ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

In-vitro Antioxidant Capacity And Membrane Protection By Leaf Aqueous Extract of *Piper betle* L.

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ABSTRACT

Antioxidants are natural or man-made substances that can prevent cell damage. Vitamin C, Vitamin E, Vitamin K, quercetin, kaempferol, catechin-gallate, catechin, hydroxybenzoic acids, hydroxycinnamic acids, and carotenoids are some of the crucial antioxidants, which help in preventing diseases. The present study is aimed to evaluate the antioxidant and membrane protection ability of leaf aqueous extract (LAE) of leaves of *Piper betle* L. Initially, LAE was prepared from shade dried leaves by employing maceration process for 5 to 7 days. Later, antioxidant activity assays were performed to measure the percent-scavenging efficacy of LAE. In this view, ABTS radical scavenging activity of LAE revealed that at a concentration of 40 µM, LAE possessed maximum ABTS radical scavenging activity with an IC-50 of 12.2 µM. In addition, ferric reducing capacity LAE has been performed using potassium ferricyanide and different concentrations of LAE, and demonstrated that LAE is showing potent ferric reducing ability with an IC-50 of 13.9 µM. Similarly, HO scavenging activity and DPPH radical scavenging activity were performed in the presence and absence of LAE. It has shown that IC-50 of LAE for HO scavenging activity and DPPH radical scavenging activity are 14.7 µM and 14.3 µM respectively. Furthermore, effect of LAE on lipid peroxidation was assessed using oxidized LDL and concluded that LAE is showing significant inhibition of lipid peroxides when compared to that of control group. Together, all these findings can conclude that LAE is showing potent antioxidant activity and also showing significant inhibition of lipid peroxides in vitro.

Keywords: *Piper betle*, Antioxidant activity, lipid peroxides, LDL.

https://doi.org/10.33887/rjpbcs/2021.12.3.10

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ISSN: 0975-8585

INTRODUCTION

Antioxidant activity is defined as the scavenging of free radicals and protecting the human body from the adverse effects induced by oxidative damage. Free radicals are highly reactive atoms with unpaired electrons that can react with the surrounding biomolecules and cause biomolecular alterations. Commonly known free radicals are hydroperoxyl radical (HO_2), superoxide anion (O^{2-}), nitric oxide ($NO^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), etc. These are also known as reactive oxygen species (ROS).

Inside the human body, during metabolism ROS will be produced within the mitochondria by many processes like physical exercise, phagocytosis, xanthine oxidase, inflammation, peroxisomes and ischemia. These unstable molecules react mainly with DNA, proteins, lipids and other neighboring tissues of the body [1]. Internal antioxidant system balances these high ROS levels by neutralization phenomenon. If this reaches beyond the threshold level, internal antioxidant system becomes insufficient and not able to handle the critical situation, leading to many adverse consequences [2]. Proteins can by oxidatively modified by ROS in three distinct ways, namely modification of specific amino acid, peptide cleavage and protein cross-linkage due to peroxidation products of lipids [3]. Besides, free radicals can damage DNA indirectly by inducing chemical and structural modifications. One of the important reactions involved with the damage to DNA is Fenton reaction in which hydroxyl radicals are generated. These radicals will react with all the components of the DNA such as deoxyribose, purines and pyrimidine bases. In addition, the peroxy radicals also interact and disturb the DNA molecule causing oxidation [4].

GSH (reduced form of glutathione) plays a major role to control all these consequences as an endogenous tripeptide, by protecting against free radical oxidative damage. It also helps in the regeneration of other antioxidants in the body like ascorbate [5]. Despite its applications, the endogenous antioxidants are not suffice and so humans basically depend on different antioxidants present in the diet [6]. The important antioxidants are Vitamin C [7], Vitamin E [8], Vitamin K [9], flavonoids like quercetin, kaempferol, catechin-gallate and catechin [10,11], phenolic acids like hydroxybenzoic and hydroxycinnamic acids [12,13], and carotenoids [14].

Synthetic antioxidants are developed to compare with natural antioxidants as a standard antioxidant measurement system. They can withstand many conditions and treat many diseases once they added to the food. Pure synthetic antioxidants also help in increase in the shelf life of food. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the main extensively used chemical antioxidants. According to the European food safety authority (EFSA), 0.25 mg/kg body weight per day of BHT and 1.0 mg/kg body weight per day of BHA are the acceptable daily intakes [15,16].

