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Comparison of Antioxidant Potential, Total Flavonoid and Phenolic Contents of Different Extracts of *Kalanchoe daigremontiana* Leaves

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ABSTRACT

In present study, different assays have been used to evaluate antioxidant potential of various extracts of *K. daigremontiana* leaves prepared in different solvents and compared with their phenolic and flavonoid contents. The results of various antioxidant assays suggest that the butanol extract exhibits highest antioxidant potential as compared to other solvents (ethanol, dichloromethane, hexane and water). Results of $ABTS^{•+}$ radical decolourization activity show that butanol extract of *K. daigremontiana* leaves exhibited strong activity with IC_{50} value 8.43 μg mL^{-1}. As for lipid peroxidation inhibition studies again butanol extract exhibits highest potential (IC_{50}, 0.97 μg mL^{-1}) which is better than one of the standards gallic acid (IC_{50}, 2.20 μg mL^{-1}) and comparable to *n*-propyl gallate (IC_{50}, 0.40 μg mL^{-1}) and Trolox (IC_{50}, 0.10 μg mL^{-1}). Same fraction possessed maximum total phenolic contents (72 mg/100 g dry weight), while highest total flavonoid contents are observed in case of dichloromethane extract.

KEYWORDS

Antioxidant potential, Lipid peroxidation, *K. daigremontiana*.

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INTRODUCTION

Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as reactive oxygen species (ROS). Many such reactive species are free radicals and have a surplus of one or more free-floating electrons and are, therefore, unstable and highly reactive. Free radicals are being produced in the body during metabolic reactions. Imbalance between the production of free radicals and the ability of biological system to scavenge them, results into a serious condition known as oxidative stress. Oxidative stress may cause chronic damages like, atherosclerosis, myocardial infarction, acute respiratory distress syndrome and rheumatoid arthritis [1]. In addition to this, oxidative stress may enhance the aging process [2]. Types of free radicals include the hydroxyl radical ($OH^{•}$), the superoxide radical ($O^{•2}$), the nitric oxide radical ($NO^{•}$) and the lipid peroxyl radical ($LOO^{•}$) [1]. Lipid peroxidation is important molecular mechanism which is responsible for the oxidative damage to cell structures that lead to cell death. Lipid peroxidation proceeds by a free-radical mediated chain reaction. Methylene group

of poly unsaturated fatty acids is the main target of free radicals. Free radicals abstract hydrogen from the unsaturated fatty acids and produce alkyl radicals finally addition of oxygen to the alkyl radicals generates $ROO^{\bullet}$ [3].

Plants produce many beneficial compounds which mostly protect them from different environmental factors. These compounds or secondary metabolites are mostly responsible for the medicinal properties of plants [4]. Commonly found secondary metabolites present in different plant extracts are flavonoids and phenolic acids [5,6] which are reported as powerful radical scavengers and antioxidants [7,8]. *Kalanchoe daigremontiana* syn. *Bryophyllum daigremontianum* also called Alligator Plant. This plant is distinguished by its ability to propagate *via* vegetative propagation. All parts of the plants are poisonous which can be fatal if ingested by infants or small pests [9]. It is highly anti-inflammatory and promotes wound healing. As it moisturizes it also heals and restores the skin's flexibility, durability and firmness [10]. On the basis of easy availability and medicinal importance *Kalanchoe daigremontiana* was selected for the present study. For this purpose crude alcoholic extracts and its various fractions were tested for *in vitro* anti-lipid peroxidation, $ABTS^{\bullet+}$ radical scavenging activity as well as total phenolic and total flavonoid were also determined.

EXPERIMENTAL

2,2'-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), thiobarbituric acid (TBA), 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), Trolox (6-hydroy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (USA). All other chemicals and solvents used during the study were of analytical grade.

