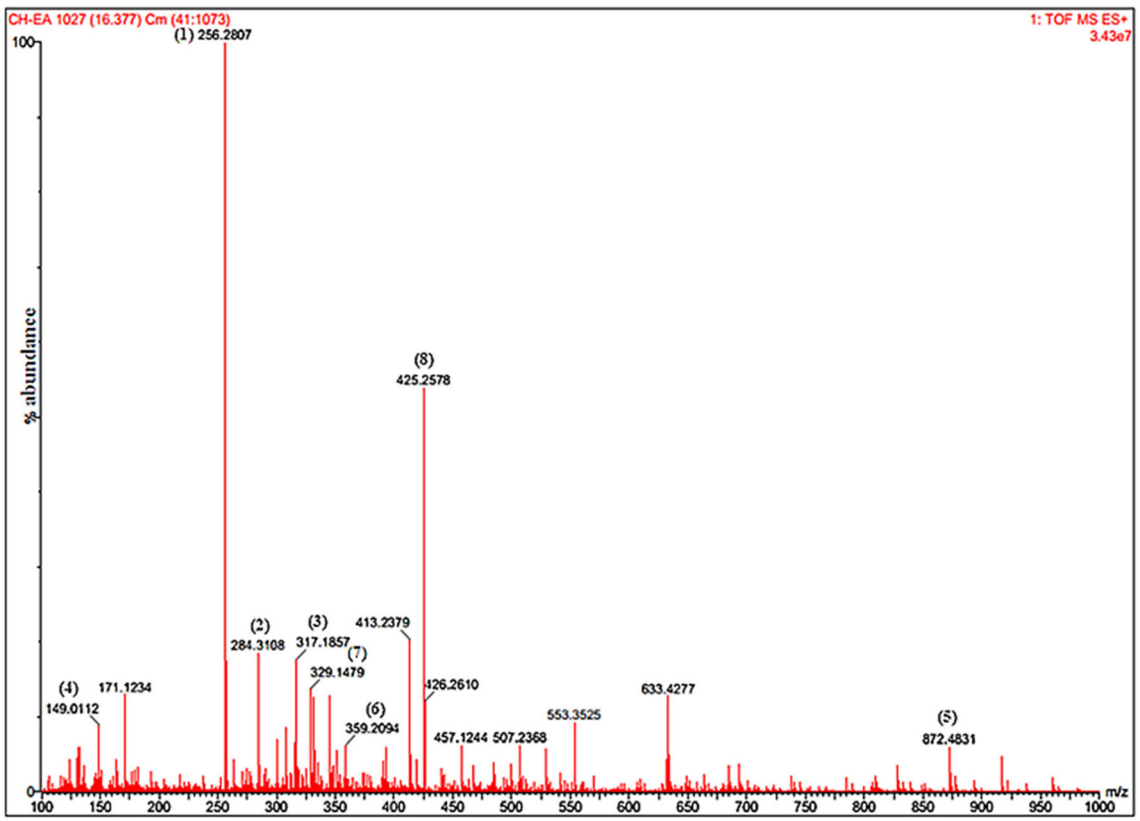


Fig. 11 UPLC-MS/MS positive ion chromatogram of ethyl acetate extract of *P. integrifolia* leaves showing peaks of identified compounds with their *m/z* values. (1) Quercetin-3-Dxyloside (256), (2) Kaempferol-3,7-O-bis-alpha-L-rhamnoside (284), (3) Isorhamnetin-3-O-glucoside (317), (4) Luteolin-3′,7-di-O-glucoside (149), (5) Eriodictyol-7-O-glucoside (872), (6) Syringetin-3-O-galactoside (359), (7) Petunidin-3-O-beta-glucopyranoside (329) and (8) Vitexin-2′′-O-rhamnoside (425)

solvents showed steady increase in percentage inhibition by DPPH radicals with increasing concentration (Table 1). The degree of reduction in absorbance is the marker of the increased antioxidant capacity of the extract. Ethyl acetate extract of leaves and roots of rainy season depicted greater antioxidant activity compared to stem. Nearly similar

Table 7 Drug likeness properties of compounds

Compound name	Molar mass	Hydrogen bond donor	Hydrogen bond acceptors	LOGP	Molar refractivity	Status
Eriodictyol-7-O-glucoside	450.00	7	11	0.311	104.59	Rejected
Campestrol	400.00	1	1	7.63	123.59	Accepted
Stigmasterol	412.00	1	1	7.80	128.12	Accepted

