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Free radical scavenging potential and antihaemolytic activity of methanolic extract of *Pouteria campechiana* (Kunth) Baehni. and *Tricosanthes tricuspidata* Linn

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ABSTRACT

Antioxidants from natural sources exhibits high standards concerning their obligatory roles in the improvement of nutraceuticals, pharmaceuticals and cosmetic products. The present study was carried out to evaluate the *in vitro* antioxidant, anti haemolytic activity and FT – IR analysis of two medicinal plants namely, *Pouteria campechiana* and *Tricosanthes tricuspidata*. *In vitro* antioxidant activity of PCBE and TTME extract was screened by various antioxidant assays such as 1,1- diphenyl–2-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiozoline-6-sulfonic acid cation decolorization (ABTS)test, hydroxyl radical scavenging, inhibition of lipid peroxidation, superoxide scavenging assay, reducing power capacity and antihaemolytic activity assay. PCBE exhibited high anti radical activity in DPPH, ABTS, hydroxyl radical scavenging, lipid peroxidation and superoxide radical scavenging activity with the IC_{50} value of 41.1, 9, 220, 82 and 48.2 μg/ml respectively where as the TTME showed moderate activity with the IC_{50} value of 63.3, 47, 130, 161, 84 μg/ml respectively. Linear regression analysis of phenolic and flavonoid content and antioxidant assays for PCBE and TTME displayed a positive correlation ($R > 0.931$; $R2 > 0.614$) which indicate that polyphenols are considered as main antioxidants. The FT-IR spectroscopy analysis confirms the presence of alcohols, phenols, alkanes, amides, amino acids, carboxylic acids, nitro compounds and amines in both the extract. The results suggested that the phytochemicals in both the plants have potent antioxidant activities in correlation with their polyphenolics content.

1. Introduction

Reactive oxygen species such as superoxide anion (O_2^-), hydroxyl peroxide (H_2O_2), peroxyl radicals (ROO^-), reactive hydroxyl ($^{\cdot}OH$) and nitric oxide (NO) radicals are continuously generated in our body system during metabolism. Antioxidants are the agent that can neutralize the free radicals by chelating, catalytic metals and by quenching the singlet oxygen (Wanasundara and Shahidi, 2005). Nevertheless, all aerobic organisms, including human beings have antioxidant defenses (enzymatic and non enzymatic antioxidant system) that protect against oxidative damage to remove or repair damaged molecules (Ridha et al., 2007; Mayakrishnan et al., 2012). But in cases of excessive generation of these reactive oxygen species/free radicals can damage lipids, proteins, DNA and extra cellular matrix ultimately leads to many chronic diseases, such as cancer, diabetes, aging and neurodegenerative diseases in human (Samak et al., 2009).

Haemolysis persuaded oxidative stress by ROS & RNS which play a vital role in the development of various deadly diseases such as thalassemia, glucose -6- phosphate dehydrogenase deficiency and sickle cell anaemia. RBCs are the key target of radical synthesis due to its high polyunsaturated fatty acids in the membrane. It encompasses in oxygen transference coupled with effective ROS promoters. Further more oxidation leads to depletion of protein content and microcirculation depletion (Khalili et al., 2014). Hence intake of dietary antioxidant compounds plays a vital role in reducing such free radical induced inflammations. Previous reports have shown that polyphenolic compounds in medicinal plants scavenge oxygen species and effectively prevent oxidative cell damage (Divya and Mini, 2011). Moreover, the synthetic antioxidants such as BHT, BHA are reported to be carcinogen. On the other hand, investigations for bioactive compounds in medicinal plants add importance due to the presence of antioxidant rich compounds. Recently, the capability of phenolic substances, including

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flavonoids and phenolic acid acting as antioxidants has been comprehensively investigated. So far, many researchers have shown much interest in plants due of their safety and their wide acceptance by consumers (Jeyadevi et al., 2013). Therefore, use of herbal products as dietary products could be a better option to meet the objective of finding a suitable treatment for reducing the free radical generation.

Medicinal plants selected for the present investigations were *Pouteria campechiana* and *Tricosanthes tricuspidata*. *Pouteria* is a genus of tropical fruit trees belonging to the family Sapotaceae. The pulp is yellow with one to four hard seeds. The fruit can be eaten fresh or after baking and also can be used in making custards, ice creams, milkshakes, jam, and marmalade. The *Pouteria species* was reported to possess many biologically active polyphenolic antioxidants including gallic acid, (+)-gallocatechin, (+)-catechin, (−)-epicatechin, dihydromyricetin, (+)-catechin-3-O-gallate, and myricitrin (Ma et al., 2004). The leaves are reported to be anti-inflammatory. Bark decoction of *P. campechiana* are used as antipyretic in Mexico as well as to treat skin eruptions in Cuba. Unripe fruits used to control diarrhoea. *Pouteria campechiana* seeds are also used as a remedy for ulcers. (Morton, 1987).

