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Original Article

Antioxidant and anticancer activities of *Plectranthus stocksii* Hook. f. leaf and stem extracts

Kasipandi Muniyandi,[a] Elizabeth George,[a] Vekataramana Mudili,[b] Naveen Kumar Kalagatur,[c] Allen Joseph Anthuvan,[b] Kadirvelu Krishna,[b] Parimelazhagan Thangaraj,[a,*] Gopalan Natarajan[c]

[a] *Department of Botany, Bharathiar University, Coimbatore, India*
[b] *DRDO-BU Centre for Life Sciences, Coimbatore, India*
[c] *Defence Food Research Laboratory, Mysore, India*

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ABSTRACT

The properties of *Plectranthus stocksii*—a well-known folk medicinal plant—were investigated. The plant extracts were successively extracted and tested for phytochemicals using high performance liquid chromatography, while antioxidant and anticancerous properties were assessed using MCF-7, Caco-2 and RAW 264.7 cancerous cell line models. The methanolic extract of leaves showed higher concentrations of total phenolics (415.41 mg gallic acid equivalents/g extract) and tannins (177.53 mg gallic acid equivalents/g extract) contents than other studied extracts. In the case of flavonoids, ethyl acetate extract of leaf (LEA) showed a higher concentration (777.11 mg rutin equivalents/g extract) and was also found to have better antioxidant activity against stable radical 2,2-diphenylpicrylhydrazyl (3.46 μg/mL), 2,2′azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical (27.41 mM Trolox equivalent/g extract) and superoxide (24.16 μg/mL) radicals and showed better IC_{50} (the concentration of the sample at which the inhibition rate reaches 50%) values on MCF-7 (48.874 μg/mL) and Caco-2 (36.088 μg/mL) cancerous cell line models. The immense anti-oxidant potential of *P. stocksii* leaf and stem extracts could be utilized as a good source of natural, anti-oxidant supplement in food to defend against oxidative-stress-related disorders and more generally in the food safety industry.

Introduction

Natural products, especially plant products are the most important source of new chemical substances and the phytoconstituents have been analyzed and used as potent antioxidants, drugs and various pharmaceutical products (Petlevski et al., 2013). Among plant constituents, secondary metabolites including, phenolic compounds such as simple phenolics, flavonoids, phenolic acids and anthocyanins are of special interest due to their pronounced health-related properties such as antioxidant potential, anti inflammatory, antibacterial and anti diabetic properties (Petlevski et al., 2013). One of the areas where phenols are considered to play an important role is in the protection of the organism from oxidative stress—a state of oxidant/antioxidant imbalance in the organism—where unstable molecules (free radicals) become stable by electron pairing with other molecules and often these molecules are associated with oxidative stress, tissue damage and chronic diseases like cancer (Aslan et al., 2011). As a result, the accumulation of free radicals from environmental causes or by production within the body can alter the pro-oxidant and antioxidant balance leading to oxidative stress. Among the synthetic antioxidants, butylated hydroxyanisole, butylated hydroxytoluene (BHT), propyl gallate and tert-butyl hydroquinone are the most frequently compounds used in the food industry. Due to toxicity and the manufacturing cost of synthetic antioxidants, there is a need to identify alternative natural and probably safer sources of food antioxidants. Furthermore, the antioxidant activity also depends on the solvent polarity, the extraction procedure, the extracted compound purity and the method adopted to evaluate the antioxidant activity (Wanasundara and Shahidi, 1998).

Cancer results from a single cell beginning to divide uncontrollably and forming a tumor and these cells differ from normal

* Corresponding author.
E-mail address: drparimel@gmail.com (P. Thangaraj).

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cells in that they are no longer responsive to normal growth-controlling mechanisms (Bertram, 2001). The cytotoxic activities of plant extracts can be tested in various cell lines using cell-viability assays such as MTT and Trypan blue among others. Major drawbacks of the current chemo-preventive practices (chemotherapy and radiation therapy) are the side effects and suppression of the immune system (Devasagayam and Sainis, 2002). Plants contain abundant quantities of less toxic and more effective substances (Bertram, 2001). Therefore, efforts are being made to identify naturally occurring anticarcinogens which would prevent, slow and/or reverse the cancer induction and development (Devasagayam and Sainis, 2002). Apoptosis is a specific process that leads to programmed cell death which is essential in maintaining the stability of normal body tissues and occurs in various physiological and pathological situations (Hengartner, 2000). The secondary products of plant phenolics, flavonoids, tannins, terpenoids and coumarins act as potent natural antioxidants and are found in fruits, leaves, seeds and oils (Jeong et al., 2004). Recently, there has been a rising awareness of separating these plant compounds using different solvent systems for extraction (Wijngaard et al., 2012).

Among the plant species, members of the Lamiaceae family are widely used as a source of bioactive compounds such as antioxidants and other pharmacologically active substances. The genus *Plectranthus* with 300 species has 62 species that have been reported to treat various ailments and for ethnobotanical uses (Cook, 1995). Previously various phenolics and abietane diterpenoids have been reported as the most diverse from a variety of *Plectranthus* species (Abdel-Mogib et al., 2002).

The present study investigated whether *P. stocksii* could serve as an effective free-radical inhibitor that may be a good source for plant-derived pharmaceutical products. This research work also aimed to identify naturally occurring chemo-preventive agents, particularly those present in medicinal plants, by evaluating the antioxidative activity of the ethyl acetate extract of the *P. stocksii* and examining its anticancer effects on various cell lines, particularly in cancerous cells.

Materials and methods

Chemicals, solvents, reagents

2,2-Diphenyl-1-picryl-hydrazyl, 2,2′azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, hydrogen peroxide, 2,4,6-Tris (2-pyridyl)-*s*-triazine, butylated hydroxytoluene, dimethyl sulfoxide, butylated hydroxyanisole, Folin-Ciocalteau phenol reagent, pBR322 plasmid DNA from *E. coli* RRI, ferulic acid, caffeic acid, quercetin, gallic acid, rutin, *p*-coumaric acid, chlorogenic acid, catechin, naringenin and *trans*-cinnamic acid were purchased from Sigma-Aldrich (Bangalore, India). Fetal bovine serum, Dulbecco's modified Eagle's medium, minimal Eagle's medium, 3′-(4,5-dimethyl-2- thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide, penicillin G and streptomycin were obtained from HiMedia (Mumbai, India). All other chemicals (analytical grade) and solvents (high performance liquid chromatography; HPLC grade) were purchased from Merck Millipore (Bangalore, India). All studies carried out in this research used Milli-Q water with resistivity of 18.2 MΩ cm^{-1} at 25 °C and a total oxidizable carbon value below 5 ppb (Merck Millipore; Billerica, MA, USA).

Collection and identification of plant material

Aerial parts of the plant samples were collected on 13 November, 2013 from Kattapattu, in the Nilgiri mountain region, Western Ghats, India. The taxonomic identity of the samples was confirmed by the Botanical Survey of India (Coimbatore, India) and the type specimen was deposited in the Bharathi Herbarium, Department of Botany, Bharathiar University (accession number: BUBH-006243). Leaf and stem samples were separated and dried under shade at 37 °C for 2 wk and then finely ground through an 80-mesh grid and stored at 4 °C until further analysis.

