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Research Article

ANTIOXIDANT ACTIVITY EVALUATION OF *KALANCHOE PINNATA* (LAM.) PERS. FRESH LEAF JUICE

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ABSTRACT

Objective: The objective of this study was to determine the antioxidant and free radical scavenging properties of *Kalanchoe pinnata* (Lam.) Pers. fresh leaf juice for standardized doses using different types of assays.

Methods: The investigation of the antioxidant activity was carried out for the previously standardized doses (by cytotoxicity and genotoxicity analysis) – 50 µl (low dose - LD) and 70 µl (high dose-HD) by Phosphomolybdenum (PM), Ferric Reducing Antioxidant Power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity assays.

Results: The activity measured by PM assay for LD and HD of the extract was equivalent to 109 µg/ml and 141 µg/ml of ascorbic acid, respectively, while by FRAP assay, it was equivalent to 17.5 µg/ml and 47 µg/ml of ascorbic acid, respectively. The activity measured by DPPH assay was found equivalent to 83 µg/ml and 177.5 µg/ml of ascorbic acid for LD and HD, with the percent scavenging activity 4.55% and 17.04%, respectively. Similarly, the activity of LD and HD by ABTS assay was found equivalent to 277.5 µg/ml and 308 µg/ml of ascorbic acid with the percent scavenging activity 39.01% and 47.93%, respectively.

Conclusion: The changes observed in the absorbance values for all the assays reflected the increasing antioxidant activity of the plant extract in dose-dependent manner, which justifies the traditional use of this herb in prevention of diseases induced by oxidative stress. The antioxidant activity of *K. pinnata* fresh leaf juice has been conducted for the 1st time and has not been previously reported.

Keywords: *Kalanchoe pinnata* (Lam.) Pers, Fresh leaf juice, Antioxidant activity, Reactive oxygen species, Radical scavenging activity.

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INTRODUCTION

The Reactive oxygen species (ROS) or free radicals are generated in the body by exogenous chemicals and various endogenous metabolic processes. The excess amount of ROS leads to imbalance between oxidants and antioxidants, resulting in oxidative stress which can oxidize the biomolecules, namely, nucleic acids, proteins, lipids, and DNA. This can cause conditions such as cancer, cardiovascular diseases, muscular degeneration, neurological disorders, and other inflammatory processes. Hence, the balance between free radicals and antioxidants is believed to be a critical concept for maintaining a good biological system [1]. The increment in intake of exogenous antioxidants would ameliorate the damage caused due to oxidative stress by inhibiting the initiation or propagation of oxidative chain reaction, acting as free radical scavengers, quenchers of singlet oxygen, and reducing agents [2,3]. Plant derived materials have now acquired great importance owing to their multipurpose applications, and an enormous variety of plants have been studied for new source of natural antioxidants [4]. Moreover, the phenolic and flavonoid compounds derived from plants have proved to be potent antioxidants and free radicals scavengers [5].

Pharmacognostical and phytochemical analysis of *Kalanchoe pinnata* was done previously in our laboratory using four types of extracts. The qualitative and quantitative phytochemical analysis showed presence of all major components, especially flavonoids and phenolics which are responsible for antioxidative effects of the plant. Fresh leaf juice showed maximum content of primary and secondary phytoconstituents and was selected as the best extract amongst four [6]. Furthermore, in our further study, we used to evaluate the cytotoxicity and genotoxicity of fresh leaf juice on human peripheral blood lymphocyte cultures for safety analysis and with that, standardized two doses as safe dose [7]. Therefore, to contribute further to the knowledge of Indian traditional plants, our present study is focused on *K. pinnata* fresh leaf juice to determine its antioxidant and free radical scavenging properties for its standardized safe doses.

METHODS

Authentication and extract preparation

The leaves of the plant were collected from Gandhinagar, Gujarat, India and the authenticated voucher specimen PH/14/009 was deposited in K.B.I.P.E.R, Gandhinagar, Gujarat, India.

The extract (fresh leaf juice) was prepared as reported earlier [6] and the two doses: 50 µl (low dose - LD) and 70 µl (high dose - HD)-standardized as final concentrations in our previous study [7] were used in this study. In the present study, all the experiments were performed in triplicate.