The present study is aimed to evaluate the potentiality of leaf aqueous extract (LAE) of *Piper betle* L. in protecting from free radicals. Initially, we prepared the crude extract using water as solvent system and maceration as an extraction procedure. Further, free radical scavenging efficacy against the ABTS, DPPH, HO radicals has measured in terms of free radical scavenging assays, and metal reducing power in terms of ferric reducing method respectively. In addition, inhibition efficacy of oxidized LDL-induced lipid peroxides by LAE was also measured.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Ascorbic acid, ABTS, potassium ferricyanide, Folin– Ciocalteu's reagent, Thiobarbituric acid and trichloroacetic acid were purchased from Himedia (Mumbai, India). All other minor chemicals were procured from the local manufacturers with an analytical grade.

Preparation of LAE

The leaves of *Piper betle* L. were collected from local market of Tirupati and confirmed by a botanist of Department of Botany, Sri Venkateswara University, Tirupati. Shade dried leaves of *Piper betle* L. were subjected to maceration using water as solvent system for 5-7 days. The solution thus obtained at the end of 7th day is subjected to rotatory evaporator (Make: Shimadzu) to remove solvent traces, if any. The LAE thus prepared is stored in a refrigerator (4 ^{o}C) until further analysis.

Antioxidant assays

ABTS radical scavenging activity

Using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical assay, the antioxidant effect of the LAE was measured according to the method [17]. Initially, ABTS radical cations ($ABTS^{\cdot+}$) were generated in dark by reacting 7 mM of ABTS solution with 2.45 mM of potassium persulphate for 12 to 16 hrs. Before performing experiment, radical solution was diluted for an initial absorbance of about 0.700 (±0.02) with 1 X PBS by taking absorbance at 734 nm. Later, different concentrations of LAE (10µl) were made up to 1ml with ABTS solution and incubated for 6 minutes at room temperature. The absorbance was taken at 734 nm using a spectrophotometer.

% ABTS scavenging activity = [(Abs of control) – (Abs of standard) / (Abs of control)] × 100

Reducing power assay

According to the method of Oyaizu 1986 with slight modifications, the reducing power of LAE was determined [18]. In brief, various concentrations of LAE (1 ml) (0–40 µM) was taken and mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$ (1%) respectively. This mixture was then incubated at for 20 min at 50 °C. After incubation, to this mixture, 2.5 ml of 10% Trichloroacetic acid was added and centrifuged at 3,000 rpm for 10 min. The supernatant was collected in a separate tube and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%) and the absorbance was taken at 700 nm. For comparison, the antioxidant capacity of Vitamin C was used as the standard.

H_2O_2 scavenging activity

Hydrogen peroxide radical scavenging activity was assessed as described by Ruch, Cheng and Klaunig 1989 [19]. Briefly, reaction mixture (0.6 ml) contains a solution of hydrogen peroxide (2 mmol/l) in phosphate buffer (pH 7.4) and different concentrations of LAE (0–40 µM). After incubation for 10 min, absorbance of hydrogen peroxide at 230 nm was taken against a blank solution of phosphate buffer without hydrogen peroxide. For comparison, the antioxidant capacity of Vitamin C was used as the standard. The percent scavenging activity of LAE was calculated using the following formula,

% H_2O_2 scavenging activity = [(Abs of control) – (Abs of standard) / (Abs of control)] × 100

DPPH radical scavenging activity

DPPH radical scavenging activity was performed in triplicate following the procedure described earlier [20]. Reaction mixture comprises of 1 ml of various concentrations of LAE (0–40 µM) and 1 ml of DPPH solution (0.1 mM). An equal amount of DPPH and ethanol was used as control. For comparison, the antioxidant capacity of Vitamin C was used as the standard. The reaction mixture was allowed to incubate for 20 minutes in dark and the absorbance was taken at 517 nm.

% DPPH scavenging activity = [(Abs of control) – (Abs of standard) / (Abs of control)] × 100

Protection of lipid membrane

TBARS assay

Isolation of LDL was done as described earlier [21] and the protein content was estimated following the method [22]. Oxidation of isolated native LDL (500 mg protein/ml) *in vitro* was done by incubating in PBS in the presence of $CuCl_2$ (2.5 mmol/l) at 37°C for 48 h [31]. This induces elevation of lipid peroxides. Lipid peroxidation levels were determined as TBARS in Oxidized LDL in the presence of LAE according to method of Ohkawa et al. (1979) with some modifications [23]. The thiobarbituric acid reactive substances (TBARS) calculates the levels of lipid hydroperoxide. This method was based on the reaction of lipid hydroperoxide with thiobarbituric acid leading to the formation of pink colour adduct that was measured at 532 nm. The results were expressed as nmol MDA/mg LDL-protein.

