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ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *PEPEROMIA PELLUCIDA* (L.) KUNTH: AN *IN VITRO* STUDY

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ABSTRACT

Aim: The study was designed to investigate the phytochemical constituents and antioxidant activity of ethanolic extract of whole plant of *Peperomia pellucida* (L.) Kunth. **Methods:** Antioxidant activity of ethanolic extract was estimated using superoxide radical scavenging and hydroxyl radical scavenging assay. Hydroxyl radical scavenging activity was measured out by the ability of different concentrations of ethanolic extract to scavenge hydroxyl radicals generated from the Fe^{3+}/ ascorbate /EDTA/H_2O_2 system. Superoxide anions are derived from dissolved oxygen by riboflavin/NADH system and reduce NBT in this system. Superoxide radical scavenging assay was carried out by the competition between NBT and ethanolic extract for superoxide anions. Ascorbic acid and Gallic acids were used as the standards. **Results:** Preliminary phytochemical screening revealed that the ethanolic extract of *P.pellucida* possesses flavonoids, glycosides, carbohydrates, steroids, triterpenoids, proteins and phenolic compounds. At 500µg/ml concentration, ethanolic extract showed 72.1% inhibition in hydroxyl radical, with an IC_{50} value of 191.67µg/ml whereas that of Gallic acid with an inhibition of 84.05% and IC_{50} value of 116.46µg/ml. Also at 500µg/ml concentration, ethanolic extract and Ascorbic acid showed 71.17% and 90.65% inhibition in superoxide radical with an IC_{50} value of 273.51 and 192.55µg/ml. Ethanolic extract showed significant antioxidant activity in both hydroxyl radical scavenging and as well as in superoxide radical scavenging activity in dose dependent manner. **Conclusion:** Findings in the present study indicated that ethanolic extract of *Peperomia pellucida* (L.) Kunth extract could be a potential source of natural antioxidant and can be explored as a therapeutic agent in free radical induced diseases.

KEYWORDS: *Peperomia pellucida* (L.), Ethanolic, hydroxyl radical.

1. INTRODUCTION

Oxidative stress, which reflects an imbalance of oxidants and antioxidants in favor of the former, is believed to result in oxidative damage to various cellular components. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage long with lipid peroxidation.[1] These changes contribute to the pathogenesis of diseases, including, cancer, cardiovascular diseases, metabolic syndrome, xenobiotic/drug toxicity and neurodegenerative diseases.[2]

All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase (SOD) and catalase, or compounds such as ascorbic acid, tocopherol and glutathione. Sometimes these protective mechanisms are disrupted by various pathological processes, and antioxidant supplements are vital to combat oxidative damage. Recently, much attention has been directed towards the development of ethno medicines with strong antioxidant properties but low cytotoxicity's.[3]

Peperomia pellucida (L.) Kunth is a herbaceous plant that can be found easily at almost all over the world especially in the tropical region. It is also known as shiny bush or silver bush belonging to family Piperaceae. Whole plant or parts of plant are used for different purposes. It has reported uses as antimicrobial, antifungal, antiprotozoal, anti-inflammatory, analgesic, antipyretic, anticancer, antiulcer and antioxidant.[4]

Phytochemical screening revealed the presence of alkaloids, cardenolides, saponins and tannins. Stem also contain alkaloid, tannins, flavonoids and steroids, except saponins. The roots of *Peperomia pellucida* (L.) Kunth also had shown the presence of alkaloid, tannins, steroids and carbohydrates etc. Flavonoids, phytosterols, arylpropanoids (e.g., apiols), substituted styrenes, and pellucidin A have been isolated. Sesquiterpenes appear to be the major chemical constituents in the essential oils. Many studies have indicated towards the free radical scavenging and antioxidant potential of *Peperomia pellucida* both *in vitro* as well as *in vivo*.[4]

2. MATERIALS AND METHODS

2.1. Chemicals

Ascorbic acid, EDTA, Potassium dihydrogen orthophosphate, Ferric chloride (Nice Chemicals), Gallic acid (CDH, Cochin), TCA, NADH, Deoxyribose, TBA, NBT, PMS (Sigma Aldrich, USA).

2.2. Plant material

Fresh, disease free whole plants of *Peperomia pellucida* (L.) Kunth were collected from Erattupetta, Kottayam district, Kerala, India in the month of September and identified by Mr. Rojimon P Thomas, Assistant Professor, Department of Botany, CMS College, Kottayam and the specimen was deposited in college herbarium, with a vouch number 1247.