Antioxidant activity

Phosphomolybdenum (PM) assay

This assay was performed according to Prieto *et al.* (1990) [8]. 50 µL and 70 µL of the extract was dissolved in water and added to 1 ml of 0.6 M Sulfuric acid, 28 mM Sodium Phosphate, and 4 mM Ammonium Molybdate and incubated at 95°C for 90 min, cooled at room temperature, and the absorbance measured at 695 nm. Same procedure was performed for ascorbic acid (10–300 µg/ml) and a calibration curve was prepared. The antioxidant activity of the extract was expressed as the equivalent numbers of ascorbic acid.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was done according to Oyaizu (1986) [9]. Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M,

pH 6.6), 2.5 ml potassium ferricyanide (1%), and 50 µl and 70 µl of the extract. The reaction mixtures were incubated at 50°C in water bath for 30 min, allowed to cool at room temperature (28°C) and 2.5 ml of 10% Trichloro acetic acid was added to each reaction mixture, and then centrifuged at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in a test tube, and 2.5 ml of distilled water was added followed with 0.5 ml $FeCl_3$ (1.0%) and allowed to react for 10 min at room temperature, and absorbance measured at 700 nm. Ascorbic acid solution (10–350 µg/ml) was used as standard.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

This assay was done by the method of Brand-Williams *et al*. (1995) [10]. The different concentrations of standard solution (ascorbic acid) (50–350 µg/ml) were prepared. To 1 ml of DPPH (0.3 mM in 100% ethanol) solution, 50 µl and 70 µlof the extract were added. For serving as control, instead of the extract sample, equivalent amount of standard phosphate buffer was added in the reaction mixture. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance of reaction mixtures measured at 517 nm. The percentage scavenging activity of different concentrations was determined and the formula for percent scavenging activity is:

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The scavenging assay by ABTS assay was done according to the method of Miller and Rice-Evans (1997) [11]. ABTS radical cation was prepared by mixing 200 µl of potassium per sulfate (70 mM) and 50 ml of ABTS (2 mM). 0.3 ml of ABTS radical cation and 1.7 ml of phosphate buffer (pH 7.4) were added to 50 µl and 70 µl of the extract. The same procedure was done for the standard (Ascorbic acid) with different concentrations (50–500 µg/ml) and water was used as control. The absorbance was measured at 734 nm and calculated according to following formula:

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

RESULTS

The antioxidant activity of the extract was calculated from the standard curve of ascorbic acid (10–300 µg/ml) and the absorbance was found to be increased with the higher dose of extract. The activity by LD and HD of the extract was equivalent to 109 µg/ml and 141 µg/ml of ascorbic acid, respectively (Table 1 and Fig. 1).

The antioxidant activity of the extract was calculated from a standard curve of ascorbic acid (10–350 µg/ml) and the absorbance was found to be decreased with the higher extract dose. The activity by LD and HD of the extract was equivalent to 17.5 µg/ml and 47 µg/ml of ascorbic acid, respectively (Table 1 and Fig. 2).

The total antioxidant activity of the extract was calculated from a standard curve of ascorbic acid (50–350 µg/ml). The activity by LD and HD of the extract was equivalent to 83 µg/ml and 177.5 µg/ml of ascorbic acid, respectively (Table 1 and Fig. 3). The percent scavenging activity was 4.55% for LD and 17.04% for HD (Table 2).

The total antioxidant activity of the extract was calculated from a standard curve of ascorbic acid (50–350 µg/ml). The activity by LD and HD of the extract was equivalent to 277.5 µg/ml and 308 µg/ml of ascorbic acid, respectively (Table 1 and Fig. 4). The percent scavenging activity of LD was 39.01% and 47.93% for HD (Table 2).

DISCUSSION

In the living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis [12]. Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines [13] and many natural antioxidants have been isolated from different plant materials [14].

Jaiswal *et al*. (2014) studied the ethanolic extract of *K. pinnata* and concluded that it has strong protective potential than standard antioxidants against oxidative stress in both aqueous and lipid phases [15]. Tatsimo *et al*. (2012) studied the antioxidant activities of ethyl acetate and methanolic extracts of the plant and reported that the methanolic extract showed highest activity by scavenging of free radicals along with inhibition of microorganisms [16]. Sindhu and Manorama (2013) evaluated the hexane, chloroform, ethyl acetate, acetone, and ethanol extracts of the plant leaves which showed varying degrees of antioxidant activity in different test systems in a dose-dependent manner [17]. Singh *et al*. (2019) also confirmed the antioxidant activity of *K. pinnata* extracts prepared with various doses [18]. However, this plant has never been studied for the antioxidant activity of fresh leaf juice and this is the first attempt to report such investigation.