ISSN: 0975-8585

RESULTS AND DISCUSSION

LAE scavenged free radicals significantly

The percent ABTS scavenging activity of different concentrations of LAE (0, 5, 10, 20, 40 µM) was measured. From Figure 1, it is inferred that control group (0 µM), devoid of LAE, is showing zero percent ABTS scavenging ability which was increased to maximum at 40 µM in a dose dependent manner. Values of percent ABTS scavenging activity of LAE and Vitamin C were represented as Mean ± SD in the Table 1. This study is in line with the findings of Jyothi et al. [24].

Similarly, percentage of ferric reducing power of LAE (0, 5, 10, 20, 40 µM) was calculated and the % values of reducing power of LAE and Vitamin C were represented as Mean ± SD in Table 2. Figure 2 depicts an idea about the percent ferric reducing power of LAE. It has shown that on increasing the concentration of LAE, the percent ferric reducing ability was increased in a concentration dependent manner when compared to that in control group (0 µM). Similar findings were done by Jamuna et al. [25] and Hemalatha et al. [26].

In addition, the percent H_2O_2 scavenging activity of LAE (0, 5, 10, 20, 40 µM) was measured and the percent values of LAE and Vitamin C were represented as Mean ± SD in Table 3. Figure 3 has demonstrated that the control group (0 µM) is showing zero reducing power. However, on treatment with LAE (0, 5, 10, 20, 40 µM), H_2O_2 scavenging ability is increased and observed maximum percent activity at 40 µM. It was also observed that the increase in the scavenging ability is in a dose dependent manner. Similar report was given by a study by Patil et al. [27] and Baburao et al. [28] respectively.

Percentage of DPPH scavenging ability of LAE (0, 5, 10, 20, 40 µM) was determined by using DPPH radical solution. The % scavenging values of LAE and Vitamin C were represented as Mean ± SD in Table 4 and the Figure 4 demonstrated that LAE increased the scavenging of DPPH in directly proportional to its concentration, compared with the control group (0 µM). A study by Sathaye and Redkar demonstrated that essential oil of Ocimum sanctum L. significantly inhibited DPPH radicals [29].

LAE inhibited lipid peroxides significantly

Furthermore, lipid peroxidation levels were measured in terms of nmol MDA/mg LDL-protein in the presence of LAE (0, 20, 40 µM) and oxidized LDL. In control group (0 µM), lipid peroxidation is less which was elevated in the oxidized LDL group alone. However, on treatment with LAE, lipid peroxides of oxidized LDL were inhibited when compared to the oxidized LDL alone group in a dose dependent manner as represented in Figure 5. Our finding in this study is in congruence with the report submitted by Arefeh et al. [30].

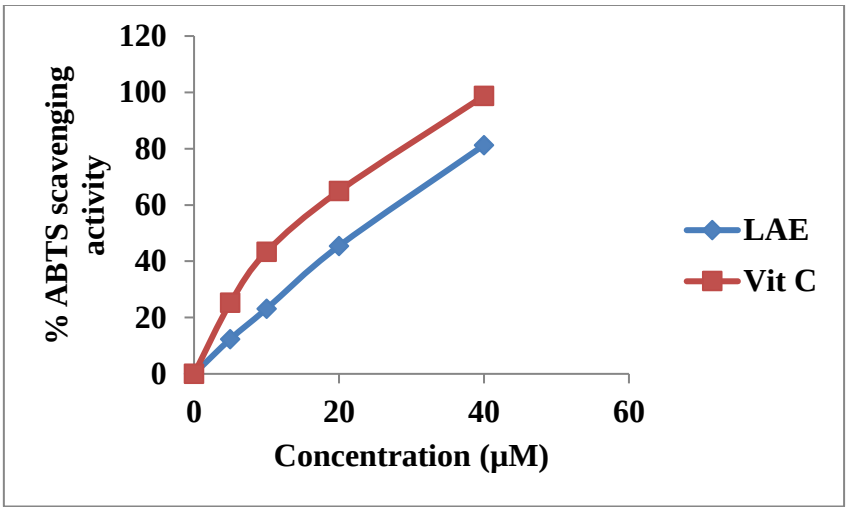

Figure 1: ABTS scavenging activity of LAE with Vitamin C as standard.