2.3. Preparation of plant extract

The whole plants of *P.pellucida* (L.) Kunth were washed with water several times to remove dirt and allowed to shade dried for 2 weeks and coarsely powdered. The powdered materials were stored in an air tight container. The shade-dried, powdered whole plants (326g) were extracted exhaustively by Soxhlet apparatus (6h) with 95% ethanol. The total ethanol extract was then concentrated in vacuo to a syrupy consistency (32g).

2.4. Preliminary phytochemical screening

Preliminary phytochemical screening for alkaloids, flavonoids, steroids, tannins, saponins, proteins, amino acids, tannins, carbohydrates and terpenoids carried out on the ethanolic extract of *P.pellucida* (EPP)(3).

2.4.1. Tests for Carbohydrates

a. *Molisch's Test*. Solutions of extracts were mixed with few drops of Molisch's reagent (α-Naphthol) and conc. sulfuric acid was added from side of test tube. Formation of purple color ring at junction indicated the presence of carbohydrates.
b. *Fehling Solution Test*. One mL each of Fehling A and Fehling B solutions were mixed with 2mL of different extracts. The mixtures were boiled for 5–10 minutes on water bath. Reddish brown color was obtained due to formation of cuprous oxide which indicated the presence of reducing sugar.
c. *Benedict's test*. Two mL of the extract is mixed with equal volume of Benedict's reagent. The mixture is heated on a water bath for 2 minutes, formation of reddish brown precipitate indicate the presence of reducing sugars.

2.4.2. Tests for Alkaloids

a. *Dragendroff's Test*. One mL of Dragendroff's reagent was added to different extracts. Formation of reddish brown precipitate indicated the presence of alkaloids.
b. *Mayer's Test*. One mL of Mayer's reagent was added to different extracts. Formation of cream color precipitate indicated the presence of alkaloids.
c. *Wagner's Test*. One mL of Wagner's reagent was added to different extracts. Formation of reddish brown precipitate indicated the presence of alkaloids.
d. *Hager's Test*. One mL of Hager's reagent was added to different extracts. Formation of yellow colour precipitate indicated the presence of alkaloids.

2.4.3. Tests for Flavonoids

a. *Alkaline Reagent Test*. To the extract samples few drops of sodium hydroxide solution were added. Formation of intense yellow color, which turned colorless after addition of few drops of dilute hydrochloric acid, indicated the presence of flavonoids.
b. *Shinoda Test*. To the extract samples few magnesium turnings and few drop of conc. hydrochloric acid were added after few minutes' appearance of crimson red color indicated the presence of flavonoids.

2.4.4. Tests for Cardiac Glycosides

a. *Keller-Killiani Test (Test for Deoxy Sugars)*. This test was carried out by extracting the drug with chloroform and the extract was evaporated to dryness then 0.4mL glacial acetic acid containing trace amount of ferric chloride was added. After transferring to a small test tube, 0.5mL of conc. sulfuric acid was added by the side of test tube. Appearance of blue color of acetic acid layer indicated the presence of cardiac glycosides.
b. *Legal Test*. The extract samples were treated with pyridine and then alkaline sodium nitroprusside solution was added. Appearance of blood red color indicated the presence of cardiac glycosides.
c. *Baljet Test*. The extract samples were treated with sodium picrate. Appearance of orange color indicated the presence of cardiac glycosides.

2.4.5. Tests for Saponin Glycosides

Froth Formation Test. Two mL of each extract sample was placed with water in a test tube and shaken well. Formation of stable froth (foam) indicated the presence of saponin glycosides.

2.4.6. Tests for Phenolics

a. *Ferric Chloride Test*. Different extract samples were treated with ferric chloride solution; appearance of blue and green colors indicated the presence of hydrolysable and condensed tannins, respectively.

b. *Lead acetate Test.* To one mL of extract in distilled water, added 3 mL of 10% lead acetate solution. A bulky white precipitate indicates the presence of phenolic compounds.

2.4.7. **Tests for Proteins and amino acids**

a. Millon's Test. To the extract samples, few drops of Millon's reagent were added. Formation of a white precipitate indicates the presence of aromatic amino acids.

b. *Biuret Test*. An aliquot of two mL of extract was treated with one drop of 2% copper sulphate solution. To this 1 mL of 95% ethanol was added followed by addition of potassium hydroxide pellets in excess. Pink colour in the ethanolic layer indicates the presence of proteins.

c. *Ninhydrin Test*. About 2 drops of ninhydrin solution was added to few mL of extract. A characterstic purple colour indicates the presence of amino acids.

