South African Journal of Botany 103 (2016) 49–53

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Photoprotective, antioxidant screening and new ester from dry root extracts of *Potentilla atrosanguinea* (Himalayan cinquefoil)☆

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ARTICLE INFO

Article history:
Received 6 May 2015
Received in revised form 20 July 2015
Accepted 24 August 2015
Available online xxxx

Edited by AR Ndhlala

Keywords:
Potentilla atrosanguinea
Antioxidant
Photoprotective
Esters

ABSTRACT

In vitro photoprotective and antioxidant activities of dried aqueous-methanolic (H_2O/MeOH) crude extract and ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) as well as aqueous (H_2O) fractions of roots of western Himalayan plant *Potentilla atrosanguinea* (Himalayan cinquefoil) were evaluated. The ability of protection against UVB region followed the trend EtOAc > *n*-BuOH > H_2O/MeOH > H_2O. Total phenol content of crude H_2O/MeOH extract was found to be 429.808 mg GAE/g. The H_2O/MeOH crude extract showed highest antioxidant of DPPH radical scavenging, superoxide anion radical scavenging and cupric ion reducing of 90.04, 78.86 and 88.64% respectively at 200 μg/mL. One new compound methyl pentatetraconta-30,32,34,36,38,40,42-heptaenoate (**PA-1**) along with a known pentadecylbutyrate (**PA-2**) was isolated by column chromatography. Results indicated the importance of root extracts as photoprotective agents in sunscreen preparation in the pharmaceutical industry and considered as a natural source of antioxidants.

1 . Introduction

In the preceding few years the awareness of the effects of ultraviolet (UV) radiation on human health, especially in some skin pathological conditions, and also in immunosuppression and eye damage has grown strongly (Afaq, 2011). Oxidative stress and inflammatory response provoked by UV radiation can cause a variety of intolerable effects in skin, including premature photo aging and skin carcinogenesis (de Oliveira Junior et al., 2013). The total radiant energy emitted from sun consists of about 5% UV range that is divided into three regions, UV-A (320–400), UV-B (280–320) and UV-C (200–280). Solar UV radiation is a potent mutagen and regarded as the leading cause of cancer that is confirmed by both epidemiological and molecular evidence (Afaq, 2011). Among all regions of UV, UV-C is most dangerous and can induce genotoxic stress in human beings who are under direct exposer to UV-C radiation because of its short wavelength and high energy. Fortunately, UV-C radiation is filtered by the atmospheric ozone layer before reaching the earth. However, UV radiation, especially UV-A and UV-B, which reaches the earth and penetrates the skin, causes a variety of adverse effects (Afaq and Mukhtar, 2006; Afaq, 2011). Only about 4–5% of total UV radiation emitted from sun is linked with the UV-B region that is considered to be more genotoxic and capable of causing cell damage. However, UV-B has no penetrating powers as possessed by UV-A, but acts predominantly on the epidermal basal layer of the skin (Afaq et al., 2005; Bickers and Athar, 2006; Halliday and Lyons, 2008; Timares et al., 2008). The efficacy of sunscreen can be defined in terms of sun protection factor (SPF), higher the SPF, more effective is the product in preventing sunburn. *In vitro,* SPF determination may play a vital and rational tool in defining the photoprotective activity of sunscreen preparation which in turn reduces UV exposure of human subjects and its consequences. Moreover, it also reduces the number of *in vivo* experiments, when technical test parameters are adjusted and optimized. Reactive oxygen species (ROS) which comprise superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are regarded primarily responsible, for UV-induced injurious effects. It is a notable fact that, majority of the cellular constituents absorbs UV-A, which ultimately induces oxidative damage indirectly, and UV-B catalysis the formation of dipyrimidine photoproducts in DNA by a direct photochemical mechanism (Longstreth et al., 1998). For preventing the occurrence and reducing the severity of UV-related deleterious effects, (Fryer, 1993; Reddy and Bhat, 1999; Erden and Kahraman, 2000; Dreher and Maibach, 2001; Bickers and Athar, 2006) topical and systemic administration of natural or synthetic antioxidants emerged as a successful strategy.