T. tricuspidata is a creeping herb found throughout India, China and tropical Australia. *T. tricuspidata* belongs to the family cucurbitaceae which is commonly known as red ball snake gourd. It has been known as a traditional herbal medicine for its anti-inflammatory property, migraines, treatment for opthalmia, epilepsy, lung disease, diabetic carbuncles, stomatitis, cold and influenza headaches (Kanchanapoom et al., 2002). In India, the leaf extract of *T. tricuspidata* is used for curing snake bite poisoning (Duvey et al., 2012). Recently the leaf extract of *T. tricuspidata* was proved to show antipyretic activity (Manivel et al., 2011). The present study was undertaken to evaluate the protective effect of *Pouteria campechiana* methanolic bark extract and *Tricosanthes tricuspidata* methanolic leaf extract on free radicals generated in various assay models.

2. Materials and methods

2.1. Chemicals and supplies

Diphenyl picryl hydrazyl (DPPH), 2, 2′-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS), butylated hydroxyl toulene (BHT), thiobarbituric acid (TBA) and ferrozine were obtained from Sigma, St. Louis, MO, USA. Potassium ferricyanide was obtained from Qualigens, India. Gallic acid, nitro blue tetrazolium (NBT) and hydrogen peroxide were obtained from Merck Limited, Mumbai, India. All other chemicals used were of analytical grade obtained from commercial source.

2.2. Sample collection and extraction

The bark of *Pouteria campechiana* were collected from Nagercoil, Kanyakumari district, and the leaves of *Tricosanthes tricuspidata* were collected from Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The leaves and bark were washed well with water, air dried at 25 °C in the absence of sunlight. The dried plant material was blended in a blender and was then subjected to successive extraction in soxhlet apparatus using methanol as solvent (1:5 ratio (w/v)) at 90 °C. The extract was then evaporated to dryness by rotary flash evaporator (Buchi type Rotavapor, Mumbai, India) and the resultant crude methanol extract was used for further analysis.

2.3. Determination of total phenolic content (TP)

Total phenolic content was determined by using Folin-Ciocalteu method (Sadasivam and Manickam, 1996). Briefly, an aliquot of the crude extract was made up to 3 ml with distilled water and allowed to react with 0.5 ml of Folin-ciocalteu reagent. After 3 min of reaction, 2 ml of 20% sodium carbonate was added to the reaction mixture and incubated for 30 min at room temperature. The absorbance was read at 765 nm using UV-Visible spectrophotometer (Shimadzu UV-1601, Columbia, MD, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/gram of the extract. The estimation of total phenolics in the extract was carried out in triplicate and the results were averaged.

2.4. Determination of total flavonoid content (TF)

A modified aluminium chloride method was used for determining the total flavonoid content in the plant extract (Shen et al., 2009). A total of 100 µl of sample (1 mg/ml) was mixed with 600 µl of methanol, 40 µl of 10% aluminium chloride, 40 µl of 1 M potassium acetate and made up to 2 ml with distilled water. The tubes were kept at room temperature for 30 min and the absorbance was measured at 420 nm using UV–visible spectrophotometer. The total flavonoid content was obtained from extrapolation of the calibration curve, which was made by preparing various concentrations of a quercetin solution in methanol. The total flavonoid content was expressed as mg of quercetin equivalents (QE)/gram of the extract.

2.5. In vitro antioxidant activity assays

2.5.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

DPPH radical scavenging activity was adopted from those previously described with slight modifications of Singh et al. (2002). Total of 1 ml of 0.135 mM DPPH were prepared in methanol was mixed with 1.0 ml of aqueous extract ranging from 20 to 100 µg/ml. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Changes in the absorbance of the samples were measured at 517 nm. The inhibition percentage was calculated using the following formula:

$$\% \text{ Radical scavenging activity} = [(\text{Abs con} - \text{Abs sample})]/(\text{Abs con})] \times 100$$

where, Abs con is the absorbance of control; Abs sample is the absorbance of test sample/standard. Butyl hydroxytoulene (BHT) was taken as reference standard. Mean values were obtained from triplicate experiments. The percentage inhibition versus concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC_{50} value.

2.5.2. 2, 2′-azinobis-3-ethylbenzothiozoline-6-sulfonic ($ABTS^{\cdot+}$) cation decolorization test

The ABTS scavenging activity of the crude extracts was performed using the method described by Re et al. (1999). The working solution was prepared by mixing stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal amounts and allowing them to react for 12 h at room temperature in the dark. The resulting solution was later diluted with distilled water, and the absorbance was read at 734 nm using a UV–visible spectrophotometer. A total of 1 ml of freshly prepared ABTS solution was added to 1 ml of the plant extracts, the reaction mixture was vortexed for 10 s and the absorbance was measured at 734 m after 6 min. The percentage of the extract's ABTS scavenging inhibition activity was calculated using the above said formula and compared with that of the standard BHT.

2.5.3. Hydroxyl radical scavenging activity

The hydroxyl scavenging activity was determined according to the method described previously (Klein et al., 1981). Briefly, different concentrations (50–250 µg/ml) of extract were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80–90 °C for 15 min in a water bath. The reaction was then terminated by the

addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm against reagent blank. BHT was considered as the reference standard. The hydroxyl radical scavenging activity was calculated using the above said formula and compared with that of the standard BHT. IC_{50} was calculated as 50% reduction in absorbance brought about by sample.