The methods used for antioxidant activity determination differ in terms of their assay principles and experimental conditions; consequently, in different methods, particular antioxidants have varying contributions to

Table 1: The total antioxidant activity of extract doses in different assays showing results equivalent to the standard solution

Doses	Activity equivalent to ascorbic acid (µg/ml)			
	PM	FRAP	DPPH	ABTS
LD – 50 µl	109	17.5	83	277.5
HD – 70 µl	141	47	177.5	308

LD: Low Dose (50 µl), HD: High dose (70 µl), PM: Phosphomolybdenum, FRAP: Ferric reducing antioxidant power, DPPH: 2, 2-diphenyl-1-picrylhydrazyl, ABTS: 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

Table 2: Percent scavenging activity (%) of *Kalanchoe pinnata* fresh leaf juice

Doses	% Scavenging Activity	
	DPPH	ABTS
LD-50 µl	4.55	39.01
HD-70 µl	17.04	47.93

LD: Low dose (50 µl), HD: High dose (70 µl)

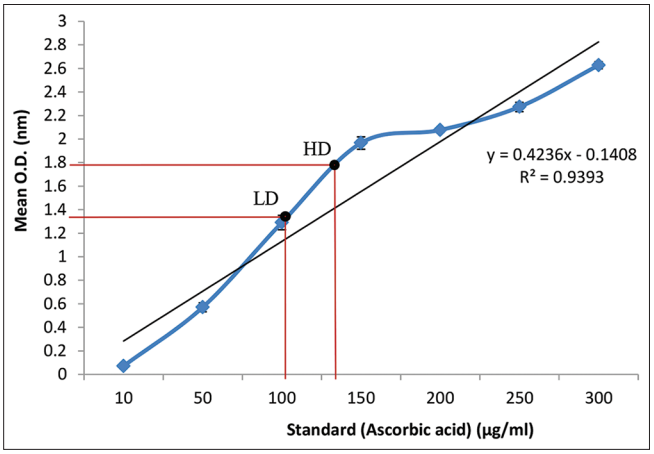

Fig. 1: Phosphomolybdenum (PM) assay. LD: Low dose (50 µl), HD: High dose (70 µl)

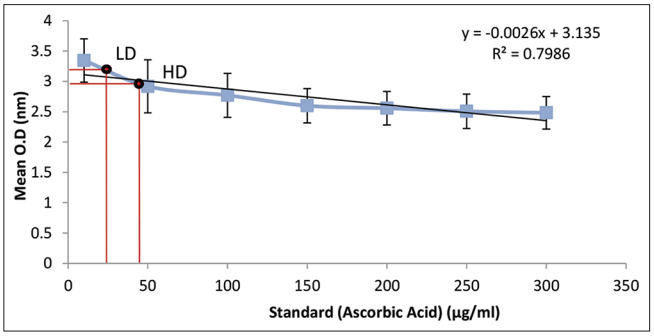

Fig. 2: Ferric reducing antioxidant power (FRAP) assay. LD: Low dose (50 µl), HD: High dose (70 µl)

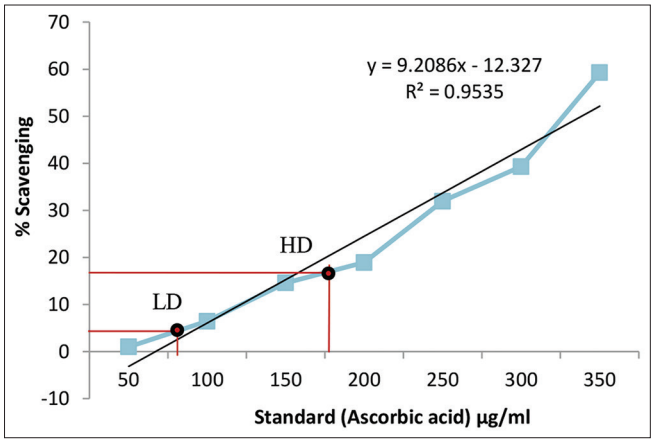

Fig. 3: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%). LD: Low dose (50 µl), HD: High dose (70 µl)

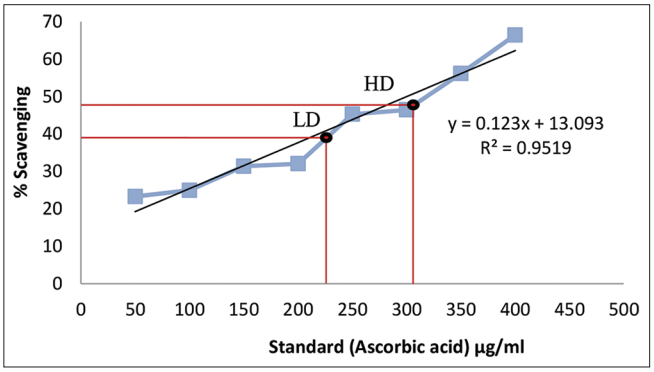

Fig. 4: 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (%). LD: Low dose (50 µl), HD: High dose (70 µl)