Potentilla atrosanguinea Lodd. (*Rosaceae*), commonly known as Himalaya cinquefoil is a perennial herb native to western Himalayas (Sahoo et al., 2001; Kalia et al., 2008). Traditionally, *P. atrosanguinea* has been used for wound healing, diarrhea, influenza and bleeding

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http://dx.doi.org/10.1016/j.sajb.2015.08.007
0254-6299/

(Tomczyk and Latté, 2009). Recent research so far has been done on only aerial parts of several *Potentilla* species including antioxidant activity (Miliauskas et al., 2004; Kalia et al., 2008; Jaitak et al., 2010b; Chu et al., 2013; Negro et al., 2013; Sapirstein et al., 2013), but antioxidant as well as photoprotective activities of root extracts of *P. atrosanguinea* are remaining to be explored which is of outmost importance as its roots served as human food (Kalia et al., 2008). The objective of the present study was to evaluate photoprotective potential, total phenol content and antioxidant activity of root extracts of *P. atrosanguinea.*

2 . Materials and methods

2.1. Plant materials

P. atrosanguinea was collected from Kunzum Pass (light intensity, 2500 μEinstein/m^2/s; daytime air temperature, 3–10 °C; altitude, 4517 m; 32° 24′ 20″ N; 077° 38′ 40″ E) in Lahaul and Spiti district of Himachal Pradesh in Western Himalayas of India in the month of September 2012 (Voucher specimen number CUPB 08/2012). Roots were dried for 10–15 days under shade until roots seem brittle or ready for grinding and stored at 15 °C, were subjected to grinding in a Willey grinder (Arthur H. Thomas type) to 60 mesh size and stored at 4 °C.

2.2. Chemicals and instruments

Solvents used for extraction were purchased from Sisco Research Laboratory, Mumbai, India. 2, 2'-Diphenyl-1-picryl hydrazyl (DPPH), 2-thiobarbituric acid (TBA), Bromophenol blue, Ethylene diamine tetra-acetic acid (EDTA), Tris (hydroxymethyl) aminomethane, folin ciocalteu reagent, trichloroacetic acid (TCA) and gallic acid were obtained from Sigma Chemical Co (St. Louis, MO, USA). NMR spectra were recorded in $CDCl_3$, using Bruker Advance 400 spectrophotometer (Punjab University, Chandigarh India) operating at 400 MHz (^{1}H) while ^{13}C was recorded at 100 MHz in the same instrument.

2.3. Extraction and isolation

Air dried root powder (3.8 kg) of *P. atrosanguinea* was extracted with H_2O/MeOH (20:80, v/v) (3 × 3 L) for 12 h at room temperature (Cold extraction). Combined H_2O/MeOH extracts (mother liquor) were filtered using Whatman filter paper number 42 and dried on rotavapor yielding 1.8 kg of the extract which was further partitioned with PE (6 L), EtOAc (4 L), *n*-BuOH (4 L) and H_2O (4 L). All fractions were concentrated under reduced pressure at 50 ± 5 °C yielding PE extract (7 g), EtOAc extract (21.5 g), *n*-BuOH extract (141.4 g) and H_2O extract (52 g), respectively. Since ethyl acetate extract (15 g) showed highest photoprotective activity, it was subjected to column chromatography (CC) over silica gel (60–120 mesh) using gradient elution of PE: EtOAc with increasing proportion of EtOAc (5, 10, 20%) to give a total of 50 fractions. Fractions 48–50 (10 mL each) were combined on the basis of a single spot on precoated silica gel 60 F_{254} TLC plates. Combined fractions were dried on a rotavapor yielding **PA-1** (93 mg), as a yellow semi-solid. Further, the column was eluted with an increasing proportion of EtOAc (25–50%) to give a total of 72 fractions. Fractions 32–50 were combined and dried on rotavapor under reduced pressure yielding 140 mg of sea green solid mixture, which was further subjected to column chromatography (silica gel, 60–120 mesh) and eluted with 20% EtOAc in PE. Subfractions 2–8 were combined on the basis of a single spot on the precoated silica gel 60 F_{254} TLC plates. The combined fractions were dried on rotavapor yielding **PA-2** (25 mg) as a yellowish liquid.