2.5.4. Lipid peroxidation assay (LPO)

Thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid rich medium (Ruberto et al., 2000). Egg homogenate (0.5 ml of 10% v/v) and 0.1 ml of extract were added to a test tube and made up to 1 ml with distilled water. 0.005 ml of $FeSO_4$ (0.07M) was added to induce lipid peroxidation and incubated for 30 min. To the mixture, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of (w/v) TBA (0.8%) in 1.1% sodium dodecyl sulphate (SDS) and 0.5 ml TCA (20%) were added. The resulting mixture was vortexed and then heated at 95 °C for 60 min. After cooling, 5.0 ml of butan-n-ol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was read at 532 nm. Inhibition of lipid per oxidation by the extract was calculated as follows

% of inhibition = [(1-E/C) x 100]

where C is the absorbance value of the fully oxidized control and E is (Abs 532 with TBA – Abs532 without TBA).

2.5.5. Super oxide radical scavenging activity

Super oxide anion radicals (O2·-) are generated in the phenazine methosulfate-reduced form of nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium chloride (NBT) by the extract (Yu et al., 2006). The super oxide anion were generated in 1.25 ml of Tris–HCl (16 mM, pH 8.0), 0.25 ml of NBT (150 µM), 0.25 ml of NADH (468 µM) and different concentrations (20–100 µg/ml) of the sample and standard BHT. The reaction was initiated by addition of 0.25 ml of phenazine methosulfate (60 µM) to the mixture. Following incubation at ambient temperature for 5 min, the absorbance was read at 560 nm. The percentage of inhibition of the sample and the standard was calculated using the above said formula. IC_{50} was calculated as 50% reduction in absorbance brought about by sample compared with blank.

2.5.6. Ferric reducing power capacity

The reducing power capacity of the crude extract was determined by the method of Oyaizu (Oyaizu, 1986). A mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of the various concentration of plant extract dissolved in distilled water (100–300 µg/ml). The resulting mixture was incubated at 5 °C for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 5000 rpm for 10 min and to the supernatant (2.5 ml), equal amount of distilled water along with 0.5 ml of $FeCl_3$ (0.1%, w/v) were added. Increased absorbance of the reaction mixture was then measured at 700 nm against a blank sample.

2.6. Inhibitory effects on H_20_2 induced erythrocyte hemolysis

The anti-haemolytic activity of the extract was examined by the method described previously by Naim, (1976). Erythrocytes from cow blood were separated by centrifugation which was washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless. It was then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of sample (100–500 µg mL^{-1}) with saline or buffer were added to 2 mL of the suspension of erythrocytes and finally the volume was made up to 3.5 mL with saline or buffer. This mixture was pre incubated for about 5 min after which 0.5 mL of H_20_2 solution was added appropriately. The concentration of H_20_2 in the reaction mixture was adjusted so as to bring about 90% hemolysis of blood cells after 120 min of incubation. The samples were subjected to centrifugation at 1000*g* for 5 min for concluding the reaction. The extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation.

2.7. FT-IR analysis

FT-IR analysis is used for analysis of identifying the types of chemical bonds. The dried samples were ground with potassium bromide powder and pressed into pellet form. The processed sample was processed in FT-IR spectrometer (Perkin Ellmer 200, Thermofisher scientific Inc, USA) in the frequency range of 4000–400 cm^{-1} (Muruganantham et al., 2009).

2.7. Statistical analysis

The results were presented as the mean and standard deviation (±) of three parallel measurements. Linear regression analysis was performed, quoting the correlation coefficient. One-way analysis of variance (ANOVA) were carried out to determine significant differences $P < 0.005$ (DMRT) between the means of assays by SPSS (version 10 for Windows 98, SPSS Inc).

3. Results

3.1. Total phenolics and flavonoid estimation

The total phenolics and flavanoid content of PCBE and TTME were determined and the results are presented in Table 1. Total phenolic content of PCBE was found to be 39.45 ± 0.89 mg of GAE/g of extract where as the TTME showed the presence of 6.0 ± 2.31 mg of GAE/g of extract with reference to the gallic acid standard curve ($y = 0.009x + 0.064$; $R^2 = 0.996$). Similarly, the flavonoid content of PCBE and TTME was found to be 12.56 ± 2.1 and 4.7 ± 0.016 mg of quercetin equivalents/gram of extract with reference to standard curve ($y = 0.091x + 0.039$; $R^2 = 0.991$). Normally, most of the bioactive compounds fall under the classification of phenolics and flavonoids, which possess antioxidant activity.

3.2. In vitro antioxidant activity assays

3.2.1. DPPH radical scavenging activity

The antioxidant activity of PCBE and TTME against DPPH radical is shown in Fig. 1A. Radical scavenging potential of PCBE and TTME on DPPH radicals increased with increasing concentration. DPPH radical scavenging activity of PCBE and TTME was found to be 88.54% and 73.45% at the concentration of 100 µg/ml in a concentration dependant manner respectively. Comparatively PCBE was found to be more effective radical scavenger than TTME.