total antioxidant potential [19]. Therefore, no single method is sufficient and more than one type of antioxidant capacity measurement needs to be performed to take into account the various modes of action of antioxidants [20]. Another important aspect is the selection of appropriate reference compounds to compare the antioxidant activity of plant samples. Consequently, extracts showing poor antioxidant properties with one concrete method should not be discarded as poor source of antioxidants without having been tested with other methods and compared with different reference standards. An approach of utilize multiple assays for evaluating the antioxidant potential of extracts is more informative and necessary [21]. Therefore, we used four different methods for the evaluation of the antioxidant properties of *K. pinnata* fresh leaf juice. Methods used in this study were selected as they are most appropriate for rapid screening and estimation of the radical scavenging potential.

PM assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate/Mo (V) complex at acidic pH, which usually detects antioxidants such as some phenolics, ascorbic acid, α-tocopherol, and carotenoids [8]. Due to its simplicity and the low cost of the reagents used, the PM assay is a good alternative to the methods already available for evaluation of total antioxidant capacity [22]. In the present study, the antioxidant activity of the extract was found to be increased with the increase in concentration which was indicated by increasing absorbance.

In the FRAP assay, the antioxidant compound presents in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid, and ferric chloride and the absorbance decreases as the antioxidant activity increases, which is measured at 700 nm by UV-Spectrophotometer. This assay represents a very important parameter for estimation of antioxidant activity. The plant extracts examined in this study demonstrated good reducing power, thereby acting as efficient reductones. Reductones are reported to be terminators of free radical chain reactions [23]; thus, the antioxidant activity of plant extracts may be related to their reducing power. Furthermore, the absorbance was found to be increased with the dose increase which indicates the increase in reducing power. Bhatti *et al*. (2012) also have done similar studies for *K. Pinnata* extracts of leaves and stems by both PM and FRAP assays and confirmed that scavenging capacity increases with increase in concentration, which supports our findings [24].

In this study, we selected the DPPH method as it is one of the most effective assays for evaluating radical-scavenging capacity of plant extracts by the chain-breaking mechanism. A freshly prepared $DPPH^{\bullet}$ solution exhibits a deep purple color with an absorption maximum at 517 nm [25]. DPPH radical scavenging activity assay uses the stable radical DPPH as a reagent [26]. The hydrogen atom or electron donating abilities of the compounds and some untainted compounds can be measured from the bleaching of purple colored methanol solution of DPPH [27]. This purple color generally disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench $DPPH^{\bullet}$ free radicals and convert them to a colorless product (2, 2-diphenyl-1-picrylhydrazine), resulting in a decrease in absorbance [21]. The scavenging activity measured by this assay was observed to increase with higher dose.

The ABTS free-radical scavenging assay overcomes limitations of the DPPH method such as solubility and problems of spectral interference and is more versatile as both the polar and non-polar samples (lipophilic and hydrophilic antioxidants) can be assessed for their scavenging activity. This assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with ascorbic acid. The ABTS is generated by reaction of a strong oxidizing agent (e.g., potassium permanganate or potassium per sulfate) with the ABTS salt. Reduction of blue-green ABTS radical colored solution by hydrogen-donating antioxidant was measured by the suppression of its characteristic long wave (734 nm) absorption spectrum [11]. In particular, the spectral interference is minimized as the long wavelength absorption occurs maximally at 734 nm and eliminates color interference in plant extracts [28]. The dose-dependent increase was observed in scavenging activity by this assay also.

Thus, the results of this study reveal that the analyzed doses of *K. pinnata* fresh leaf juice are having potential antioxidant activity which is observed to increase at higher dose, but should be used at standardized doses and durations as it can show genotoxic potential at higher doses as per our previous study [7].

CONCLUSION

This indicated that the fresh leaf juice of *K. pinnata* could be a potential source of natural antioxidant and validated the herb for traditional use in prevention of oxidative stress-induced diseases. The antioxidant

activity study of fresh leaf juice of this plant has been conducted for the 1st time and has not been previously reported.

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The plant was authenticated by Dr. M. N. Zaveri, Head, Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar.

AUTHORS CONTRIBUTIONS

All authors read and approved the final manuscript.

1. Shruti Bhavsar carried out all the practical work and drafted the manuscript.
2. Divya Chandel conceived the study, supervised the practical work, and helped to draft the manuscript.

CONFLICTS OF INTREST

There is no conflicts of interest.

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