2.4. Photoprotective activity

The absorbance values of each dilution of extract were determined at 290–320 nm, at 5 nm intervals, taking MeOH/H_2O (80:20, v/v) as blank using the UV-visible spectrophotometer (Shimadzu, Central University of Punjab, Bathinda). SPF was calculated according to the equation developed by Mansur et al., 1986.

$$\text{SPF spectrophotometric} = CF \times \Sigma\ EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where EE (λ) indicates erythemal effect spectrum; I (λ) indicates solar intensity spectrum; Abs (λ) indicates absorbance of sunscreen product and *CF* stands for correction factor (=10). The values of EE × I are constant (Sayre et al., 1997).

2.5. Determination of total phenol content

The total phenol content of *P. atrosanguinea* was quantified spectrophotometrically as per the procedure given by Yu et al., 2002. The absorbance of blue colored mixture, formed due to the reaction of Folin Ciocalteu reagent (1:1) and 20% sodium carbonate solution with extract and fraction solution, was measured at 765 nm (Systronics 2202 UV–VIS Spectrophotometer). The amount of total phenol was calculated as mg GAE/g (Gallic Acid Equivalents) dry weight of extract from the calibration curve of gallic acid (120 μg/mL) standard solution (refer supplementary data).

2.6. Evaluation of antioxidant activity

The measurement of hydrogen donating capability of extract was assessed using DPPH radical as substrate, following the method described by Kaur et al., 2008. In this assay, 0.3 mL of extract solution was added to 2.7 mL of 0.1 mM methanolic DPPH solution in a cuvette and absorbance was read at 517 nm. The decrease in absorbance at ambient temperature was correlated with the scavenging action of the test compound and compared with rutin, IUPAC name: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl] oxan-2-yl]oxychromen-4-one (used as standard phenolic compound). The radical scavenging activity was calculated using the equation:

$$\%\ \text{DPPH radical scavenging} = (1 - A_S/A_C) \times 100$$
$$A_S = \text{Absorbance of sample solution};\ A_C = \text{Absorbance of Control solution}$$

For assessing the superoxide anion scavenging ability of different extract/fractions, the method described by Nishikimi et al., 1972 was followed with slight modifications. Briefly, 1 mL of plant extract or fractions of different concentrations (20–200 μg/mL) was mixed with 156 μM NADH (1 mL), 60 μM NBT (1 mL) and 468 μM phenazine methosulphate (PMS) (1 mL) in phosphate buffer (pH = 8.3). The reaction was initiated by the addition of PMS. The reaction mixture was incubated at 25 °C for 10 min. The absorbance of colored complex was measured at 560 nm and the inhibition percentage was calculated using the formula $(1 - A_S/A_C) \times 100$; A_C = absorbance of control, A_S = absorbance of sample solution.

The cupric ion reducing potential of different fractions was determined spectrophotometrically (Apak et al., 2004). To the mixture of 1 mL (10 mM) $CuCl_2$, 1 mL (7.5 mM) neocuproine and 1 mL (1.0 M, pH 7) ammonium acetate buffer solution, added 100 μL of extract solution with 1 mL of distilled water as a dilution factor of different concentrations of extract/fractions. The reaction mixture was allowed to stand for 30 min at room temperature and absorbance was measured at 450 nm. An increase in absorbance indicates the increased reduction ability. The percentage reduction of the sample as compared to the standard, *i.e.* rutin was calculated by using the formula $[1 - (1 - A_S/A_C)] \times 100$. Here, A_C = absorbance of the standard at maximum concentration tested and A_S = absorbance of the sample.