Extraction of plant material

Powdered plant parts were extracted for 12 h with 150 mL of petroleum ether, chloroform, ethyl acetate, acetone and methanol in increasing order of polarity using a Soxhlet extractor. The Soxhlet system was pre-cleaned using petroleum ether for 2 h. The thimble was dried in a hot air oven at below 40 °C before the extraction using the different solvents and the final stage involved extraction with water by incubating the flask on an orbital shaker at 30 °C and agitating at 120 rpm for 15 h. All extracts were concentrated using a rotary vacuum evaporator (Equitron; Mumbai, India) at 40 °C until the solvent had been completely evaporated. The extract was stored in an amber glass vial at 4 °C until further analysis. The amount of extract recovered after successive extractions was weighed and the percentage yield was calculated using Equation (1):

$$\text{Extract recovery\%} = \{\text{Amount of extract(g)} \div \text{Amount of plant sample(g)}\} \times 100 \quad (1)$$

Determination of total phenolic, tannin and flavonoid contents of the extracts

The total phenolic content of compounds in the plant extracts was determined using the Folin-Ciocalteu colorimetric method with gallic acid as a reference standard (Makkar, 2003). The triplicates of plant extracts (200 μL) were made up to 1 mL with distilled water and then mixed with 500 μL of Folin-Ciocalteau phenol reagent (1 N). After 5 min incubation, 1.250 mL of 5% Na_2CO_3 solution was added to the mixture. After incubation for 40 min in the dark at room temperature, the absorbance was read at 725 nm in opposition to the reagent blank (distilled water). Gallic acid was used for the calibration curve and a concentration range of 5–50 μg/mL was prepared and analyzed as above. The total phenolic contents of extracts were expressed as milligrams gallic acid equivalents (GAE)/g extract.

The total tannin content was determined using the Folin-Ciocalteu procedure as above, after removal of tannins by their adsorption on an insoluble matrix (polyvinylpolypyrrolidone; PVPP) according to Makkar (2003). Insoluble, cross-linked PVPP (100 mg) was weighed into test tubes and 500 μL of plant extracts added. After 15 min at 4 °C, the tubes were vortexed and centrifuged for 10 min at 4350×*g*. The resultant supernatant containing non-tannin phenolics was transferred into test tubes and the non absorbed phenolics were determined as described in the total phenolics estimation. The calculated values were subtracted from the total phenolics contents and the total tannin contents were expressed as milligrams gallic acid/g extract. The tannin content of the plant extracts was calculated using Equation (2):

$$\text{Tannins} = \text{Total phenolics} - \text{Non tannin phenolics} \quad (2)$$

The flavonoids content was determined using the aluminum chloride method with rutin as a reference compound (Zhishen et al., 1999). This method is based on the formation of a complex flavonoid aluminum having its absorption maximum at 415 nm. About 500 μL of extract was mixed with 2 mL distilled water. After 6 min incubation, about 150 μL of 5% $NaNO_2$ and 10% $AlCl_3$ were

added to the mixture at room temperature. An amount of 2 mL of 4% NaOH was added to all the test tubes which were made up to 5 mL using distilled water. The pink color formation was read at 415 nm after 15 min against a blank prepared using 500 μL of plant extracts and a drop of acetic acid, and then diluted to 3 mL with methanol. The amount of flavonoids in plant extracts was expressed in rutin equivalents (RE)/g extract from a standard curve, which was prepared using rutin in methanol under the same conditions.

High performance liquid chromatography analysis and quantification

The phenolic compounds were identified using a liquid chromatography system Nexera HPLC system (Shimadzu, Kyoto, Japan) equipped with a C18 column, 5 μm, 250 × 4.6 mm (Phenomenex; Torrance, CA, USA). Data were processed using LC Solution for Windows Basic Edition 2.1 (Spinco; Columbia, MD, USA). Detection was carried out using an ultraviolet/visible light diode array detector at 280–60 nm. Solvent solutions were vacuum degassed using ultrasonic cleaner with a frequency of 30 ± 3 KHz and a nominal power of 50 W (Rivotek; Mumbai, India) prior to use. The composition of mobile phases and the profile of gradient for HPLC analyses were as follows: solvent A, 3% aqueous acetic acid (volume per volume; v/v); solvent B, water:acetonitrile: acetic acid (47:50:3 all v/v); commencing with 0% B, rising to 35% after 25 min, then to 50% after 45 min and ending with 100% B at 65 min. The flow rate was set at 1 mL/min. The injection volume for all samples was 20 μL. All chromatographic experiments were performed at 35 ± 2 °C. The reference compound standard solutions (1 mg/mL) were prepared by dissolving an accurate amount of the individual compound in dimethyl sulfoxide (DMSO) and water (1:9 v/v) and filtering through a 0.45 μm Durapore membrane filter (Millipore; Billerica, MA, USA). Quantification was performed independently for each extract from the standard graphs using Equation (3):

$$C(sa) = [C(st) \times A(sa)] \div A(St) \quad (3)$$

where, C (sa) is the concentration of compound in sample, C (st) is the concentration of standard, A (sa) is the area under the peak in the sample and A (st) is the area under the peak in the standard.

In vitro antioxidant assays

2,2-Diphenylpicrylhydrazyl radical scavenging activity

The scavenging effect of plant extracts on stable radical 2,2-diphenylpicrylhydrazyl ($DPPH^{\bullet}$) was studied, employing the spectrophotometric method described by Braca et al. (2001). Briefly, 3 mL of $DPPH^{\bullet}$ solution (0.1 mM, in methanol) was incubated with varying concentrations of the different extracts. The reaction mixture was shaken well and incubated for 30 min in the dark at ambient temperature. The changes in color (from deep violet to light yellow) were measured at 517 nm on an ultraviolet (UV)/visible light spectrophotometer (Shimadzu; Kyoto, Japan) against a blank of methanol to estimate the radical scavenging capacity of each extract, while methanolic $DPPH^{\bullet}$ solution was used as a negative control. The positive controls were $DPPH^{\bullet}$ solution with rutin and BHT. The radical scavenging activity of the extracts was expressed as IC_{50} (the concentration of the sample required to inhibit 50% of the $DPPH^{\bullet}$ concentration). The IC_{50} values were calculated using a linear regression of plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percentage of scavenging capacity from the three replicates.

2,2′Azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical scavenging activity

The total antioxidant activity of the samples was measured using 2,2'azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical ($ABTS^{\bullet+}$) decolorization assay according to the method of Re et al. (1999). Initially the $ABTS^{\bullet+}$ was generated by reacting 7 mM aqueous ABTS solution with 2.4 mM potassium persulfate in the dark for 12–16 h at ambient temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30 °C to give an absorbance of 0.700 ± 0.02 at 734 nm. Radical scavenging reaction was started by adding 10 μL of different extracts to 1 mL of diluted $ABTS^{\bullet+}$ solution and incubating in the dark for 30 min at room temperature. Triplicate determinations were made at each dilution of the standards and plant extracts, and the absorbance was read against the blank at 734 nm. A Trolox calibration curve was constructed by measuring the reduction in absorbance of the $ABTS^{\bullet+}$ solution in the presence of different concentrations of Trolox (0–2000 μM). The $ABTS^{\bullet+}$ radical scavenging activity of the extracts and standards was measured by comparing the ratios of the gradients of the concentration plot of the extracts with that of Trolox over a linear concentration range. Results were expressed as mM Trolox equivalent (TE) antioxidant capacity per gram of sample extracts. BHT and rutin were used as positive controls.