Table 8 ADMET properties of compounds

Compound name	Blood brain barrier (BBB)	Human intestinal absorption (HIA)	AMES toxicity	Carcinogenicity	Status
Eriodictyol-7-O-glucoside	Average	Poor	Toxic	Carcinogenic	Rejected
Campestrol	Poor	Excellent	Nontoxic	Noncarcinogenic	Accepted
Stigmasterol	Poor	Excellent	Nontoxic	Noncarcinogenic	Accepted

variation pattern of EC50 value was obtained for stem and root sample extracted in ethanol methanol and aqueous extract. It was evident that season of collection of plant parts greatly affects the antioxidant activity towards DPPH free radical. Superoxide radical is very harmful species to cellular components because they are precursor of ROS (Buonocore et al. 2010). Current study revealed that all extracts are observed as effective scavenger of singlet oxygen generated in riboflavin-NBT-light system and their activity increased with increase in concentration of extracts (Table 2). In this assay ethyl acetate extract of leaves of rainy season has greater scavenging potential compared to other plant part extracts of different seasons. Ferric reducing antioxidant power (FRAP) is a useful assay for the measurement of antioxidant activity. FRAP was determined based on the colorimetric detection of the reaction mixture. Increase in the absorbance is the indication of the improved antioxidant activity (Mishra et al. 2016). In the present investigation, reducing potential of all extracts increased with increase in concentration, which was in close correlation with its observed antioxidant activity (Figs. 3, 4, 5, 6, 7, 8). Thus, this reducer might be responsible for antioxidant potential of extracts. Lipids are responsible for maintenance of cellular integrity. Results revealed that the rainy season ethyl acetate extract of leaves has higher inhibitory potential for lipid peroxidation compared to other plant parts (Table 3) The GC–MS analysis of ethyl acetate leaf extract of rainy season of *P. integrifolia* showed the presence of 26 compounds with seven major compounds (Table 4; Fig. 9). UPLC-Q-TOF–MS/MS analysis of above extract illustrated the presence of eight polyphenolic compounds whose antioxidant properties were reported earlier (Kähkönen and Heinonen 2003; Wu et al. 2012; Hu et al. 2012) (Table 5; Fig. 10).

Alanine amino transferase (ALT) test is normally used to check the hepatic damage such as hepatitis and cirrhosis. When the liver is damaged, the level of ALT rises in the liver and thereby in the bloodstream. Therefore, in present study, ALT was taken for the in-silico docking analysis against ligands. Among all compounds, three compounds stigmasterol, campesterol, and eriodictyol-7-O-glucoside were showing maximum binding energies against ALT (Table 6, Figs. 12 and 13). The residues were showing van der Walls, hydrophobic, hydrogen bond interactions which is presented in Figs. 12 and 13. Thus, it indicates that these three compounds have potential to protect hepatic damage. The two compounds stigmasterol and campesterol were satisfying the drug likeness properties according rule of the Lipinski's five, while the eriodictyol-7-O-glucoside was rejected by Lipinski's criteria (Table 7). Both compounds stigmasterol and campesterol were showing best absorption, distribution, and were non-toxic and non-carcinogenic (Table 8). While, eriodictyol-7-O-glucoside was showing poor intestinal absorption, and was observed as toxic and carcinogenic while ADMET scoring. This is the first report of the in-silico evaluation of hepatoprotective evaluation of stigmasterol and campesterol. There are reports supporting hepatoprotective activity of quercetin-3-D-xyloside, kaempferol-3,7-O-bis-alpha-L-rhamnoside, isorhamnetin-3-O-glucoside, vitexin-2''-O-rhamnoside, eriodictyol-7-O-glucoside (Table 5) but these compounds were not binding efficiently during in silico docking, which is evident from their binding energies (Table 6).