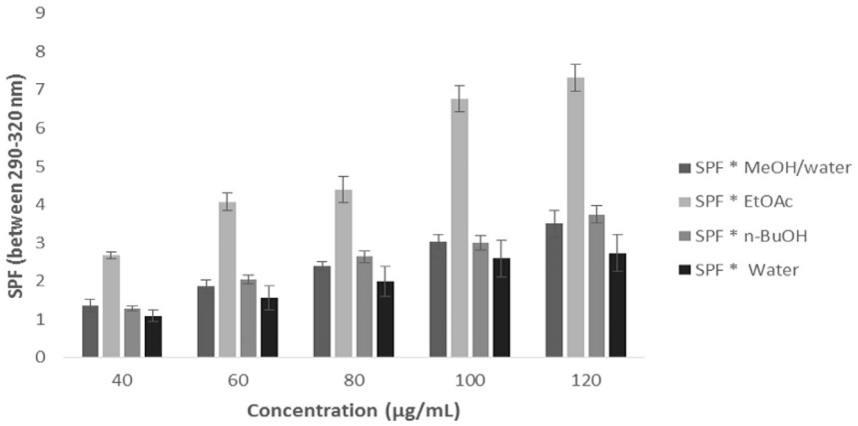

Fig. 1. SPF activity of the extracts of *P. atrosanguinea.*

2.7. Statistical analysis

Statistical analysis was carried out using Sigma Plot 11.0 software; the measurements were taken thrice, and the values of absorbance were expressed in mean ± standard deviation.

3. Results and discussion

In vitro SPF is useful for screening test during pre-formulation of a sunscreen product, as a supplement of *in vivo* SPF measure. *In vitro,* SPF was determined by the spectrophotometric method developed by Mansur et al., 1986, using the UV-B region. In the present study different extract/fractions of *P. atrosanguinea,* e.g., H_2O/MeOH, EtOAc, *n*-BuOH and H_2O were evaluated for their photoprotective activity by UV spectrophotometry. SPF values were determined for each extract/fractions are depicted in Fig. 1. The SPF ranged from 1.35 to 3.49, 2.67 to 7.31, 1.27 to 3.74 and 1.08 to 2.72 for H_2O/MeOH, EtOAc, *n*-BuOH and H_2O extracts, respectively. Measurements were taken thrice, and values were represented as mean ± S.D. It was observed that the EtOAc extract showed higher SPF (7.319 ± 0.353) at 120 µg/ mL that was very appreciative in the terms of concentration of other reported SPF values of extracts. SPF of *Aloe vera* has been reported in the range of 1.29 to 3.49 and 1.37 to 9.97 at concentration 4000 µg/mL of crude lyophilized and MeOH extracts respectively (Kumar et al., 2009). Photoprotective activity (SPF value) of *P. atrosanguinea* extract is more as compared to *Boerhavia diffusa* (3.539 ± 0.0213 to 7.1747 ± 0.0038) and of *Aloe vera* gel (0.995 ± 0.221) (Ashawat et al., 2006).

The ability of protection against UV-B region followed the trend EtOAc > *n*-BuOH > H_2O/MeOH > H_2O and their calculated SPF value at 120 µg/mL concentration were found to be 7.139 ± 0.353, 3.740 ± 0.223, 3.494 ± 0.362 and 2.725 ± 0.479 respectively. The increment in the photoprotective activities of all studied extract/fractions were concentration dependent (Fig. 1). It was observed that on increasing the concentration of extract/fractions, there was an increase in photoprotective activity. *n*-BuOH fraction showed respectable SPF at all studied concentrations and could be utilized as an ingredient of the photoprotective topical formulation. However, H_2O fraction has the lowest SPF value among all studied extract/fractions.