Plant material collection and extraction: Leaves of *Kalanchoe daigremontiana* were collected from the botanical garden of Lahore College for Women University, Lahore and identified from Dr. Tahira Aziz Mughal (Botany Department, Lahore College for Women University, Lahore, Pakistan). Collected leaves were washed and air dried under shadow. After drying they were chopped and soaked in ethanol and covered with aluminum foil to reduce the rate of evaporation. After 8 days solvent was filtered and evaporated with the help of rotary evaporator to get crude ethanolic extract. Crude extract was further extracted into hexane, dichloromethane, butanol and water extracts and dried. All these extracts were used in different assays.

$ABTS^{\bullet+}$ decolourization assay: $ABTS^{\bullet+}$ radical cation decolourization potential of samples and reference compounds was evaluated by applying reported method [11] after slight modifications. ABTS stock solution (7.0 mM) was oxidized by 2.45 mM $K_2S_2O_4$ to generate $ABTS^{\bullet+}$ radical cation. Absorption of the resultant solution was adjusted at 0.70 (± 0.10) (734 nm). Reaction mixture contained 2.5 mL $ABTS^{\bullet+}$ and 0.5 mL sample solution was incubated at room temperature for 5 min and percentage quenching was estimated as:

$$\text{Inhibition } (\%) = \left(1 - \frac{\text{Absorbance}_{(\text{sample})}}{\text{Absorbance}_{(\text{control})}}\right) \times 100$$

Total antioxidant activity: Total antioxidant activity of each extract was evaluated by method of Robbins [12]. A green phosphate molybdenum/Mo(V) complex at acidic pH was formed when Mo(VI) reduced to Mo(V) by the plant extract. 0.5 mL of each extract was mixed with 4 mL of reagent solution containing 0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated at 95 °C for 90 min. The absorbance of the solution was measured at 695 nm using a UV-visible spectrophotometer (T80, PG- Instruments Limited, UK) against blank (methanol) after cooling to room temperature. Total antioxidant activity is expressed as gram equivalence of ascorbic acid per gram dry weight.

Anti-lipid peroxidation assay: *In vitro* anti-lipid peroxidation activity of samples and standards was assessed by a modified Halliwell *et al.* [13] protocol. KCl (1.5 mL, 1.15 %) and egg yolk (1.0 mL, 10.0 %) were added to series of sample's concentration. 0.2 mM ferric chloride (0.5 mL) was added in the reaction mixture to induce lipid peroxidation. After 1 h, incubation at 37 °C, reaction was stopped by adding ice-cold HCl (2.0 mL, 0.25 N) containing 15 % trichloroacetic acid (TCA), 0.38 % thiobarbituric acid (TBA) and 0.5 % butylated hydroxytoluene (BHT). Resultant mixture was then heated for 1 h at 80 °C, followed by centrifugation at 3000 rpm. Pink adduct of malondialdehyde (MDA) and thiobarbituric acid was detected spectrophotometrically at 532 nm.

DPPH radical scavenging assay: DPPH radical scavenging activity of various extracts was estimated by slightly modified standard DPPH assay protocol [14]. The reaction mixture contains 0.5 mL of different fractions of sample and 2.5 mL of DPPH (100 µM in methanol). After incubation at 37 °C in dark the absorbance was measured at 517 nm by using UV-visible spectrophotometer.

Total phenolic contents: Reaction mixture contains 20 µL sample solution, 1.58 mL deionized water and 100 µL Folin-Ciocalteu reagent. After 10 min, 300 µL of 25 % sodium carbonate solution was added in it and incubated at 40 °C for 0.5 h. Absorbance was recorded at 765 nm and results were documented as gallic acid equivalent (GAE mg g^{-1} dry wt.) [15].

Total flavonoid content: 250 µL of Sample solution was added in 1.5 mL deionized water and 90 µL of $NaNO_2$ solution (5 %). After 5 min 180 µL of $AlCl_3$ solution (10 %) and 0.6 mL of 1 M NaOH solution was added to this mixture and volume was made up to 3 mL by deionized water. Absorbance of final solution was recorded at 510 nm. Results were documented as quercetin equivalent (QE mg g^{-1} dry wt.) [16].