2.4.8. Tests for Steroids and Triterpenoids

a. *Salkowski's Test*. Different extract samples were treated with few drops of concentrated sulfuric acid. Appearance of red and yellow color at the lower layer indicated the presence of steroids and triterpenoids, respectively

Antioxidant activity

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of ethanolic extract of *P.pellucida* (L.) Kunth was measured according to the method described by Ozyurek Bektasoglu, Guclu and Apak.[5] The reacting mixture for the deoxyribose assay contained in a final volume of 1mL the following reagents: 200µL KH_2PO_4–KOH (100mM), 200µL deoxyribose (15mM), 200µL $FeCl_3$ (500µM), 100µL EDTA (1mM), 100µL ascorbic acid (1mM), 100µL H_2O_2 (10mM) and 100µL sample. Reaction mixtures were incubated at 37 ◦C for 1h. At the end of the incubation period, 1mL of 1% (w/v) TBA was added to each mixture followed by the addition of 1mL of 2.8% (w/v) TCA. The solutions were heated on a water bath at 80 ◦C for 20 min to develop the pink coloured malondialdehyde–thiobarbituric acid: MDA–TBA_2 adduct. The absorbance of the resulting solution (total volume = 3.0 mL) was measured at 532 nm against a blank containing phosphate buffer. Gallic acid used as reference standard**.**

The percentage inhibition and IC_{50} were calculated. The percentage inhibition of herbal extract was calculated using the following formula

$$\text{Percentage inhibition} = \left[\frac{A_0 - A}{A_0}\right] \times 100$$

Where A_0 is the absorbance of control and A is the absorbance of test /standard.

The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the hydroxyl free radical was calculated using linear regression analysis and used to indicate antioxidant capacity

Superoxide radical scavenging assay

The superoxide anion scavenging activity was measured by the method of Liu, Ooi and Chang.[6] A 0.75 ml of 120 µM PMS solution was added to 3 ml of 100 mM Tris–HCl buffer (pH 7.4) containing 0.75 ml of 300 µM NBT solution, 0.75 ml of 936 µM NADH solution and 0.3 ml of different concentrations of test compounds. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured. The results were expressed as percentage (%) of superoxide anion scavenging calculated. The percentage inhibition of herbal extract was calculated using the following formula

$$\text{Percentage inhibition} = \left[\frac{A_0 - A}{A_0}\right] \times 100$$

Where A_0 is the absorbance of control and A is the absorbance of test / standard.

The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the superoxide free radical was calculated using linear regression analysis and used to indicate antioxidant capacity.

3. RESULTS AND DISCUSSION

Reactive oxygen species cause damage to cellular biomolecules such as proteins, nucleic acids, lipids and carbohydrates. Antioxidants interfere with the generation of ROS and also play a crucial role in their inactivation. Although, human cells protect themselves against oxidative damage by antioxidants, these are occasionally not sufficient to prevent ROS induced cellular damage.[7] Under stressful condition, human body produces reactive oxygen species (ROS) which leads to cell damage and health related problems.[8]

The present study investigated antioxidant and free radical scavenging potential of ethanolic extract of *Peperomia pellucida* (L.) Kunth based on their ability to (i) scavenge hydroxyl radical anion, (ii) scavenge superoxide radical anion

Preliminary phytochemical analysis

Phytochemical analysis of ethanolic extract of whole plant of *Peperomia pellucida* (L.) Kunth revealed the presence of various active constituents such as glycosides, carbohydrates, proteins, flavonoids, phenolics, sterols and terpenoids which may be responsible for the antioxidant activity of the plant.

Table 1: Phytochemical screening.

Phytoconstituents	***Results***
Glycosides	++
Carbohydrates	+++
Proteins and amino acids	+
Flavonoids	+++
Phenolics	++
Steroids and triterpenoids	+

Hydroxyl radical scavenging assay

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules such as all proteins, DNA, PUFA, nucleic acid, and almost any biological molecule it touches. This damage causes aging, cancer and several diseases. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases.[9]

Hydroxyl radical, generated by the Fenton reaction, attacks deoxyribose, degrades into fragments that react with TBA on heating at low PH to form a pink color. We examined the inhibitory action of ethanolic extract of *P.pellucida* on deoxyribose degradation which gives an indication of hydroxyl radical scavenging action and iron chelating activity.[10] The results showed that EPP neutralized hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner (100-500mcg/ml).