Table 1
Total phenolics and flavonoid content of *Pouteria campechiana* methanolic bark extract (PCBE) and *Tricosanthes tricuspidata* methanolic leaf extract (TTME).

S.No	Assay model	Total Phenolics[a] (mg GAE/g dw)	Total Flavonoids[a] (mg QE/g dw
1	PCBE	39.45 ± 0.89	12.56 ± 2.1
2	TTME	6.0 ± 2.31	4.7 ± 0.016

[a] Each value in the table was obtained by calculating the average of three experiments (n = 3), ± standard deviation.

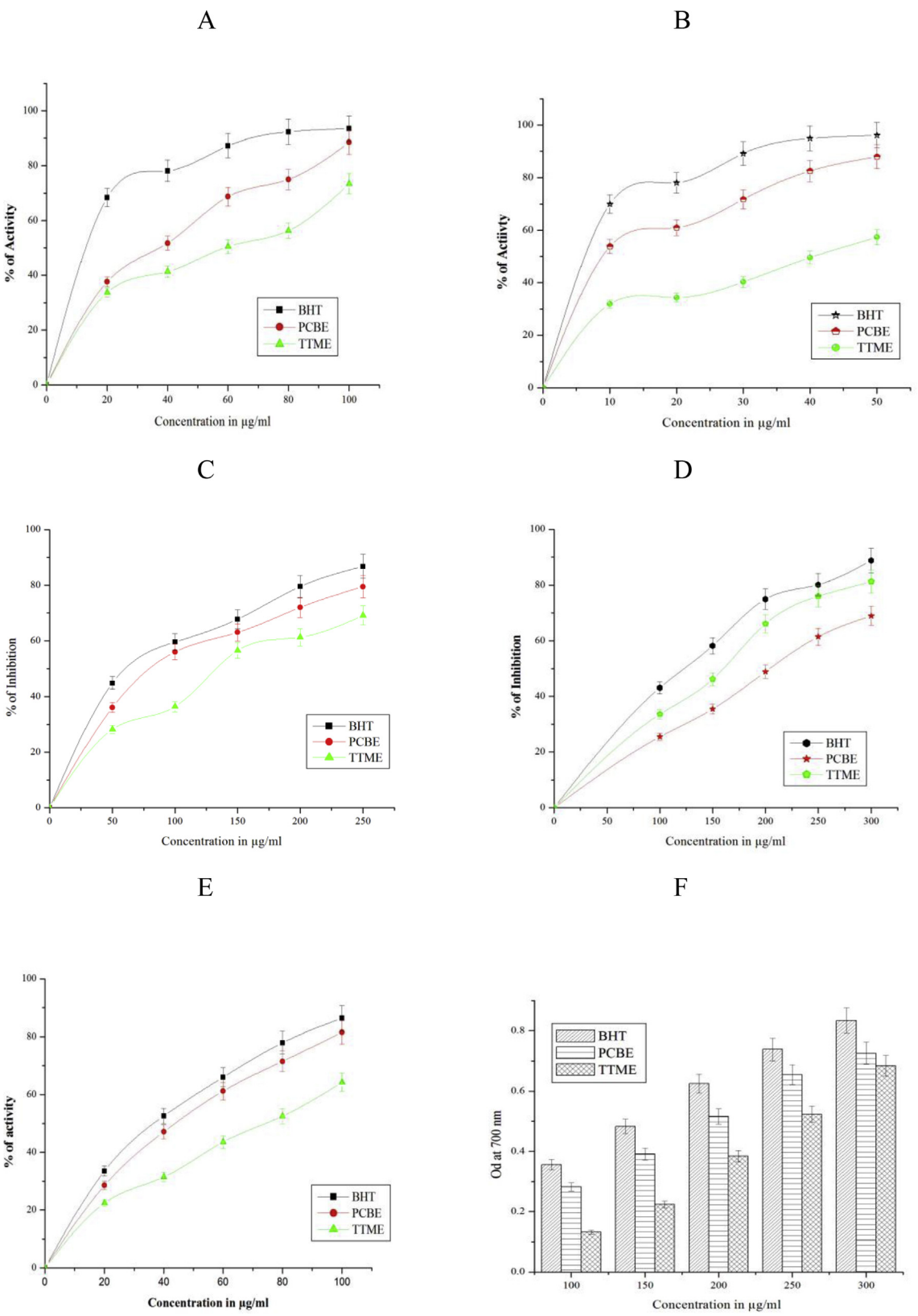

Fig. 1. Free radical scavenging potential of PCBE and TTME compared to standard butylated hydroxytoluene (BHT). Each value is expressed as mean ± standard deviation (n = 3). Fig. 1A. DPPH radical scavenging activity of PCBE and TTME; Fig. 1B. ABTS radical scavenging activity of PCBE and TTME; Fig. 1C. Hydroxyl radical scavenging activity of PCBE and TTME; Fig. 1D. Lipid peroxidation activity of PCBE and TTME; Fig. 1E. Super oxide radical scavenging activity of PCBE and TTME; Fig. 1F. Reducing power activity of PCBE and TTME.