Statistical analysis: Data were represented as mean value (± SD) of triplicates from three independent experiments. IC_{50} values were calculated by linear regression analysis. Data were statistically examined using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Antioxidant compounds present in different plants play an important role as a health promoting factors. These important molecules reduced the risk of chronic diseases including inflammation and heart diseases. Pharmacological properties of plant are not because of a single mechanism but it exhibits its protective effect through a combination of mechanisms. In present study antioxidant potential of extracts in different solvents of *Kalanchoe daigremontiana* leaves was determined

through different assay including ABTS$^{•+}$ decolourization, DPPD radical scavenging and anti-lipid peroxidation assay.

ABTS$^{•+}$ decolourization: This method is rapid and very sensitive for the detection of both lipophilic and hydrophilic antioxidants, including carotinoids, flavonoids and plasma antioxidants. ABTS$^{•+}$ is a nitrogen centered radical with a characteristic blue-green colour, which become colourless upon reduction by antioxidants [11]. Results of ABTS$^{•+}$ decolourization are presented in Table-1. Among different extracts of leaves, butanol fraction has shown highest de-colourization potential (8.43 µg mL^{-1}) which is quite near to one of the standards Trolox (3.86 µg mL^{-1}). Radical decolourization potentials of CH_2Cl_2, C_2H_5OH and water extracts were in decreasing order as shown in Table-1.

TABLE-1
COMPARISON OF *in vitro* ANTI-RADICAL AND ANTI-LIPID PEROXIDATION PROPERTIES OF VARIOUS FRACTIONS OF *Kalanchoe daigremonitana* LEAVES

Fractions/ Standard	50 % Inhibitory concentration (IC_{50})		
	ABTS$^{•+}$ Decolorization assay (µg mL^{-1})	Anti-lipid Peroxidation assay (mg mL^{-1})	DPPH radical scavenging assay (µg mL^{-1})
Ethanol	28.40 ± 0.09[a]	1.65 ± 0.32	61.9 ± 0.17
Hexane	48.00 ± 0.42	5.86 ± 0.06	198.9 ± 0.22
Dichloromethane	13.52 ± 0.02	4.41 ± 0.08	200.0 ± 0.32
Butanol	8.43 ± 0.33	0.97 ± 0,12	23.0 ± 0.27
Water	39.66 ± 0.21	8.36 ± 0.50	72.4 ± 0.33
n-Propyl gallate	1.71 ± 0.03	0.40 ± 0.02	2.30 ± 0.04
Trolox	3.86 ± 0.03	0.10 ± 0.01	6.20 ± 0.02
Gallic acid	0.68 ± 0.08	2.20 ± 0.33	2.40 ± 0.03

[a]Data represented here as mean of three experiments (n = 3) ± SD.

DPPH radical scavenging: This is an easy, sensitive and rapid tool to determine the antioxidant potential of extracts and compounds. DPPH is a stable radical with an odd electron. Free radical which is purple colour, gives strong absorption maximum at 517 nm. Antioxidants reduce this radical by donating hydrogen which is indicated by the colour change from purple blue to yellow [14]. Greater the phenolic contents of the fraction more will be the decolourization. Analysis of data indicates that almost all extracts of *Kalanchoe daigremontiana* leaves have shown DPPH radical scavenging potential (Table-1). The butanol extract of *Kalanchoe daigremontiana* leaves has shown highest radical scavenging potential (23.0 µg mL^{-1}), followed by EtOH (61.9 µg mL^{-1}) and water (72.4 µg mL^{-1}) as shown in Table-1. Among all the tested extracts CH_2Cl_2 fraction was least active (200 µg mL^{-1}) in this assay.

