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In Vitro Antioxidant Properties of Phloretin—An Important Phytocompound

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Abstract

Reactive oxygen species [ROS] cause oxidative damage to the tissues and protection from such damages is provided by endogenous and exogenous antioxidants. Much research work has been carried out in recent years on the beneficial effect of phenolic compounds which act as natural antioxidants and help to neutralize free radicals. So, this study was aimed to evaluate the *in vitro* antioxidant capacity of one of the phenolic compounds phloretin. Phloretin was used at four different concentrations like 20, 40, 60 and 80 µg/ml to determine the antioxidant activity by different methods such as total antioxidant capacity, reducing power, DPPH radical scavenging, superoxide anion radical scavenging and metal chelating assays. In addition to that the ascorbic acid was used as reference compound. The results showed that the phloretin displayed potent *in vitro* antioxidant capacity. It was able to scavenge different *in vitro* free radicals in all tested concentrations. Among the different concentrations, 80 µg of phloretin has maximum activity when compared to other concentrations in all *in vitro* antioxidant assays. High antioxidant property and maximum protective effect of phloretin were observed in a concentration dependent manner. The results were expressed as IC_{50} value. The lowest IC_{50} value indicates the highest scavenging activity. The reducing power of the phloretin was also found in concentration dependent. According to the results of this study, we concluded that the phloretin possesses antioxidant property. Therefore, phloretin is a powerful antioxidant phytocompound which can protect biological systems against the oxidative stress. From this study, we suggest that the phloretin may be used as a dietary natural antioxidant supplement for preventing free radical related diseases.

Keywords

Antioxidant Activity, *In Vitro*, Phloretin, DPPH, Free Radicals

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1. Introduction

The reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are produced within the body or from the environment have a great effect on human system. They are continuously produced during cell metabolism, or sometimes the immune cells produced them to neutralize the foreign bodies. Moreover, environmental factors such as radiation, pollution, herbicides, cigarette smoke and certain foods can also create free radicals [1]. ROS play an important role in the physiological process; however because of their toxicity, their levels must be controlled by the endogenous antioxidant system. But when the ROS production is increased, an imbalance is promoted between these and the antioxidant molecules and this phenomenon is known as oxidative stress (OS). It can adversely affect various cellular biomolecules like protein, RNA and DNA causing serious damage to tissues and organs resulting in chronic disease such as cancer, heart disease, diabetes mellitus, arthritis and neurodegenerative disease [2].

Antioxidants are act as free radical scavengers, reducing agents and quenchers of singlet oxygen molecule, and activators for antioxidant enzymes to suppress the damage induced by free radicals in biological system [3]. Human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally or externally supplied through foods and/or supplements. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS and therefore can also enhance the immune defenses and lower the risk of cancer and degenerative diseases [4]. In recent years, there is an increasing interest in the field of determination of antioxidant phytochemicals, because they can inhibit the propagation of free radical mediated oxidative stress and protect the human body from diseases [5]. Numerous natural and synthetic ROS scavengers and antioxidants have been developed and studied to protect biomolecules against the attack of ROS and/or to suppress the resultant damage. Some proteins from jellyfish *Rhopilema esculentum* [6], processed cowpea seed extracts [7] and an enzymatic hydrolysate from brown algae *Ecklonia cava* [8] have been reported to possess a noticeable antioxidant activity. Natural flavonoids are known for their significant scavenging properties on oxygen radicals by both *in vivo* and *in vitro*. In addition to these important effects, they have membrane stabilizing properties and also affect some processes of intermediary metabolism [9]. Flavonoids are a diverse group of compounds, which are widely distributed in the plant kingdom. These agents and related synthetic analogues mediate a broad spectrum of biological responses, such as antiallergic, antiinflammatory, antioxidant, gastro protective, antiviral, antimutagenic and anticarcinogenic activities [10].

Among the natural products, phloretin is attracting much interest because of its beneficial effects. Phloretin, a natural active compound belongs to flavonoids, exists in sap of apple, pear and other fruits and vegetables. Phloretin has been studied as a possible penetration enhancer for skin-based drug delivery [11], attenuates inflammation by antagonizing prostaglandins [12] and protects the skin from UV light-induced photodamage [13]. It can serve the purposes of antioxidation, antitumor, antidiabetes, antibiosis and para-hormone under physiological context [14]-[16]. There are many pharmacological studies on phloretin, but no studies on *in vitro* antioxidant activity of phloretin. So, the present study was aimed to examine the *in vitro* antioxidant activity of phloretin.

2. Materials and Methods

2.1. Chemicals

Phloretin was purchased from sigma chemicals company (St. Louis, MO, USA). Nitro blue tetrazolium (NBT), Ethylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and Potassium hexa cyano ferrate [$K_3Fe(CN)_6$] were used in this study are of analytical grade and were purchased from Himedia Laboratories, Bangalore, India.