3.2.2. $ABTS^{\cdot+}$ radical scavenging activity

ABTS is a well accepted method for the determination of the antioxidant activity as hydrogen donating antioxidant. The crude extract of PCBE and TTME were fast and effective scavengers of the ABTS radical and was comparable with that of BHT. The inhibition percentage of ABTS radicals in PCBE extract increased from 53.78% at 10 µg/ml to 87.98% at 50 µg/ml. Whereas, the TTME showed a moderate activity of 36.28% at 10 µg/ml to 57.31% at 50 µg/ml concentration.

3.2.3. Hydroxyl radical scavenging activity

In the present investigation, both the extract showed antioxidant activity in a concentration dependent manner (Fig. 1C). The hydroxyl radical scavenging activity of PCBE showed maximum inhibition percentage of 76.46% at 250 µg/ml. There was a moderate inhibition of hydroxyl radical activity by TTME extract (69.11% at 250 µg/ml) as compared to BHT, which showed 86.78% inhibition at 250 µg/ml concentration.

3.2.4. Lipid peroxidation assay

Lipid peroxidation is an oxidative modification of polyunsaturated fatty acids in the cell membranes that generates malendialdehyde (MDA) as a byproduct (Abuja and Albertini, 2001) which can react with TBA, yielding a pinkish red chromogen with a maximum absorbance of 532 nm. The percentage of lipid peroxidation inhibition effect of crude TTME and PCBE increased with the increase of sample concentrations as shown in Fig. 1D. At the concentration of 300 µg/ml, the inhibition effect of PCBE, TTME and BHT was found to be 68.97%, 81.22% and 88.78% respectively.

3.2.5. Superoxide radical scavenging activity

PCBE was found to be an effective scavenger of superoxide radical (81.5% at 100 µg/ml) than TTME extract (64.25% at 100 µg/ml) in a dose depended manner. BHT was used as a positive control which showed the percentage of 86.4 at 100 µg/ml. As a result, the increasing scavenging activity thus may be a sign of superoxide anion consumption in the reaction mixture by the plant extract.

3.2.6. Reducing power activity

Reducing power assay measures the electron-donating capacity of an antioxidant. The results obtained in reducing power assay are shown in Fig. 1F. PCBE had higher reducing power (0.726 OD_{700}) potential at 300 µg/ml than the TTME (0.684 OD_{700}). The OD value of TTME increased from 0.132 at 100 µg/ml to 0.684 at 300 µg/ml respectively.

3.2.7. Antihemolytic activity

Results indicated that PCBE & TTME exhibited potent anti-hemolytic action repressed by hemolysis of cow erythrocyte in a dose dependent manner (Fig. 2). The inhibition ability of extract. The methanolic extract of PCBE and TTME exhibited increasing antihemolytic activity with increase in concentration. The membrane damage induced by H_2O_2 in the erythrocytes was inhibited by TTME (26.7%) at 500 µg/ml concentration whereas the PCBE exhibited increased activity (34.66%) at the same concentration. However maximum inhibition of hemolysis was exhibited by PCBE (83.54% RBC membrane stabilization) at 500 µg/ml, whereas TTME exhibit minimum anti-hemolytic activity as compared to the standard BHT (89.63% at 500 µg/ml) (Fig. 3). In the present study lysis of erythrocytes was demonstrated to be diminished with an increase in concentration of extract.

3.3. IC_{50} values and correlations

The antioxidant effects of the extracts were tested and the results were normalized by calculating the concentration of samples at which the inhibition was 50% which was expressed as IC_{50} values in µg/ml. The IC_{50} values of PCBE in DPPH, ABTS, hydroxyl ion scavenging, lipid peroxidation and superoxide test were 41.1 ± 0.32, 9 ± 1.12, 82 ± 0.98, 220 ± 2.71, 48.2 ± 0.55 µg/ml respectively (Table 2). Similarly, the IC_{50} values of TTME were found to be 63.3 ± 1.55, 47 ± 0.86, 130 ± 1.05, 161 ± 2.65, 84 ± 0.42 µg/ml respectively. Moreover, the standard BHT showed good IC_{50} values in all the antioxidant assays (10.5 ± 3.15, 6.50 ± 2.43, 52 ± 0.83, 128 ± 0.95, 33 ± 3.11 µg/ml). Lower IC_{50} values indicate that the extracts were effective in antioxidant properties.

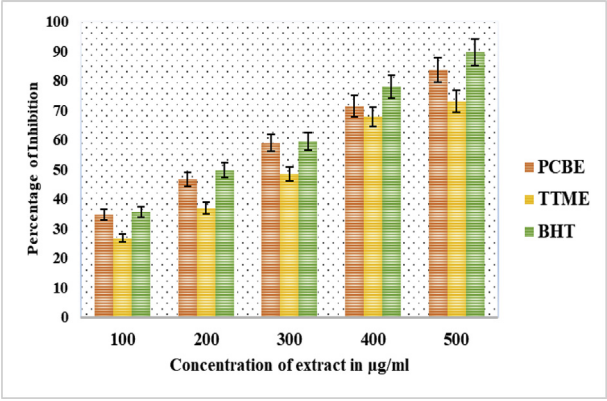

Fig. 2. Inhibition of erythroyte hemolysis of PCBE and TTME compared to standard butylated hydroxytoluene (BHT). Data are mean ± SD (n = 3).