Antilipid peroxidation: Lipid peroxidation is actually oxidative degradation of lipids, where free radicals grab the electrons from the lipids in the cell membrane, resulting in cell damage. Poly unsaturated lipids are especially susceptible to this type of damage under oxidizing environment and form lipid peroxides. These peroxides further converted into malondialdehyde (MDA) which has become one of the most widely reported analytes for estimating oxidative stress effect on lipids. In this study, slightly modified thiobarbituric acid reactive species (TBARS) assay was used to measure *in vitro* lipid peroxidation using egg yolk homogenate as lipid rich media [17]. Initially all samples were screened at 5 mg/mL, after that IC_{50} (50 % inhibitory concentration) was determined at different concentrations of each sample (Table-1). BuOH sample showed maximum inhibitory potential with IC_{50} value 0.97 mg mL^{-1} which compare able to one of the standards *n*-propyl gallate (IC_{50}, 0.40 mg mL^{-1}) and better than gallic acid (IC_{50}, 2.20 mg mL^{-1}). Crude (EtOH) extract also showed good ability to retard lipid peroxidation (IC_{50}, 1.65 mg mL^{-1}). Least activity was exhibited by water extract (IC_{50}, 8.36 mg mL^{-1}), while hexane (IC_{50}, 5.86 mg mL^{-1}) and dichloromethane (IC_{50}, 4.41 mg mL^{-1}) showed moderate activity when compared with different standards.

Total antioxidant activity: Total antioxidant activity of different extracts of *Kalanchoe daigremontiana* was determined by the formation of phosphomolybdenum complex. In presence of sample containing antioxidants, Mo(VI) is reduced to green colour Mo(V) complex which shows absorption at 695 nm [12,18]. Different fractions showed various levels of activity (Table-2). Highest level was achieved by dichloromethane extracts while the order of activity of other fraction is as follows: water > hexane > ethanol > BuOH.

TABLE-2
COMPARISON OF TOTAL ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENTS OF DIFFERENT EXTRACTS OF *Kalanchoe daigremonitana* LEAVES

Fractions	Total phenolic contents (mg/100 g dry weight)[c]	Total flavonoids (mg/g dry weight)[b]	Total antioxidant activity[a] (ascorbic acid equal activity)[d]
Ethanol	27.0 ± 0.05	660.0 ± 0.12	240.0 ± 0.40
Hexane	13.0 ± 0.03	925.0 ± 0.08	195.0 ± 0.15
Dichloromethane	28.8 ± 0.11	935.0 ± 0.16	150.0 ± 0.24
Butanol	72.1 ± 0.21	800.0 ± 0.20	360.0 ± 0.33
Water	26.0 ± 0.14	690.0 ± 0.02	170.0 ± 0.08

[a]mg ascorbic acid equivalent; [b]mg gallic acid equivalent 100 g^{-1}; [c]mg quercetin equivalent g^{-1}; [d]*n*-propyl gallate, gallic acid and Trolox were used as standard compounds. Values are same as given in Table-2 [Ref. 20]

Total phenolic and flavonoid contents: Phenolic acids like caffeic acid, gallic acid ferulic acid have repeatedly been implicated as natural antioxidants widely distributed in plant kingdom. Phenolics with two phenolic rings join together are flavonoids and are reported as strong antioxidants because of their ability to scavenge free radicals [19]. Total phenolic contents of crude as well as various extracts of leaves of *K. daigremontiana* were assayed as gallic acid equivalent (mg/ 100 g dry weight) using Folin-Ciocalteu reagent. The blue compound formed between extracts and Folin-Ciocalteu reagent was identified by spectrophotomeric method. Total flavonoid content was also spectrophotomerically measured and expressed as quercetin eq. g^{-1} dry wt. From this study it is evident that there is a positive relationship between antioxidant assays as well as total phenolic contents of different fractions of *K. daigremontiana* leaves. Among all the samples, BuOH fraction has shown highest antioxidant potential and possessed high phenolic contents.

Conclusion

Present study based on *in vitro* antioxidant potential and total phenolics and flavonoid contents of *K. daigremontiana* leaves. Results showed that it is a promising source of cheap and renewable natural antioxidants. Among different fractions butanol fraction showed highest activity in all the assays which was in accordance to its high total phenolic contents. On the bases of these observations, the use of *K. daigremontiana* leaves might be helpful against oxidative damages. For future, isolation of active components from butanol fraction of crude ethanol extract of *K. daigremontiana* and further biological evaluations should be investigated.

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