Ferric reducing antioxidant power assay

The ferric reducing activity of the plant extracts was estimated based on the ferric reducing ability of plasma (FRAP) assay developed by Pulido et al. (2000). The FRAP reagent consisted of 2.5 mL of 20 mM 2,4,6-Tris (2-pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$. $6H_2O$ and 25 mL of 0.3 M acetate buffer (pH − 3.6). The assay was performed as follows: freshly prepared FRAP reagent was incubated at 37 °C for 5 min after which a blank reading was taken at 593 nm. Thereafter, 30 μL of extract or standard and 90 μL water were added to 900 μL of the FRAP reagent. Absorbance readings were recorded immediately upon the addition of the FRAP reagent after the start of the reaction. The absorbance was related to the absorbance changes of an Fe (II) standard solution tested in parallel. All results were based on three experiments and results were expressed as micro moles ferric reducing activity of the extracts per gram of dried weight. BHT and rutin were used as positive controls. Methanolic solutions of known Fe (II) concentration, ranging from 100 μM to 2000 μM, ($FeSO_4 \cdot 7H_2O$) were used for the preparation of the calibration curve. The parameter equivalent concentration was defined as the concentration of antioxidant having a ferric-TPTZ-reducing ability equivalent to that of 1 mM $FeSO_4 \cdot 7H_2O$. An equivalent concentration was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of Fe (II) solution.

Phosphomolybdenum assay

The antioxidant activities of extracts were evaluated using the green phosphomolybdenum complex formation according to the method of Prieto et al. (1999). A 100 μL aliquot of different extracts was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were covered with foil and incubated in a water bath at 95 °C for 90 min. After the samples had been cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. The reported results were mean values expressed as milligrams of ascorbic acid equivalents/g extract. BHT and rutin were used as reference standards.

Superoxide anion radical scavenging activity

The superoxide anion scavenging activity was determined as described by Beauchamp and Fridovich (1971), with minor modifications. The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in the riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 μg riboflavin, 12 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mg NBT. Briefly, 25–100 μL of the sample solution and standards (rutin and BHT) of sample extract at different concentrations, and reaction mixture were mixed and illuminated for 90 s. The absorbance was measured at 590 nm against the reaction mixture and distilled water as blank. The scavenging activity on superoxide anion generation was calculated as the IC_{50} value described as in DPPH radical scavenging activity.

Analysis of hydroxyl radical-induced DNA strand scission

In vitro protection of plant extracts on hydroxyl-radical-induced plasmid DNA breakage was determined by the conversion of supercoiled conformation to open circular and linear forms according to the method of Keum et al. (2000) with some modifications. The conversion of double-stranded, closed, circular pBR322 plasmid DNA to open, circular and linear forms by hydroxyl radicals ($OH^{\bullet}$) was generated from UV photolysis of hydrogen peroxide (H_2O_2). Briefly, the reaction mixture (10 μL) consisted of 25 μg pBR322 plasmid DNA, 10 mM Tris–HCl, 1 mM EDTA, 10 mM H_2O_2 and the plant extracts at different concentrations (1–100 μg). Plasmid DNA pBR322 exposed with plant extract alone was used as a control. The reaction mixture was exposed to a 25 W UV lamp (Philips; JG Eindhoven, the Netherlands) with a distance of 10 cm for 15 min at 37 °C. The reaction was stopped by the addition of 5 μL loading buffer (0.25% bromophenol blue and 30% glycerol) and electrophoresed on 1.5% agarose gel containing 0.5 μg/mL of ethidium bromide at 70 V for 30 min. The gel was observed and photographed under a UV transilluminator (G:BOX F3, Syngene; Frederick, MD, USA) using the GeneSys software (1.4.6.0; Syngene, Frederick, MD, USA).

Cell culture maintenance

The RAW 264.7 (mouse macrophage), MCF-7 (human breast cancer) and Caco-2 (human colon cancer) cell lines were procured from the National Centre for Cell Sciences, Pune, India. The RAW 264.7 and MCF-7 cells were maintained in Dulbecco's modified Eagle medium and the Caco-2 was maintained in minimal Eagle media supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 U/mL streptomycin in a humidified incubator in 5% CO_2 at 37 °C.

Anticancer activity

The anticancer property of the extracts was examined on MCF-7 and Caco-2 cancerous cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The dry extracts were dissolved in 0.01% DMSO and diluted in cell culture media (without FBS) to give a final concentration of 0.1–100 μg/mL. Briefly, 1×10^5 cells/well were seeded in 96-well plates and allowed to settle down for 12 h. The different concentrations of the plant extract were added to the cells and further incubated for 24 h. The culture supernatant was replaced with 100 μL of MTT solution (5 mg/mL in PBS) and incubated for 3 h at 37 °C. Following the incubation period, the formazan crystal (the reduction product of MTT) was dissolved in 100 μL DMSO and the absorbance was measured using a multi plate reader (Tecan infinite 200 pro; Männedorf, Switzerland) at 570 nm. The wells containing 5-fluorouracil with media were used as a positive control. The morphological features of the cells were photographed under an inverted microscope (Olympus; Shinjuku-ku, Japan). The cell viability was expressed as a percentage of the control and the IC_{50} value was calculated using Equation (4):

$$\%\text{Cell viability} = 100 - [100 \times (\text{Ac} - \text{At}) \div \text{Ac}]. \quad (4)$$

where A_t is the absorbance value of extracts/reference samples and A_c is the absorbance value of the control.

Statistical analyses

All experiments were carried out in triplicate independently and the results were expressed as mean ± SD. The data were analyzed using one-way analysis of variance followed by Tukey's test and mean values were considered statistically significant at $p < 0.05$. Graphical representation was performed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA).

Results and discussion

Extract recovery percentage

The Soxhlet method is very simple and inexpensive for the extraction of plant-based products (Luque de Castro and Garcia-Ayuso, 1998). The highest yield percentage was obtained from LH, (7.80%) and for stem, SM produced a maximum yield of 7.22%. Comparing both plant parts, leaf acetone extract showed low yield (0.16%) and the decreasing order of recovery percentage was: leaf hot water (LH) > stem methanol (SM) > leaf methanol (LM) > leaf petroleum ether (LP) > stem hot water (SH) > leaf chloroform (LC) > leaf ethyl acetate (LEA) > stem ethyl acetate (SEA) > stem chloroform (SC) > stem petroleum ether (SP) > stem acetone (SA) > leaf acetone (LA) as shown in Fig. 1. Using various solvents yielded different extracts with a range of compositions. However, a lower recovery may often result using the alternative solvents due to the decreased extracting affinity of the solvent and solute (Li et al., 2004).

Quantification of total phenolics, tannins and flavonoids

In the Soxhlet method for extraction, the bound phenol compounds from plant materials can be released using heat treatment (Jeong et al., 2004) with powerful antiradical activity. In this study, the total phenolics, total tannins and flavonoids constituents of the crude extracts were evaluated and the results were presented as equivalents of various standard compounds (Table 1). The total phenolics content ranged widely from 46.39 ± 2.44 mg GAE/g extract to 415.41 ± 9.18 mg GAE/g extract in both leaf and stem extracts. For the leaf extracts, the highest amount of total phenolic content was found in LM extract (415.41 ± 9.18 mg GAE/g extract) followed by LEA extract (355.41 ± 8.48 mg GAE/g extract) and the least amount was found in LP extract (56.59 ± 7.62 mg GAE/g extract). For the stem extracts, SEA, SA and SM extracts showed comparable amounts of 252.27 ± 7.83 mg GAE/g extract, 219.53 ± 7.48 mg GAE/g extract and 204.43 ± 13.37 mg GAE/g extract, respectively. The phenolic compounds present in medicinal plant extracts are important due to their multifunctional pharmacological properties as these secondary groups of phytochemicals act broadly as natural antioxidants through their oxygen quenching, reducing and metal chelating abilities so that plant extracts rich in phenolic compounds are often correlated with an abundant range of therapeutic and physiological benefits other than antioxidant properties (Obrenovich et al., 2011). Thus, medicinal plants