A positive correlation between antioxidant activity and phenolic and flavonoid content was found, and this effect might be due to their bioactive compounds. High correlation coefficient was found between phenolic and flavonoid content with superoxide radical and DPPH scavenging activity (R^2 = 0.984; R^2 = 0.988; R^2 = 0.994). Comparing with the TTME extract, PCBE showed good correlation relationship with the polyphenolic content and antioxidant assays. The R^2 values of PCBE was found to be (R^2 = 0.988, 0.978, 0.988, 0.969). Moreover, it was also found that there was a superior correlation between antioxidant assays ABTS and LPO (R^2 = 0.999). On the other hand TTME also showed better correlation with antioxidant assays except ABTS radical scavenging activity. The R^2 values of TTME with polyphenolic content and antioxidant assay were found to be R^2 = 0.996, 0.964, 0.952, 0.701, 0.642, 0.960, 0.964, 0.932, 0.983, 0.984 and 0.994 (Table 3). A very moderate correlation was found in TTME between ABTS with superoxide radical scavenging activity (R^2 = 0.584) and high correlation was found between hydroxyl with superoxide radical scavenging activity (R^2 = 0.965).

3.4. Chemical composition analysis using FT-IR spectroscopy

The FT-IR spectrum of PCBE and TTME are shown in Fig. 2 A and B. The FT-IR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The FT-IR profile confirms the presence of alcohols, phenols, alkanes, amides, amino acids, carboxylic acids, nitro compounds and amines in both the extract respectively (Table 4). A very strong absorption band observed around 3928–3788 cm^{-1}. Also there was a strong absorption band observed around 3399–1643 cm^{-1}. The O—H stretching and C—H asymmetric stretching methylene group appears near 2925 cm^{-1}. The bands observed at 2924 cm^{-1} (TTME) and at 1420 cm^{-1} (TTME) represent N—H/C—O stretching shows the presence of amino acids.

4. Discussion

Plant based foods are a budding sources of natural antioxidants (ascorbic acid, tocopherol, carotenoids, flavonoid, and phenolic acids) which possess a variety of antioxidant properties that they can act as reducing agents, hydrogen atom donators and singlet oxygen scavengers (Teepica et al., 2012) which act as a broad range of

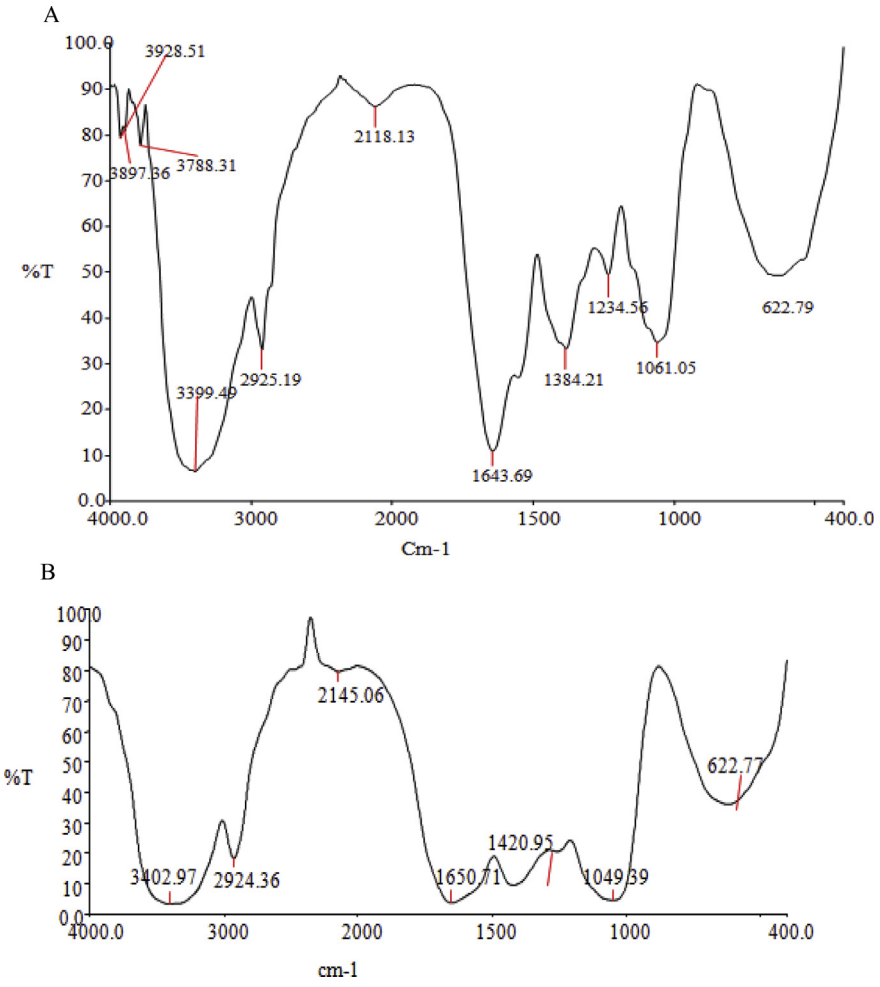

Fig. 3. FT-IR spectrum of PCBE (A) and TTME (B). The figure depicts the absorption bands and the wave number (cm $^{-1}$) of the dominant peak.