The phenolic content of *P. atrosanguinea* was determined by using the protocol given by Yu et al., 2002. The amount of total phenol was calculated as mg GAE/g dry weight of extract from the calibration curve of gallic acid. H_2O/MeOH extract exhibited higher phenol content of 429.8 mg GAE/g followed by an EtOAc (408.33 mg GAE/g), *n*-BuOH (319.87 mg GAE/g) and H_2O fraction fraction (105.12 mg GAE/g) respectively. Total phenol content of methanolic extract of aerial part of *P. atrosanguinea* was reported to be 16.86 ± 0.02 which is very less compared to its root extract (Kalia et al., 2008). Earlier reports on medicinal plants suggested that total phenol content is directly proportional to the antioxidant activity (da Silva et al., 2006; Maisuthisakul et al., 2007). DPPH radical scavenging activities of different extract/fractions of roots are shown in Fig. 2. DPPH radical is stable radical which on reduction by antioxidants produces a color change from violet to yellow (Alinezhad et al., 2012). The H_2O/MeOH crude extract showed the highest DPPH radical scavenging activity of 90.04% at 200 µg/mL concentrations whereas different fractions followed the trend EtOAc (88.10%) > *n*-BuOH (82.37%) > H_2O (42.94%) respectively at the same

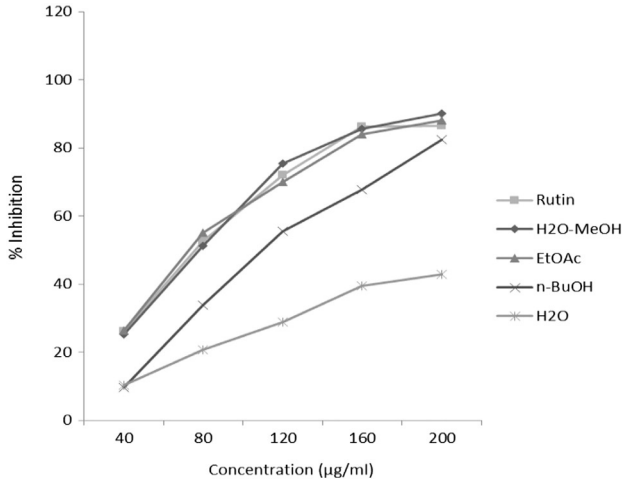

Fig. 2. Percentage inhibition of extracts/fractions by DPPH method.

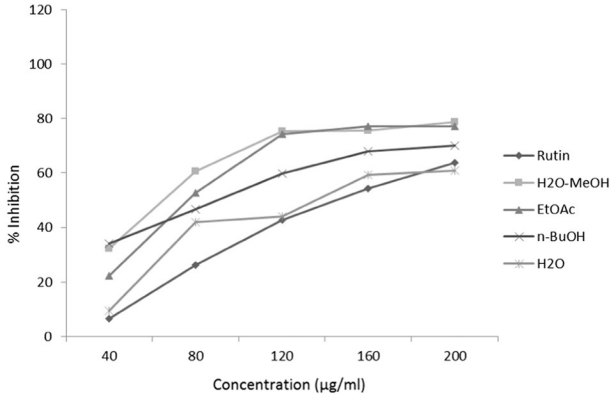

Fig. 3. Percentage inhibition of extracts/fractions by superoxide anion radical scavenging assay.

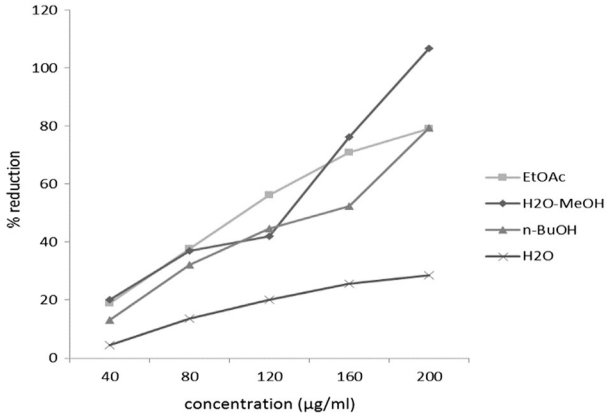

Fig. 4. Percentage reduction of extracts/fractions by cupric ion reducing potential.

concentration. Rutin was taken as a reference compound, and IC_{50} values for all fractions were calculated. IC_{50} values of H_2O/MeOH and EtOAc fraction for DPPH assay was comparable as that of rutin (80 μg/mL). However, IC_{50} value for *n*-BuOH fraction was 120 μg/mL. EtOAc extracts of *Anacardium occidentale* were able to inhibit the formation of DPPH radicals with a percentage inhibition of 46.1 at 400 μg/mL (Razali et al., 2008). In the present study, the EtOAc extract of *P. atrosanguinea* exhibited 88.10% DPPH radical inhibition at 200 μg/mL. Which clearly indicate that the antioxidant activity of *P. atrosanguinea* is better in terms of concentration and percentage inhibition.