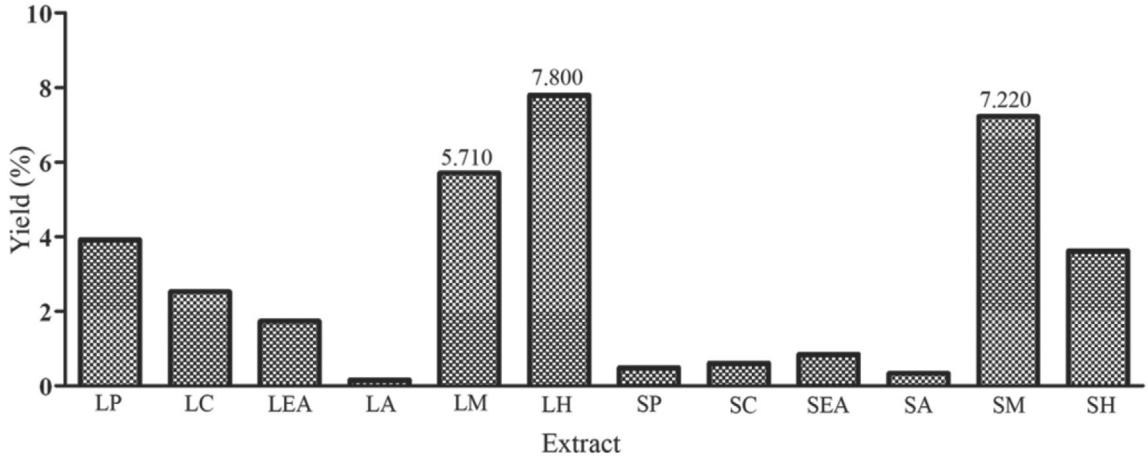

Fig. 1. Extract recovery percentage of *P. stocksii* stem and leaf extracts using leaf petroleum ether (LP); leaf chloroform (LC); leaf ethyl acetate (LEA); leaf acetone (LA); leaf methanol (LM); leaf hot water (LH); stem petroleum ether (SP); stem chloroform (SC); stem ethyl acetate (SEA); stem acetone (SA); stem methanol (SM); stem hot water (SH).

Table 1
Triplicate determination (mean ± SD, n = 3) of total phenolics, tannins and flavonoids.

	Extract*	Total phenolics (mg GAE†/g extract)	Tannin (mg GAE/g extract)	Flavonoids (mg RE‡/g extract)
LEAF	LP	$56.59 \pm 7.62^{f§}$	9.68 ± 3.11^{e}	76.44 ± 8.00^{f}
	LC	70.41 ± 4.11^{f}	16.78 ± 3.77^{e}	62.44 ± 6.19^{f}
	LEA	355.41 ± 8.48^{b}	141.92 ± 4.13^{b}	777.11 ± 6.04^{a}
	LA	176.59 ± 6.65^{e}	46.58 ± 0.93^{d}	173.78 ± 3.67^{d}
	LM	415.41 ± 9.18^{a}	177.53 ± 5.22^{a}	530.22 ± 6.04^{b}
	LH	140.90 ± 7.47^{e}	52.79 ± 0.83^{d}	181.33 ± 5.03^{d}
STEM	SP	63.18 ± 7.18^{f}	8.81 ± 2.42^{e}	107.55 ± 6.04^{e}
	SC	46.78 ± 1.79^{f}	13.52 ± 9.59^{e}	31.11 ± 8.33^{f}
	SEA	252.27 ± 7.83^{c}	70.50 ± 4.14^{c}	240 ± 4.00^{d}
	SA	219.53 ± 7.48^{d}	67.37 ± 2.91^{c}	133.33 ± 5.69^{e}
	SM	204.43 ± 13.37^{d}	66.12 ± 17.10^{c}	309.56 ± 6.67^{c}
	SH	46.39 ± 2.44^{f}	8.95 ± 3.01^{e}	38 ± 1.76^{f}

* Leaf petroleum ether (LP); leaf chloroform (LC); leaf ethyl acetate (LEA); leaf acetone (LA); leaf methanol (LM); leaf hot water (LH); stem petroleum ether (SP); stem chloroform (SC); stem ethyl acetate (SEA); stem acetone (SA); stem methanol (SM); stem hot water (SH); † gallic acid equivalents; ‡ rutin equivalents; § statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$.

could be beneficial in the treatment of diseases among human communities with one or more of complications, together with microbial infection, stress, allergy and inflammation. The extracts of *P. stocksii* which had relatively low levels of total tannin but the highest total phenolics were from LM (177.53 ± 5.22 mg GAE/g extract) followed by LEA extract (141.92 ± 4.13 mg GAE/g extract). The highest levels of flavonoids were obtained in LEA (777.11 ± 6.04 mg RE/g extract) followed by LM (530.22 ± 6.04 mg RE/g extract) extracts. The ethyl acetate extracts of both leaf and stem had the highest total phenolics and also total flavonoid contents and also showed better activities in all the assays carried out in this study. The petroleum ether and chloroform extracts from both the leaf and stem produced the lowest amounts of total phenolics, total tannin and flavonoid contents.

High performance liquid chromatography analysis

Chromatograms of *P. stocksii* leaf and stem extracts are presented in Fig. 2. The amounts of phenolic compounds detected in the samples are shown in Table 2. The HPLC analysis revealed the presence of ferulic acid, quercetin, gallic acid, rutin, *p*-coumaric acid, caffeic acid, chlorogenic acid, trans - cinnamic acid, naringenin and catechin Comparing all extracts, LEA showed higher amounts of quercetin (30.28 μg/mg of extract) and LA also showed an appreciable amount of quercetin (10.86 μg/mg of extract). The quantitative analysis showed high phenolic and flavonoid contents in the various extracts of *P. stocksii* but the specific compounds present in these were known only by the HPLC analysis. The HPLC analysis of ethyl acetate, methanol and acetone extracts showed the presence of many of the standard phenolic and flavonoid compounds. Earlier studies of aromatic plants have revealed many of the active principles as: luteolin, quercetin, ermanine, kaempferol, vanillic acid, benzyl alcohol, kumatakenin, pachypodol, flavons, pogostone, phenylethanoids (acetoside, isoacetoside, crenatoside), ombuine licochalcone and 5,7-dihydroxy-3′44-dimethoxyflavanone (George et al., 2016). Correspondingly, the present study showed the phenolic and flavonoids contents present in *P. stocksii*. The presence of these principles compounds in the leaf ethyl acetate extract is important as it is this extract which showed the best activity for almost all the assays carried out. It might be that this flavonoid could be responsible for the efficiency of this extract (Chakrapani et al., 2013).