Table 2
IC_{50} values of PCBE, TTME and standard BHT of various *in vitro* antioxidant assays.

S.No	Assay model	IC_{50} of PCBE[a](µg/ml)	IC_{50} of TTME[a] (µg/ml)	IC_{50} of BHT[a] (µg/ml)
1.	DPPH	41.1 ± 0.32^{bc}	63.3 ± 1.55^{b}	10.5 ± 3.15^{ab}
2.	$ABTS^{\cdot+}$	9 ± 1.12^{a}	47 ± 0.86^{a}	6.50 ± 2.43^{a}
3.	Hydroxyl	82 ± 0.98^{d}	130 ± 1.05^{d}	52 ± 0.83^{d}
4.	Lipid per oxidation	220 ± 2.71^{e}	161 ± 2.65^{e}	128 ± 0.95^{e}
5.	Super oxide	48.2 ± 0.55^{b}	84 ± 0.42^{c}	33 ± 3.11^{c}

[a] Each value in the table was obtained by calculating the average of three experiments (n = 3), ± standard deviation. Values in a column with different superscripts indicate significantly different at $P < 0.05$.

Table 3
Linear correlation coefficients of PCBE and TTME.

	TP	TF	DPPH	ABTS	HR	LPO	SO
Pouteria campechiana methanolic bark extract (PCBE)							
TP	1						
TF	0.996	1					
DPPH	0.988	0.994	1				
ABTS	0.988	0.978	0.969	1			
HR	0.953	0.972	0.958	0.931	1		
LPO	0.992	0.985	0.978	0.999	0.942	1	
SO	0.984	0.964	0.989	0.972	0.986	0.980	1
Tricosanthes tricuspidata methanolic leaf extract (TTME)							
TP	1						
TF	0.996	1					
DPPH	0.964	0.952	1				
ABTS	0.701	0.642	0.760	1			
HR	0.960	0.964	0.894	0.625	1		
LPO	0.932	0.983	0.849	0.614	0.993	1	
SO	0.984	0.994	0.917	0.584	0.965	0.939	1

TP- Total phenol content; TF- Total flavonoid content; DPPH- Diphenyl picryl hydrazyl; ABTS- 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); HR – Hydroxy radical scavenging; LPO – lipid peroxidation; SO – Super oxide radical scavenging.

pharmacological activities. Polyphenolics are reported to be present in a variety of plants utilized as important components of both human and animal diet. Several assay methods have been developed and applied in recent years to screen and evaluate the total antioxidant activity of herbal medicine and products. In the present study, *in vitro* antioxidant activity of the methanol extract of *P. campechiana* and *T. tricuspidata* was investigated by DPPH, ABTS, hydroxyl radical scavenging, lipid peroxidation, superoxide radical scavenging activity and reducing power capacity. The results of these analysis exhibits a proven effectiveness of the extracts against the generated free radicals in comparison to that of the reference standard BHT. DPPH is a stable free radical that accepts an electron of hydrogen radical to become a stable molecule. The scavenging activity of the DPPH radical by hydrogen donating antioxidants is characterized by a rapid decline in the absorbance at 517 nm (Balasundram et al., 2005). The radical scavenging potential of the selected two plants was thought to be their hydrogen donating ability (Sasikumar et al., 2012) which has the capacity to reduce the stable radical DPPH to the yellow colored diphenyl picrylhydrazine.

Table 4
FT-IR peak values and functional groups of different extract of PCBE and TTME.

Functional groups	Pouteria campechiana (PCBE)	Tricosanthes tricuspidata (TTME)
Carboxylic acid		
O—H stretching	2925.19	–
Alcohols, Phenols		
O—H stretching	3788.31	–
C—O stretching	3928.51	–
Amine		
N—H stretching	3399.49	3402.97
Amide		
N—H stretching	1643.69	3402.97
C—O stretching	–	1650.71
Amino Acid		
N—H stretching	–	2924.36
C—O	–	1420.95
N—H bending		
Sulfonyl		
S= O stretching	1384.21	–
Polysaccharide		
C—O—C stretching	1061.05	1049.39
Alkynes		
N—H wagging	622.79	622.77

The decolorization is based on the reduction of methanol DPPH solution in the presence of a hydrogen donating antioxidants from crude extract of PCBE and TTME by the formation of non-radical form (DPPH-H) in the reaction (Mandal et al., 2009) which was supported by good IC_{50} values. It has been suggested that the reciprocal of IC_{50} provides a clear and more direct method of radical scavenging potency of a compounds (Smilin et al., 2012).