Superoxide anion radical scavenging activities of different fractions of root extract of *P. atrosanguinea* were excellent in comparison to standard compound rutin (percentage inhibition 63.77% at 200 μg/mL concentration). The crude H_2O/MeOH extract showed highest superoxide anion radical scavenging activity of 78.86% at 200 μg/mL concentration whereas EtOAc fraction showed higher activity of 77.07% followed by *n*-BuOH (70.06%) and H_2O fraction (60.88%) at the same concentration (Fig. 3). Calculated IC_{50} values of all fractions were found to be excellent than standard rutin. H_2O/MeOH, EtOAc, *n*-BuOH and H_2O fractions were found to have IC_{50} values of 60, 70, 90 and 140 μg/mL respectively.

CUPRAC assay for the assessment of reducing power of *P. atrosanguinea* extracts was used. CUPRAC assay is based on the reduction of Cu (II) to Cu (I) by antioxidants. The cupric ion reducing potential of different fractions and the crude extract was determined, and it is represented in Fig. 4. The H_2O/MeOH extract showed the highest reducing potential of 88.64% at 200 μg/mL followed by *n*-BuOH (65.79%), EtOAc (65.48) and H_2O fraction (23.69%) at the same concentration. As per our knowledge there is not even a single report on CUPRAC assay of any *Potentilla* species in the literature except previously reported CUPRAC assay of different extracts of *Centaurea polypodiifolia*, *C. pyrrhoblephara* and *C. antalyense* showed that reducing power of the extracts (both methanol and water extracts) decreased in order of *C. polypodiifolia* var *.pseudobehen* > *C. antalyense* > *C. pyrrhoblephara*, in the presence of 800 μg mL^{-1} extract.

In continuation of our work on phytochemical investigation of high altitude medicinal plants (Jaitak, Kaul, et al., 2010; Jaitak et al., 2010b; Kaul et al., 2011), we report the characterization of a new compound, methyl pentatetraconta-30,32,34,36,38,40,42-heptaenoate (**PA-1**) together with one known pentadecylbutyrate (**PA-2**) from *P. atrosanguinea* (Fig. 5.) (Garcia-Rubio et al., 2002).

PA-I

Yellow amorphous powder; ^1H NMR (400 MHz, $CDCl_3$): 0.89 − 0.92 (m, 3H), 1.21 (m, 2H), 1.22 − 1.29 (m, 2H), 1.29 − 2.0 (m, 2 × 26H), 2.21 (t, 2H), 3.59 (s, 3H), 5.25 − 5.31 (m, 2 × 7H). ^{13}C NMR ($CDCl_3$): 13.11 (C-45), 21.56 (C-24), 21.68 (C-19), 23.91 (C-21, 23, 25, 27), 24.49 (C-8), 24.59 (C-3, 29), 26.17 (C-20, 22, 26), 28.07 (C-4, 44), 28.09 (C-18), 28.14 (C-17), 28.24 (C-16), 28.31 (C-15), 28.33 (C-14), 28.43 (C-12), 28.51 (C-10), 28.56 (C-11), 28.67 (C-8), 28.74 (C-7, 9, 13), 30.50 (C-6), 30.91 (C-5), 33.07 (C-2), 50.43 (C-1′), 126.07 (C-32), 126.68 (C-34), 126.88 (C-36), 127.02 (C-38), 127.21 (C-40), 127.23 (C-42), 128.72 (C-33), 128.96 (C-35), 129.01 (C-30), 129.18 (C-31, 37, 39), 129.23 (C-41), 130.92 (C-43), 173.36 (C-1). ESI-MS: molecular peak calculated for $(C_{46}H_{78}O_2 + H)^+$: 663.6080; found: 663.6075 corresponding to molecular formula $C_{46}H_{78}O_2$.