At 500 µg/ml percentage inhibition of Gallic acid and ethanolic extract was found to be 84.05% and 72.10. Ethanolic extract of *P.pellucida* (L.) Kunth caused a dose dependent inhibition of hydroxyl radical with an IC_{50} of 191.67µg/ml whereas that of Gallic acid was 116.46µg/ml.

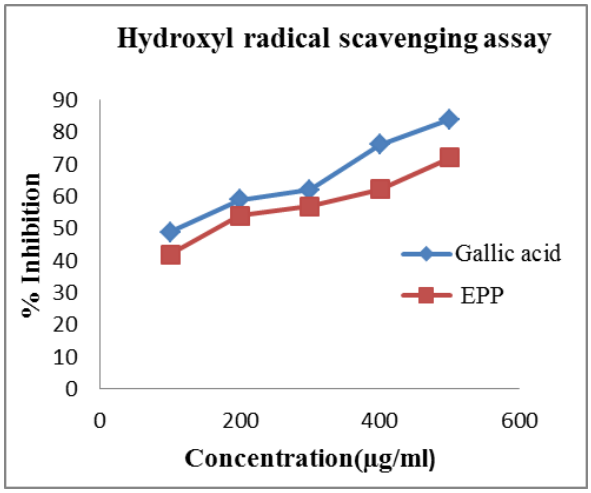

Fig. 1: Effect of Ethanolic extract of Peperomia pellucida on Hydroxyl radical scavenging assay.

Superoxide radical scavenging assay

Superoxide play an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, protein and DNA.[11] Also, superoxide has been observed to directly initiate lipid peroxidation.[12]

Superoxide anion derived from dissolved oxygen by PMS /NADH system and reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture (Ilhami et al. 2010).

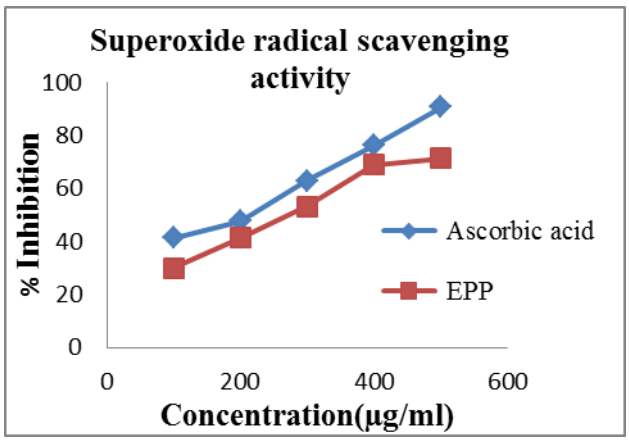

Fig. 2: Effect of Ethanolic extract of Peperomia pellucida on Superoxide radical scavenging assay.

From the investigations on the superoxide radical scavenging capacities, it was found that the ethanolic extract of whole plant of *Peperomia pellucida* (L.) Kunth showed a concentration dependent increase in percentage inhibition. At 500µg/ml, the extract showed maximum inhibition of 71.17%, whereas that of standard ascorbic acid was 90.65%. The ethanolic extract exhibited potent superoxide radical scavenging activity with an IC_{50} of 273.51µg/ml and ascorbic acid gave an IC_{50} of 192.55µg/ml.

4. CONCLUSION

Today, anti-oxidative properties of the plants have become a great interest due to their possible uses as natural additives to replace synthetic ones. The present data suggests that ethanolic extract of *Peperomia pellucida* (L.) Kunth can be used as a good source of natural antioxidants for health benefits. The results obtained have shown that the ethanolic extract contain a number of antioxidant compounds which can effectively scavenge reactive oxygen species including hydroxyl radicals and superoxide radicals under *in vitro* conditions. Ethanolic extract of *P.pellucida* possess antioxidant properties, which are concentration dependent and antioxidant activity of the extracts is compared with standard antioxidants such as ascorbic acid and gallic acid. In addition, ethanolic extract found to contain a noticeable phytochemicals (phenols, flavonoids, glycosides, tannins, steroids), which may play a role in scavenging of free radicals. Further isolation of bioactive compounds is required for identifying the unknown compounds to establish their pharmacological properties. From the results of the present study one can consider that ethanolic extract *of Peperomia pellucida* (L.) Kunth can be used as an easily accessible source of natural antioxidant in pharmaceutical industry

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