ABTS, a protonated radical has characteristic absorbance maxima at 734 nm which decreases with the scavenging of proton radicals. The decolorization of ABTS radical cation also reflects the capacity of the crude extracts to donate electrons or hydrogen atoms to deactivate these radical species (Sasikumar et al., 2010). The ABTS activity of the two plant extracts were more effective in quenching free radicals in the system at low concentration (50 µg/ml) and comparable activity to that of BHT. This may be due to variation in the types of phenolic compounds that differ significantly in their reactivity towards ABTS. Hydroxyl radical is an extremely reactive species formed in biological systems and have been implicated as highly damaging in free radical pathology. Among the reactive oxygen species, the hydroxyl radical was considered to be the most reactive radical and persuades severe damage to the adjacent molecule leading to interact with DNA, causing strand breakage that primes in contribution to carcinogenisis, mutagenesis and cytotoxicity (Babu et al., 2001). The capability of the extracts to quench hydroxyl radical seems to be directly related scavenging ability of oxygen species.

Interestingly, the TTME extract showed higher inhibition percentage than PCBE in detoxifying lipid peroxidation. The present result suggested that PCBE has moderate inhibition effect on lipid peroxidation compared with TTME. Moreover, it was also suggested that the inhibition effect on lipid peroxidation were due to their metal ion chelating activities, which could interfere with the free radical reaction chain reactions (Wang et al., 2008). The lipid peroxidation inhibition effects of PCBE and TTME were attributed to their Fe^{2+} chelating abilities as well as hydroxyl radical scavenging abilities. Superoxide is a harmful radical in cellular components since it is a precursor of more reactive oxygen species (Gulcin et al., 2010) that can damage biomolecules directly or indirectly by forming H_2O_2, OH and peroxynitrite events. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT (Sasikumar et al., 2012). Reducing power is widely used to evaluate the antioxidant activity of polyphenols. The reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by the antioxidant agents in the extracts were measured by the intensity of the resultant blue-green solution which absorbs at 700 nm, and an increased absorbance is indicative of higher reducing power (Balasundram et al., 2005). The increasing OD values (0.726 ± 0.084) of reducing power in the PCBE indicates the presence of polyphenolic compounds as electron donors which would react with the free radicals to convert them into more stable products (Guleria et al., 2010). Furthermore, it was believed that, due to the presence of polyunsaturated fatty acids membranes play a vital role in most of the susceptible free radical attack in the system. In the present study, lipid oxidation of cow erythrocyte by H_2O_2 induced peroxy radicals may leads to erythrocyte hemolysis. The *in vitro* membrane stabilizing property of the samples were witnessed by their ability to deliver protection against H_2O_2 induced cow erythrocyte hemolysis (Sasikumar et al., 2012). The antihemolytic action of the two samples on cow erythrocytes may be due to their radical scavenging property of the phenols. On the basis of the present and earlier research PCBE and TTME displayed incredible antioxidant activities due to its scavenging potential against ROS and reactive nitrogen species (RNS).

Generally, both PCBE and TTME possess good antioxidant activity, with the PCBE being more effective than the TTME in scavenging free radicals and ROS. The antioxidant effects of PCBE and TTME extracts were well correlated with phenolic and flavonoid content and different antioxidant assays using linear regression analysis quoting the correlation coefficient. There was a significant positive relationship between TP and the antioxidant assays in both PCBE and TTME. The present results were also supported by many previous reports emphasize the positive correlation between phenolic content and antioxidant efficacy (Kukic et al., 2006; Buricova et al., 2008). A positive correlation between phenolic and flavonoid content and antioxidant activity indirectly suggest that the antioxidant capacity of the plant extracts could possibly due to a great extent to their polyphenols. Our results were also supported by Sasikumar et al. (2009) and Kiselova et al. (2006). PCBE could therefore provide a useful source of antioxidants in oxidative stress related disorders and other degenerative diseases. A very strong absorption band observed in FT- IR analysis around 3928–3788 cm^{-1} may be due to the presence of bonded C—O/O—H stretching of alcohols or phenols. In addition there was a strong absorption band observed around 3399–1643 cm^{-1} which can be due to the presence of N—H/O—H stretching of amines and amides (Mohan, 2001). It was reported that vibration of NH3 group shows the presence of primary amines (Muruganantham et al., 2009). Hence the FTIR spectroscopic studies on PCBE and TTME revealed the different characteristic peak values with various functional compounds and confirmed the presence of alcohols, phenols, carboxylic acids, nitro compounds and amines.

5. Conclusion

The methanolic extract of the two plants, *Pouteria campechiana* and *Tricosnathes tricuspidata* exhibited good antioxidant activity. Hence, the potential medicinal use of these two plants was supported by the radical scavenging property can be used to attenuate oxidative stress through their antioxidant property. However, further studies are needed to identify and isolate the bioactive compounds responsible for the antioxidant property against various degenerative diseases.

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Appendix A. Supplementary data

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