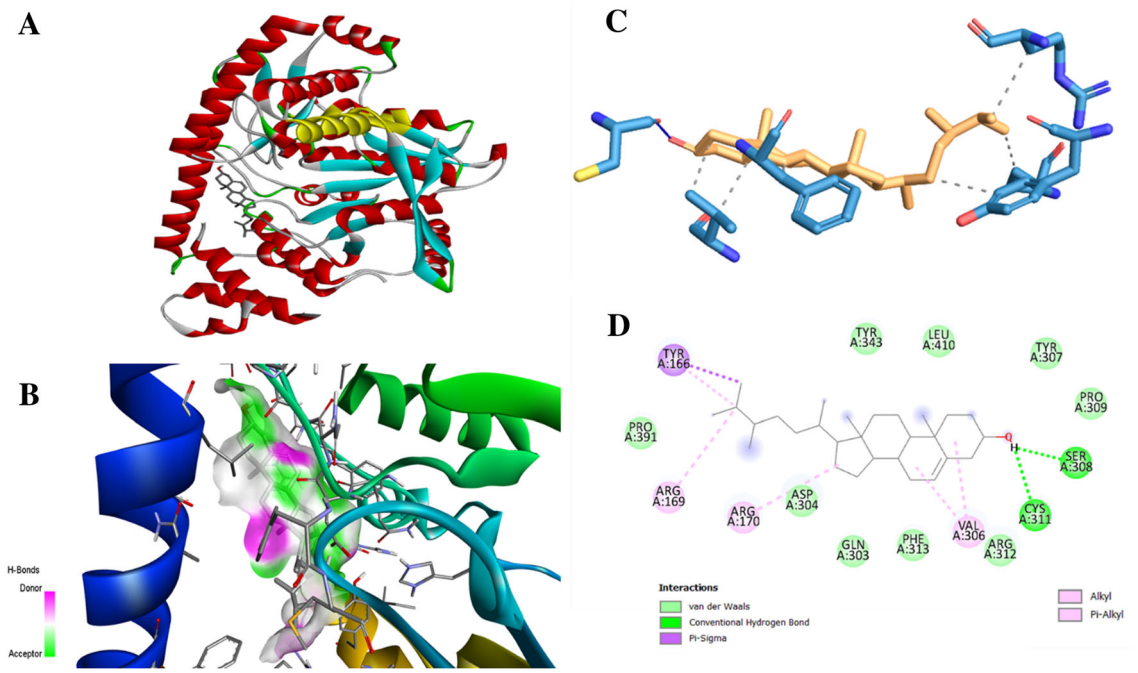


Fig. 12 In silico interaction between campesterol and receptor alanine aminotransferase **a** docked complex, **b** complex showing the active site where ligand interacted, **c** 3D level interaction between receptor and campesterol, **d** 2D level interaction between receptor and campesterol

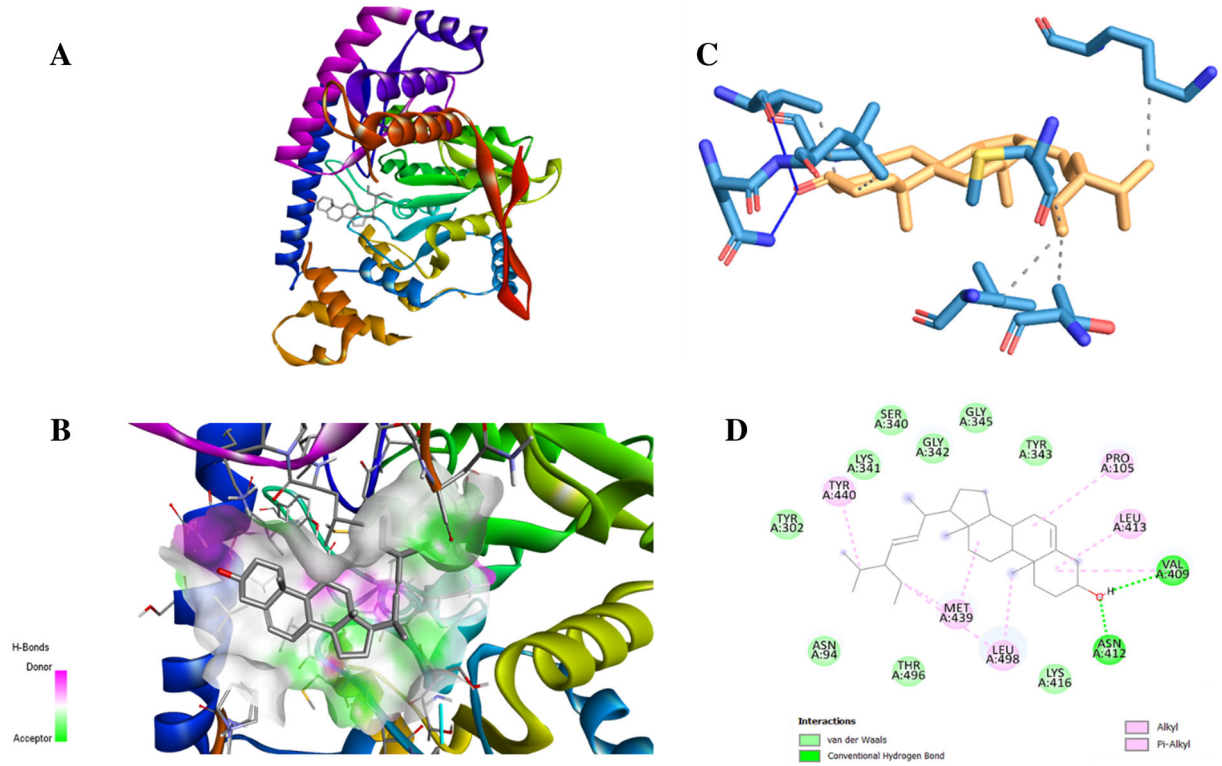


Fig. 13 In silico interaction between stigmasterol and receptor alanine aminotransferase **a** docked complex, **b** complex showing the active site where ligand interacted, **c** 3D level interaction between receptor and stigmasterol, **d** 2D level interaction between receptor and stigmasterol

Conclusion

It can be concluded that polyphenol content and antioxidant activity was dependent on the season of collection of plant materials, parts of the plant used for extraction and nature of solvents used for extraction. Lipid peroxidation activity, superoxide radical scavenging activity and reducing power varied significantly based on season, solvent as well as plant parts. Results revealed that ethyl acetate extract of leaves of rainy season, contained highest total phenol and flavonoid content and showed highest antioxidant activity. UPLC-MS/MS analysis of EAEPI confirmed the presence of eight major bioactive compounds. GC–MS analysis reported the presence of twenty-six bioactive compounds with six major compounds. In silico docking analysis of major compounds against liver enzyme revealed the presence of two hepatoprotective lead compounds that can be used in hepatic drug formulations after clinical trials.

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Author contributions CS designed the study, conducted the experiment, performs the analysis and wrote the paper. KNT supervisor of this study designed the study and reviewed drafts of the manuscript. RU performed the analysis and reviewed the final draft.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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