In vitro antioxidant assays

The phenolic compounds act as antioxidants using a mechanism based on either single electron transfer (SET) or hydrogen atom transfer (HAT); in SET reactions, a single redox reaction is involved with the oxidant as an indicator of measurement and the

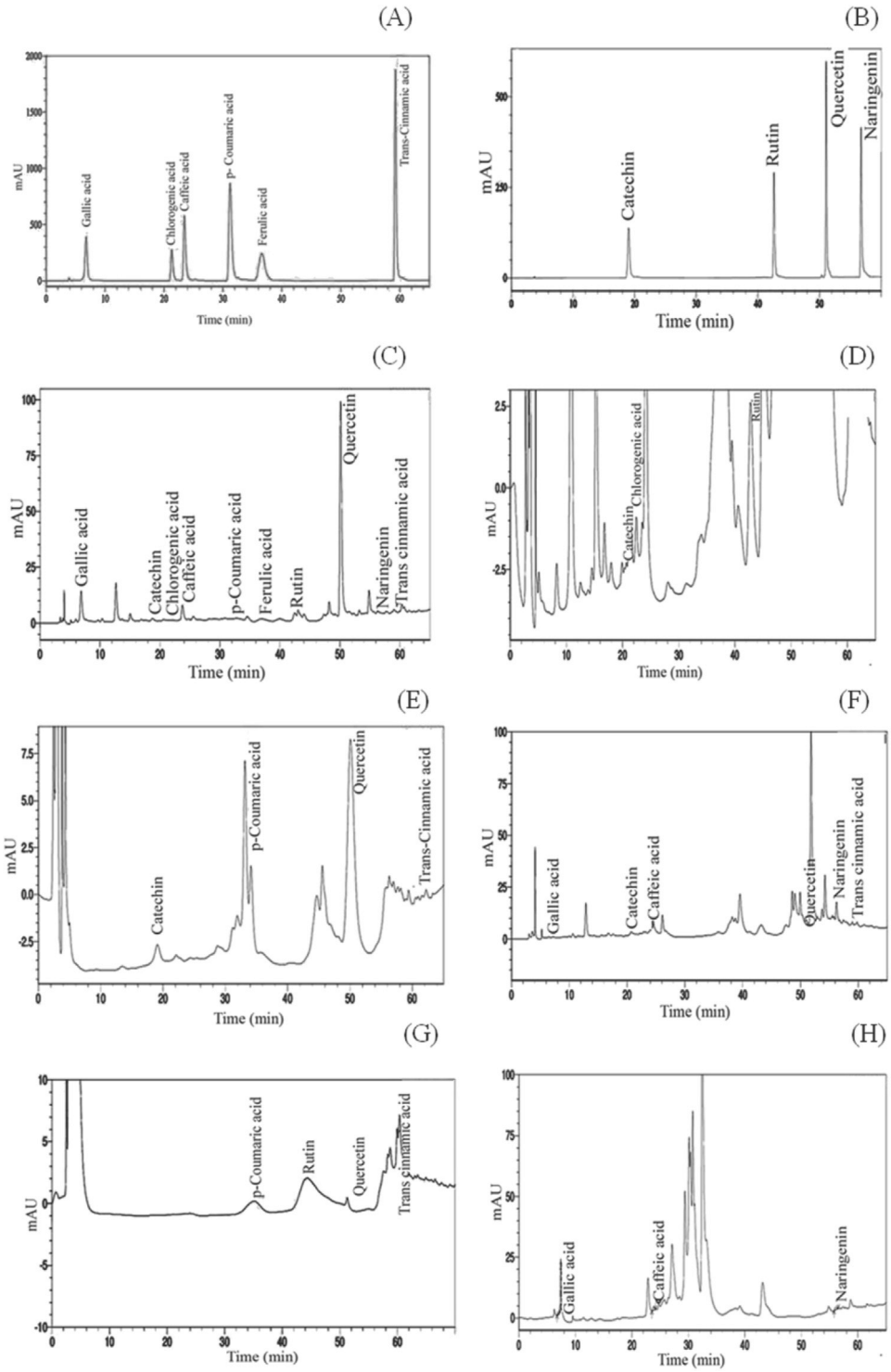

Fig. 2. High performance liquid chromatography analysis of *P. stocksii* extracts: (A) and (B) are reference compounds; (C) leaf ethyl acetate; (D) leaf methanol; (E) leaf acetone; (F) stem ethyl acetate; (G) stem methanol; (H) stem aceton

HAT-based mechanism involves the use of a synthetic source of free radicals, an antioxidant and an oxidizable molecular probe to measure radical scavenging ability (Huang et al., 2005). Therefore, it is important to use several assays to comparatively evaluate the antioxidant activity in plant extracts. Previous works by Blois (1958) and Re et al. (1999) described some assays that have been frequently used to estimate antioxidant capacities in plant extracts.

2,2-Diphenylpicrylhydrazyl radical scavenging activity

The antiradical activities of various extracts were determined using DPPH which is a stable free radical and has an absorption band at 515 nm which can be reduced markedly in the presence of proton radical scavengers (Blois, 1958). The IC_{50} value is defined as the concentration of the sample at which the inhibition rate reaches 50%. Table 3 illustrates the significant decrease in the concentration of DPPH• due to the scavenging ability of the extracts.

Table 2
High performance liquid chromatography quantification.

Compound name	Retention time (min)	Quantity* (μg/mg extract)					
		LEA	LA	LM	SEA	SA	SM
Gallic acid	6.77	2.69‡	–	–	0.05‡	0.02†	–
Catechin	19.03	1.09‡	1.49‡	0.23†	0.07†	–	–
Chlorogenic acid	21.29	0.01†	–	0.54†	–	–	–
Caffeic acid	23.45	0.97†	–	–	0.02†	0.17†	–
p-Coumaric acid	31.20	0.01†	0.75†	–	–	–	0.70†
Ferulic acid	36.58	0.55†	–	–	–	–	–
Rutin	42.61	2.43‡	–	5.50‡	–	–	9.94‡
Quercetin	51.09	30.28§	10.86‡	–	0.25†	–	0.08†
Naringenin	56.75	0.26†	–	–	1.99‡	0.37†	–
Trans-cinnamic acid	59.24	0.03†	0.01†	–	0.01†	–	0.09†

* Leaf ethyl acetate (LEA); leaf acetone (LA); leaf methanol (LM); stem ethyl acetate (SEA); stem acetone (SA); stem methanol (SM); † less amount; ‡ moderate amount; § higher amount.

Table 3
In vitro antioxidant assay results (mean ± SD of triplicate determination, n = 3) of various assays*.

	Extract†	DPPH* (IC^*_{50} μg/mL)	$ABTS^{\bullet+}$* (mM TE*/g extract)	FRAP* (μM Fe(II)E*/mg extract)	Phosphomolybdenum reduction (mg AAE*/g extract)	Superoxide radical scavenging activity (IC_{50} μg/mL)
LEAF	LP	12.96	$6.73 \pm 5.17^{d‡}$	2.88 ± 0.26^{e}	314.71 ± 2.29^{a}	91.27
	LC	72.99	9.04 ± 4.52^{e}	58.32 ± 4.36^{d}	237.64 ± 1.68^{c}	50.38
	LEA	03.46	27.41 ± 7.51^{c}	152.17 ± 1.51^{a}	276.07 ± 1.94^{b}	24.16
	LA	04.57	13.82 ± 1.59^{d}	98.75 ± 2.63^{c}	128.09 ± 1.68^{d}	48.82
	LM	05.72	26.71 ± 1.50^{c}	154.17 ± 1.81^{a}	200.91 ± 2.23^{c}	38.12
	LH	37.94	3.26 ± 1.79^{e}	67.89 ± 0.43^{d}	34.03 ± 1.32^{e}	89.87
STEM	SP	11.50	2.90 ± 9.27^{e}	6.15 ± 1.29^{e}	131.49 ± 3.01^{d}	126.50
	SC	45.55	4.61 ± 1.06^{e}	76.99 ± 2.28^{d}	183.08 ± 1.94^{c}	96.01
	SEA	03.64	24.45 ± 5.13^{c}	126.13 ± 3.37^{b}	230.85 ± 1.32^{c}	32.78
	SA	09.73	14.33 ± 4.22^{d}	102.05 ± 5.85^{c}	113.23 ± 1.60^{d}	61.34
	SM	10.39	16.47 ± 6.68^{d}	124.76 ± 4.37^{b}	145.71 ± 2.57^{d}	65.77
	SH	110.27	1.51 ± 4.89^{e}	14.56 ± 0.97^{e}	26.18 ± 4.45^{e}	30.05
	BHT	7.93	18.56 ± 5.09^{a}	155.05 ± 2.00^{a}	393.69 ± 101.05^{a}	20.61
	Rutin	6.35	15.64 ± 7.30^{b}	154.24 ± 1.49^{a}	548.05 ± 78.36^{a}	9.06