ISSN: 0975-8585

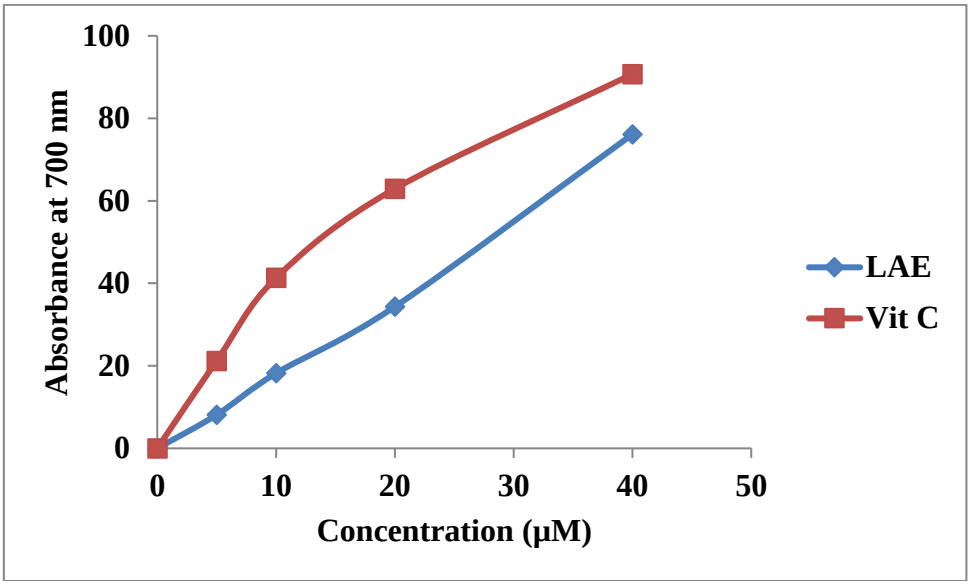

Figure 2: Ferric reducing power of LAE with Vitamin C as standard.

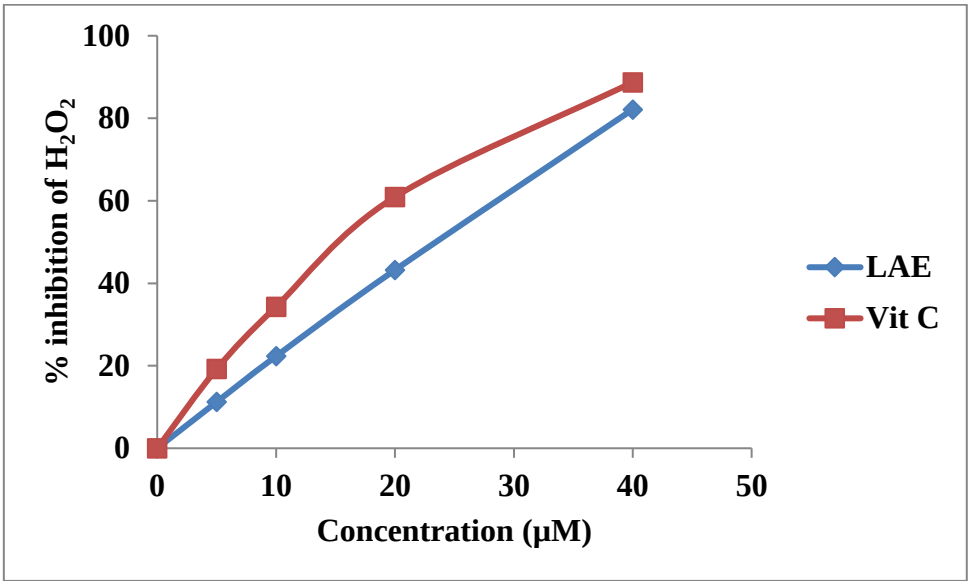

Figure 3: H_2O_2 scavenging activity of LAE with Vitamin C as standard.