Compound **PA-1**, an amorphous yellowish solid, produced a single black spot on a precoated silica gel 60 F_{254} TLC plate after spraying with 20% H_2SO_4. Its molecular peak observed at 663.6075 $[M + H]^+$ (calculated for $[C_{46}H_{78}O_2 + H]^+$, 663.6080) corresponding to molecular formula $C_{46}H_{78}O_2$, with 8° of unsaturation. IR band at 1744 cm^{-1} (C = O stretching) indicated the presence of a carbonyl group in the compound. IR bands 1466 (C═C stretching) and 804 cm-1 (C–H bending) indicated the presence of the conjugated double bond in the compound. The ^{13}C NMR spectrum, including distortionless enhancement by polarization transfer (DEPT) spectra clearly indicated the presence of 2-CH_3, 29-CH_2 and 14-CH. One signal was assigned to quaternary carbon. Furthermore, a proton at δ_H 3.59 (s, 3H) of carbon δ 50.43 (C-1′) showed correlation with δ 173.36 (quaternary carbon, C-1) which clearly indicated the presence of methoxy group δ 50.43 (C-1′) at terminal position. Proton δ_H 2.21 (t, 2H) of carbon δ 33.07 (C-2) showed correlation with δ_C 24.59 (C-3) and δ_C 173.36 (C-1) which confirmed the presence of quaternary carbon adjacent to carbon δ 33.07 (C-2). Proton δ_H 1.21(m, 2H) of carbon δ 28.07 (C-44) showed HMBC correlation with δ 13.11 (C-45) and 130.92 (C-43) which clearly indicated that carbon δ 28.07 (C-44) is attached to terminal methyl group δ_C 13.11(C-45) and alkene chain, δ 130.92 (C-43) on another side. Similarly, proton δ_H 1.22 (m, 2H) of carbon δ 24.59 (C-29) showed HMBC correlation with δ 129.01(C-30) and δ 24.49 (C-28) which indicated the presence of unsaturated aliphatic chain on one side and saturated aliphatic chain on another side respectively. The structure of **PA-1** was confirmed as methyl pentatetraconta-30,32,34,36,38,40,42-heptaenoate on the basis of NMR and other spectral assignments (Fig. 5.).

4. Conclusions

In the present study, SPF determination of the tested extract/fractions, the absorbing capacity of erythema causing wavelength (290–320 nm) was evidently maximum with EtOAc and minimum with H_2O fractions. Moreover, antioxidant activities of the H_2O/MeOH, EtOAc, *n*-BuOH, H_2O extract/fractions from the roots were evaluated by three *in vitro* experiments namely; DPPH, superoxide anion radical and CUPRAC which showed H_2O/MeOH extract have maximum and H_2O fractions have minimum activity. **PA-1** was characterized as new compound and designated as methyl pentatetraconta-30,32,34,36, 38,40,42-heptaenoate. Present findings demonstrate that roots of *P. atrosanguinea* can be used as new source of photoprotective and antioxidant.

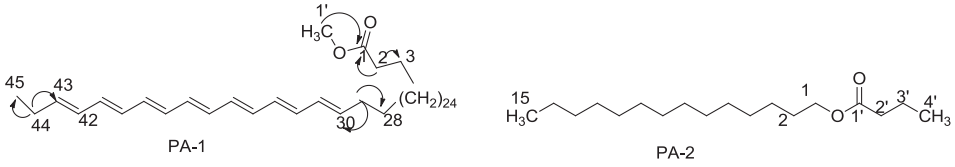

Fig. 5. Structures of **PA-1** and **PA-2**.

Acknowledgment

The authors are grateful to Prof. P. Ramarao, Dean Academic Affairs for his positive feedback during the course of the work.

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