* DPPH (2,2-diphenylpicrylhydrazyl radical scavenging activity); IC_{50} (inhibition concentration for 50%); $ABTS^{\bullet+}$ (2,2'azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical scavenging activity); TE (Trolox equivalents); FRAP (ferric reducing antioxidant power assay); Fe(II)E (ferrous ion equivalents); AAE (ascorbic acid equivalents) † leaf petroleum ether (LP); leaf chloroform (LC); leaf ethyl acetate (LEA); leaf acetone (LA); leaf methanol (LM); leaf hot water (LH); stem petroleum ether (SP); stem chloroform (SC); stem ethyl acetate (SEA); stem acetone (SA); stem methanol (SM); stem hot water (SH); ‡ statistically significant at $p < 0.05$ where in each column $^{a} > ^{b} > ^{c} > ^{d} > ^{e}$.

Among the different solvent extracts, LEA (3.46 μg/mL), SEA (3.64 μg/mL) and LM extracts (5.72 μg/mL) showed better IC_{50} values which were comparable with the positive compounds BHT and rutin (7.93 μg/mL and 6.35 μg/mL, respectively). The ethyl acetate extracts showed the most potent activity and indicated that compounds with the strongest radical-scavenging activity in this species are of medium polarity. The extract scavenging ability towards free radicals could be related to the presence and nature of the phenolics contributed by their electron transfer and hydrogen donating ability (Li et al., 2009).

2,2′azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical scavenging activity

The ABTS radical cation decolorization assay, showed quite similar results compared to those obtained in the DPPH reaction and the radical generated from the oxidation of ABTS by potassium

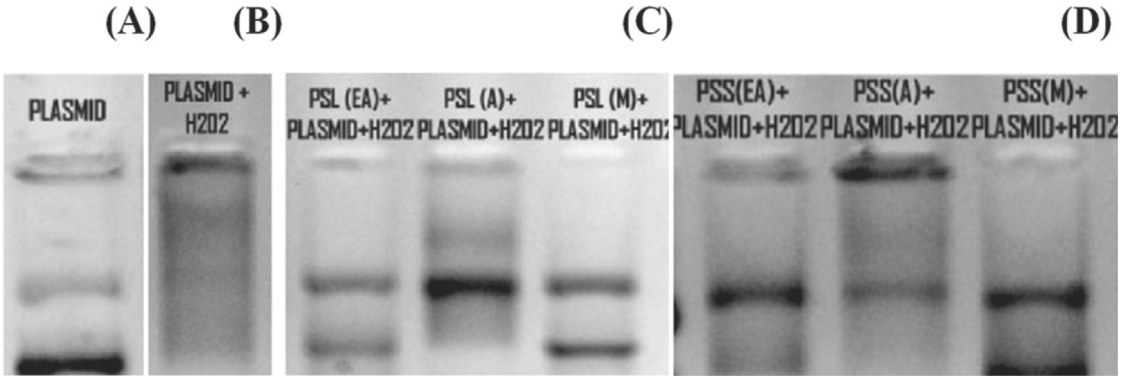

Fig. 3. Hydroxyl radical-induced DNA strand scission: (A) plasmid; (B) plasmid + H_2O_2; (C) leaf extracts (leaf ethyl acetate, leaf acetone, leaf methanol, respectively); (D) stem extracts (stem ethyl acetate, stem acetone, stem methanol, respectively).

persulfate is an exceptional tool for its hydrogen-donating and chain-breaking ability (Re et al., 1999) of a compound or crude extract. The positive reference compounds BHT (18.56 ± 5.09 mM TE/g extract) and rutin (15.64 ± 7.30 mM TE/g extract) showed the highest scavenging abilities. Among the different solvent extracts, LEA (27.41 ± 7.51 mM TE/g extract) and LM (26.71 ± 1.50 mM TE/g extract) extracts showed remarkable $ABTS^{\bullet+}$ scavenging activity, followed by SEA (24.45 ± 5.13 mM TE/g extract) and SM (16.47 ± 6.68 mM TE/g extract) extracts, which exhibited substantial activities (Table 3). However, among all the solvent extracts, ethyl acetate extracts showed better activity. Previous study on higher plants by Ardestani and Yazdanparast (2007) also reported similar findings that increased in activity in ethyl acetate extract and both the DPPH and ABTS assay results were correlated with each other.

Ferric reducing antioxidant power assay

It has been reported that antioxidant activity is associated with reducing potential and serves as an expression of sample antioxidant activity; Fe (III) reduction is an indicator of electron donating ability which is an important mechanism in phenolics as antioxidants (Hinneburg et al., 2006). The electron-donating property indicates the antioxidant compounds can act as primary and secondary antioxidants as donors and can reduce the oxidized intermediates in lipid peroxidation process (Yen and Chen, 1995). Significant differences in FRAP values were found among the leaf and stem extracts. The ferric reducing ability of the extracts revealed that all resulted in optimal FRAP activity (1704.11–2861.11 mM Fe (II)/mg extract). Among the extracts, the highest activity was noted for LM and LEA extracts (154.17 mM Fe (II)E/mg extract and 152.17 mM Fe (II)E/mg extract, respectively) followed by SEA and SM extracts (126.13 mM Fe (II)E/mg extract and 124.76 mM Fe(II)E/mg extract, respectively) (Table 3). The highest level of increase in ferric-reducing ability occurred in the methanol and ethyl acetate dissolved extracts, while the petroleum ether-based extracts of stem and leaf had the least gain in ferric reducing ability. The current findings were similar to those of Ardestani and Yazdanparast (2007) who concluded that the phenolic compounds in ethyl acetate and methanol contributed to the increase in reducing power.