ISSN: 0975-8585

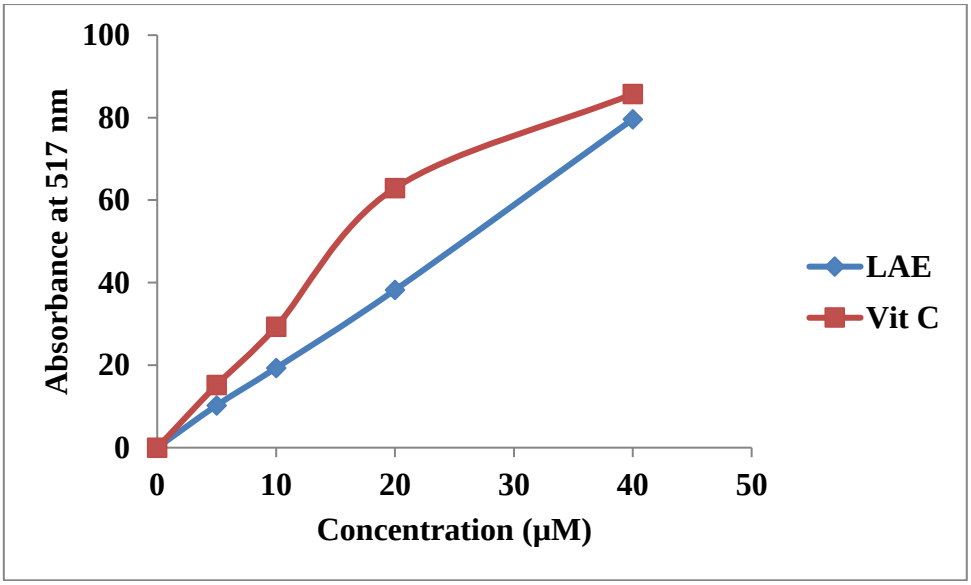

Figure 4: DPPH scavenging activity of LAE with Vitamin C as standard.

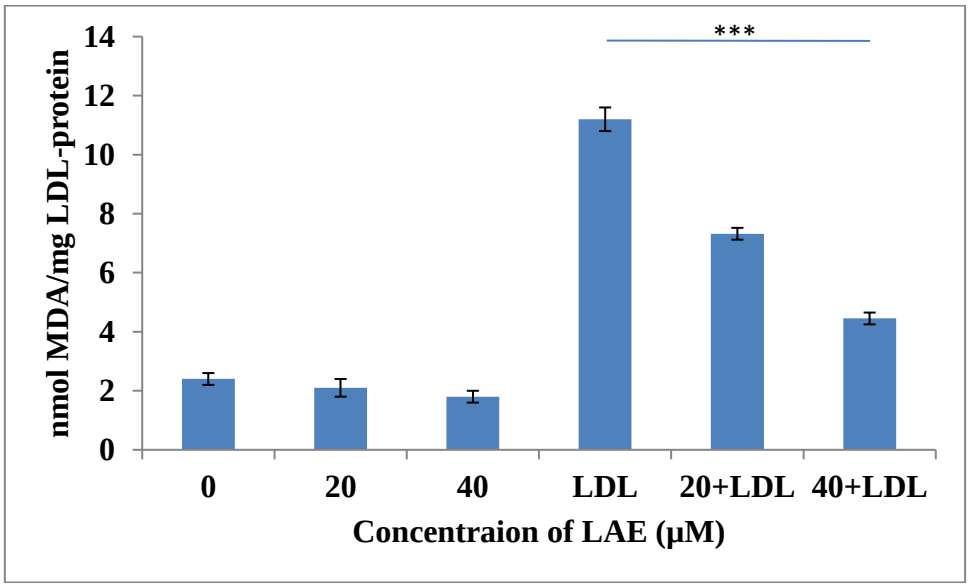

Figure 5: Inhibition of lipid peroxides of oxidized LDL by LAE

Table 1: Percent ABTS radical scavenging activity by LAE and Vitamin C

Concentration (µM)	% ABTS scavenging activity of LAE (Mean ± SD)	% scavenging activity of Vit C (Mean ± SD)
0	0 ± 0.002	0 ± 0.112
5	12.35 ± 0.002	25.21 ± 0.102
10	23.12 ± 0.001	43.32 ± 0.109
20	45.43 ± 0.012	64.93 ± 0.116

ISSN: 0975-8585

Table 2: Percent Ferric reducing activity by LAE and Vitamin C

Concentration (µM)	% ferric reducing power of LAE (Mean ± SD)	% ferric reducing power of Vit C (Mean ± SD)
0	0 ± 0.112	0 ± 0.012
5	8.13 ± 0.115	21.21 ± 0.013
10	18.21 ±0.111	41.32 ± 0.021
20	34.34 ±0.109	62.93 ± 0.002

Table 3: Percent H_2O_2 scavenging activity by LAE and Vitamin C

Concentration (µM)	% H_2O_2 scavenging activity of LAE (Mean ± SD)	% H_2O_2 scavenging activity of Vit C (Mean ± SD)
0	0 **± 0.001**	0 **± 0.102**
5	11.23 **± 0.001**	19.21 **± 0.112**
10	22.32 **± 0.012**	34.32 **± 0.12**
20	43.21 **± 0.021**	60.93 **± 0.23**

Table 4: Percent DPPH radical scavenging activity by LAE and Vitamin C

Concentration (µM)	% DPPH scavenging activity of LAE (Mean ± SD)	% DPPH scavenging activity of Vit C (Mean ± SD)
0	0 ± 0.021	0 ± 0.12
5	10.25 ± 0.034	15.21 ±0.14
10	19.32 ± 0.022	29.32 ±0.11
20	38.21 ± 0.025	62.93 ±0.12

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