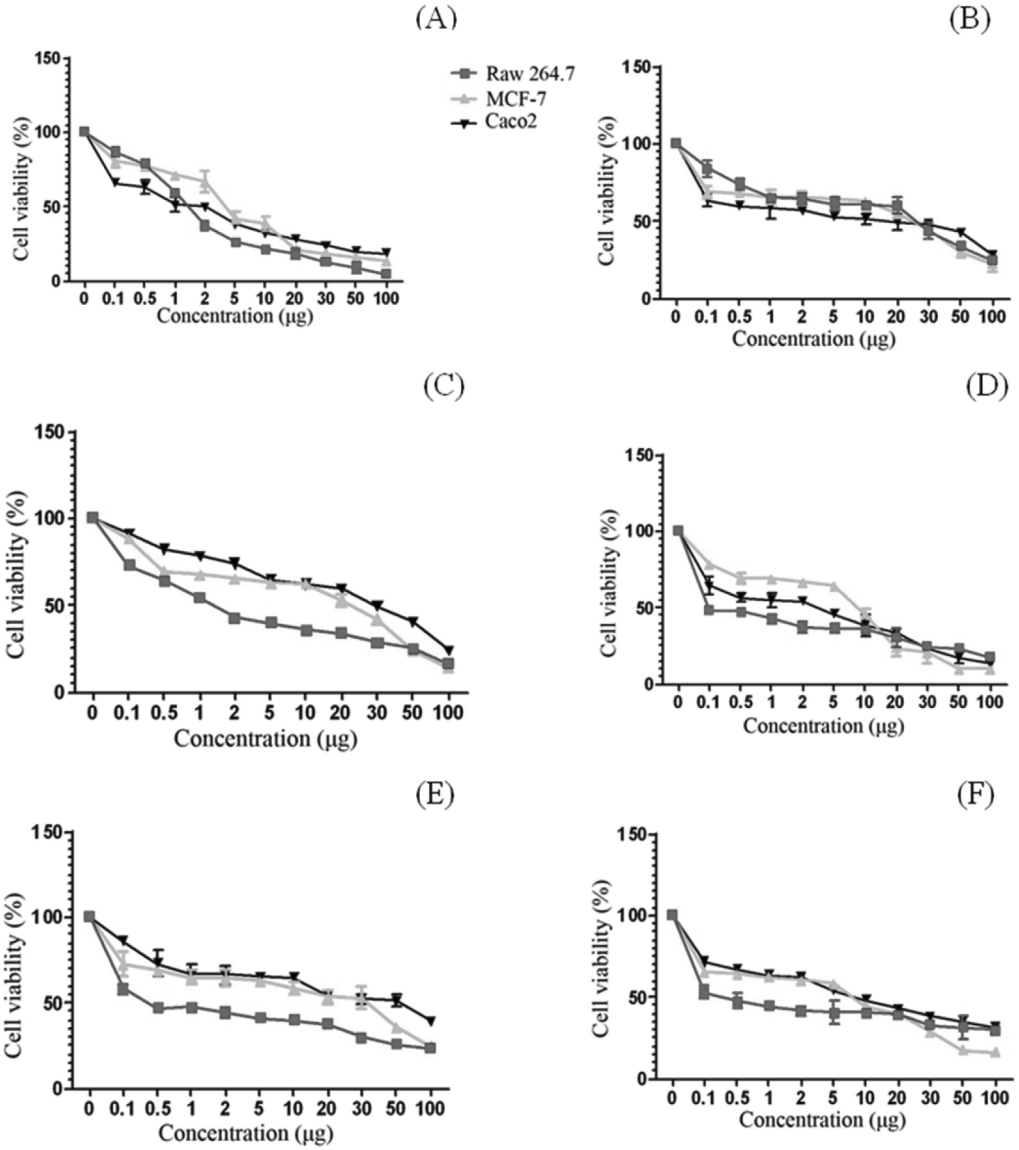

Fig. 4. Cell viability of extracts: (A) cytotoxicity of leaf ethyl acetate; (B) cytotoxicity of leaf acetone; (C) cytotoxicity of leaf methanol; (D) cytotoxicity of stem ethyl acetate; (E) cytotoxicity of stem acetone; (F) cytotoxicity of stem methanol.

Phosphomolybdenum assay (total antioxidant activity)

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm, where an increase in the absorbance indicates an increase in the total antioxidant capacity (Prieto et al., 1999). Murugan and Parimelazhagan (2014); Saravanan and Parimelazhagan (2014) also reported total antioxidant activity in some higher plant

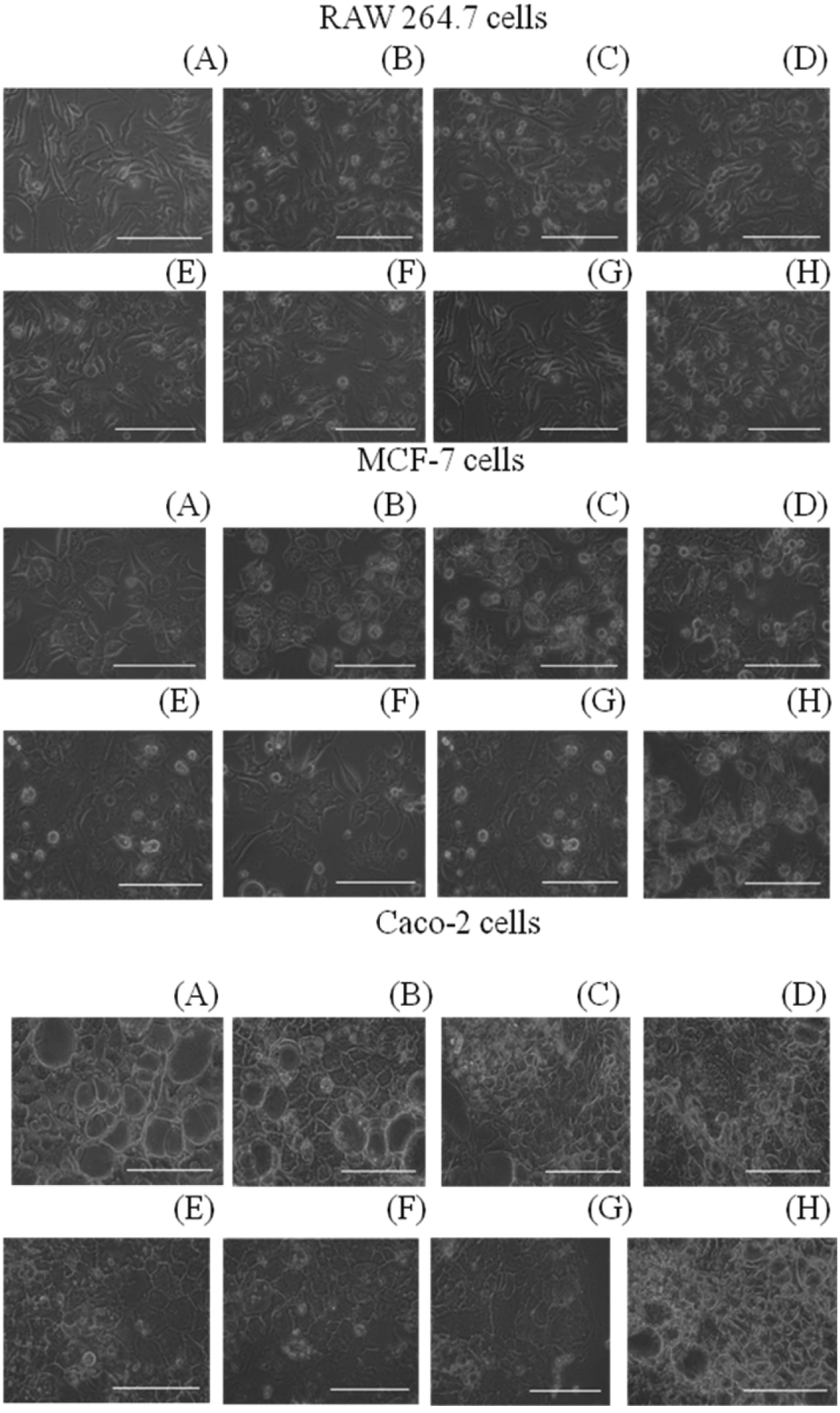

Fig. 5. Morphological analysis of cytotoxicity of extracts for RAW264.7 cells, MCF-7 cells and Caco-2 cells: (A) control; (B) leaf ethyl acetate; (C) leaf methanol; (D) leaf acetone; (E) stem ethyl acetate; (F) stem methanol; (G) stem acetone; (H) drug; All scale bars = 200 μm.

extracts. The antioxidant capacities of leaf and stem extracts of *P. stocksii* were measured spectrophotometrically through this method and BHT, quercetin and rutin were used as positive reference compounds. The results showed that rutin exhibited the highest activity (548.05 ± 78.36 mg AAE/g extract), followed by BHT (393.69 ± 101.05 mg AAE/g extract), while in *P. stocksii* extracts, LP and LEA (314.71 ± 2.29 mg AAE/g extract and 276.07 ± 1.94 mg AAE/g extract, respectively) showed higher activities when compared with all other solvents extracts in both parts (Table 3). The higher activity of the crude extracts may have been due to the presence of other compounds and also the solvents used for extraction have been reported to produce spectacular results on the reduction of Mo (VI) (Yuan et al., 2005).

Superoxide radical scavenging activity

Superoxide anion radicals are the initial radicals whose role is fundamental in the formation of reactive oxygen species (ROS) according to Stief (2003). The extracts were found to be an efficient scavenger of superoxide radicals generated in the riboflavin-NBT-light system *in vitro*. LEA, SH and SEA extracts showed higher superoxide radical scavenging activities compared to other solvent extracts. Among them, the LEA extract showed a significant result with an IC_{50} value of 24.16 μg/mL. With stem, higher activities were shown by the SH and SEA extracts with IC_{50} values of 30.05 μg/mL and 32.78 μg/mL, respectively (Table 3). The superoxide radical scavenging activities of *P. stocksii* parts followed the trend of stem < leaf. The results suggested that the extracts display scavenging effects on superoxide anion radical generation that could help to prevent or ameliorate oxidative damage and prevent the formation of ROS.

Analysis of hydroxyl radical-induced DNA strand scission

ROS and reactive nitrogen species are the major sources that can damage lipids, proteins and DNA during oxidative stress (Berlett and Stadtman, 1997). DNA is more sensitive to oxidative damage as the hydroxyl radicals easily oxidize guanosine and thymine, leading to mutation (Ames et al., 1993). The present study confirmed that the extracts were able to protect pBR322 plasmid DNA against H_2O_2 induced DNA damage (Fig. 3). In this study, the generated hydroxyl radicals were found to induce DNA strand breaks in pBR322 plasmid DNA. These results are encouraging and also may have a positive role in inhibiting several stress-induced disorders. Thus, incubation of pBR322 plasmid DNA with both H_2O_2 and UV resulted in complete destruction of plasmid DNA to smear forms by generating hydroxyl radicals (Fig. 3B). The addition of *P. stocksii* to the reaction mixture substantially diminished the DNA strand scission induced by both H_2O_2 and UV. Thus, the hydroxyl radical-quenching ability of polyphenolic compounds of extracts could account for the protection against oxidative damage to DNA (Fig. 3C and D).

Anti cancer activity

The effect of different concentrations (0.1–100 μg/mL), of *P. stocksii* extracts on MCF-7, RAW 264.7 and Caco-2 cell lines were studied. As depicted in Fig. 4, the extracts significantly inhibited the viability of all cell lines used in this study in a dose-dependent manner. For a concentration up to 5 μg/mL, all extracts inhibited RAW 264.7, MCF-7 and Caco-2 cells by 50% viability; however for a concentration higher 30 μg/mL, all cell lines were destroyed up to 90%. At lower doses, 0.1 μg/mL and 0.5 μg/mL, the extract was tolerated by the cells and its IC_{50} was calculated. In comparison with the control, the incubation with 5 μg/mL extracts significantly reduced the percentage viability in all cells by around 40–60%, by 10–60% in the case of LEA and SEA extracts and by 10–40% as a consequence of acetone and methanol extracts. MCF-7 cells were inhibited significantly by all concentrations of leaf extracts, whereas the viability of RAW 264.7 was inhibited significantly by all the extracts at an initial concentration up to 2 μg/mL. Ethyl acetate extracts caused fluctuations around 100% inhibition of cell viability until the concentration level achieved a clear inhibitory threshold (Fig. 4). The comparison between the leaf and stem extracts showed that while 5 μg/mL concentrations of ethyl acetate, methanol and acetone extracts lowered the viability of MCF-7 cells by around 60%, the Caco-2 and RAW 264.7 cell viability levels of inhibition were nearly 30 and 50%, respectively. A similar phenomenon was observed for the 50 μg/mL concentrations of all extracts which inhibited MCF-7 cells by 80% and Caco-2 cells by 60%. With RAW 264.7, a clear reduction in viability was observed until the cells were exposed to 100 μg/mL (90% inhibition). The IC_{50} values were determined to identify the concentrations of the studied substances which caused a 50% decrease in cell viability with reference to untreated controls that were regarded as 100% living cells. The *P. stocksii* extracts showed some significant IC_{50} values with all the cell lines. When compared with all the extracts SEA, SM and SA showed lower IC_{50} values (8.98, 15.58 and 25.16 μg/mL, respectively) against the RAW 264.7 cell line. However, LEA and SEA showed significant IC_{50} values (36.08 and 38.14 μg/mL, respectively) against the Caco-2 cell line. For the MCF-7 cell line, LEA exhibited a noteworthy IC_{50} value of 48.87 μg/mL when

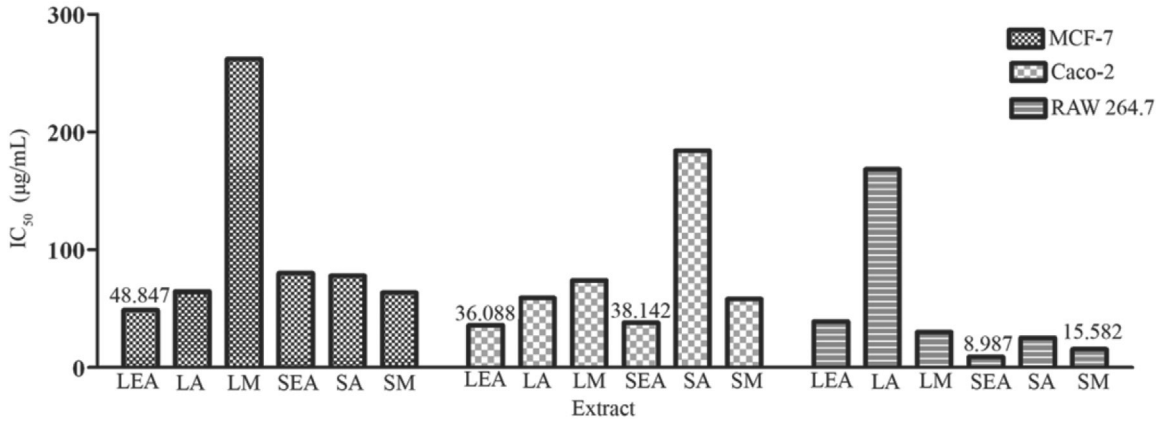

Fig. 6. Concentrations of studied substances which caused a 50% decrease in cell viability with reference to untreated controls (IC_{50}) of extracts on MCF-7, Caco-2 and RAW 264.7 cell lines (LEA = leaf ethyl acetate, LM = leaf methanol, LA = leaf acetone, SEA = stem ethyl acetate, SM = stem methanol, SA = stem acetone).

compared with other extracts (Figs. 5 and 6). Zarai et al. (2011) reported results that supported the relationship of cytotoxicity with antioxidant activity. Thus, the antioxidant activity of *P. stocksii* extracts might contribute to its cytotoxic activity. Many chemo-preventive agents appeared to target signaling intermediates in apoptosis inducing pathways and many of them are reported to have pro-oxidant or antioxidant activity (Sun et al., 2004). Finally, the current study noted that the synergistic effects of these active chemicals with other constituents of the extracts should be taken into consideration.

Hence, the prevention of oxidative stress is an important property of any medicinal plant with regard to the management of stress-related disorders. This study provided justification and encouragement for uses of plant compounds in traditional medicine. Further exploration is still necessary to scrutinize *in vivo* bioactivity and cytotoxicity in order to explore in more depth the potential beneficial uses of plant compounds against diseases and infections caused by microbes.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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