2.2. DPPH Free Radical Scavenging Assay

The free radical scavenging activity of phloretin was measured by DPPH using the method of Shimada *et al*. [17]. 0.1 mM solution of DPPH$^{\bullet}$ in ethanol was prepared and 1ml of this solution was added to 3 ml of phloretin solution at different concentrations like 20, 40, 60 and 80 µg/ml. The mixture was shaken vigorously and allowed to stand for 30 min at room temperature and then the absorbance was measured at 517 nm using spectrophotometer. Lower absorbance of the reaction mixture indicated that the higher free radical scavenging activity. The DPPH free radical scavenging activity was calculated by the following formula.

$$\%\ \text{scavenging} = \left[\left(\text{A control} - \text{A sample}\right)/\text{A control}\right] \times 100$$

where A control is the absorbance of control reaction (containing all reagents except the test compound), and A sample is the absorbance of test compound.

2.3. Determination of Total Antioxidant Capacity

The total antioxidant activity of the phloretin was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*. [18]. The assay is based on the reduction of Mo(VI)−Mo(V) by phloretin and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. 0.3 ml of phloretin solution at different concentrations like 20, 40, 60 and 80 µg/ml was mixed with 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction mixture were incubated at 95˚C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm using spectrophotometer against blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid. The scavenging activity was calculated according to the following equation.

$$\%\ \text{scavenging/Reduction} = \left[\left(\text{A control} - \text{A sample}\right)/\text{A control}\right] \times 100$$

where A control is the absorbance of control reaction (containing all reagents except the test compound), and A sample is the absorbance of test compound

2.4. Superoxide Anion Scavenging Activity

Measurement of superoxide anion scavenging activity of phloretin was carried out based on the method described by Liu *et al*. [19]. Superoxide radical is generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radical was generated in 1 ml of Tris-HCl buffer (16 mM, pH 8.0), 1ml of NBT (50 µM), 1ml of NADH (78 µM) and sample solution at different concentrations like 20, 40, 60 and 80 µg/ml. The reaction was started by the addition of 1ml of phenazine methosulphate (10 µM PMS) to the mixture. The reaction mixture was incubated at 25˚C for 5 min, and the absorbance was measured against blank at 560 nm using spectrophotometer. Ascorbic acid was used as a standard. Decrease in absorbance of the reaction mixture indicated that the increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated by the following formula.

$$\%\ \text{scavenging} = \left[\left(\text{A control} - \text{A sample}\right)/\text{A control}\right] \times 100$$

where A control is the absorbance of control reaction (containing all reagents except the test compound) and A sample is the absorbance of test compound.

2.5. Metal Chelating Activity

The chelation of ferrous ions by phloretin and standard ascorbic acid was estimated by the method of Dinis *et al*. [20]. The sample solution at different concentrations like 20, 40, 60 and 80 µg/ml was added to 0.05 ml of 2 mM $FeCl_2$. The reaction was initiated by the addition of 5mM ferrozine (0.2 ml) and the mixture was vigorously shaken and left it stands for 10 min at room temperature. The absorbance of the solution was measured at 562 nm using spectrophotometer. The percentage of inhibition of Fe^{2+}-ferrozine complex formation was calculated by the following formula.

$$\%\ \text{Inhibition} = \left[\left(\text{A control} - \text{A sample}\right)/\text{A control}\right] \times 100$$

where A control is the absorbance of control reaction (containing all reagents except the test compound) and A sample is the absorbance of test compound.

2.6. Reducing Power Assay

The reducing power of phloretin was determined by the method of Oyaizu [21]. 1ml of phloretin at different concentrations like 20, 40, 60 and 80 µg/ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50˚C for 20 min

and then 2.5 ml of TCA (10%) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and then the absorbance was measured at 700 nm using spectrophotometer. Higher absorbance of the reaction mixture indicated that the greater reducing power. Ascorbic acid was used as a standard.

2.7. Statistical Analysis

All the tests in this study were carried out in triplicates and the results were expressed as mean ± SD. The free radical scavenging activity of phloretin was expressed as percentage (%). Linear regression analysis was used to calculate IC_{50} values using Microsoft Office Excel.

3. Results and Discussion

Flavonoids are secondary metabolites with significant antioxidant and chelating activities. Biological and pharmacological properties of flavonoids depend on their antioxidant activity. Flavonoids are polyphenolic compounds and the antioxidant activity of flavonoids depends on the number, structure and substitution pattern of hydroxyl groups [22].

3.1. DPPH Radical Scavenging Activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activity in a short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. $DPPH^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [23]. Phloretin at four different concentrations like 20, 40, 60 and 80 µg/ml was used for the determination of free radical scavenging activity by DPPH method and it was compared with ascorbic acid as standard compound. The highest level of DPPH radical scavenging activity of phloretin 86.81% and ascorbic acid 99.34% was found at 80 µg/ml and the results were summarized in **Table 1** and graphically represented in **Figure 1**. The scavenging effect was increased with the increasing concentrations

Table 1. Free radical scavenging activity of phloretin using DPPH.

Concentration (µg/ml)	DPPH free radical scavenging activity	
	Phloretin (%)	Standard ascorbic acid (%)
20	19.09 ± 0.64	25.6 ± 2.04
40	33.63 ± 0.95	61.26 ± 4.90
60	67.27 ± 1.91	88.98 ± 7.11
80	86.81 ± 2.23	99.34 ± 7.94
	IC_{50} = 48.56 µg/ml	IC_{50} = 34.91 µg/ml

Values are expressed as mean ± SD of triplicates.

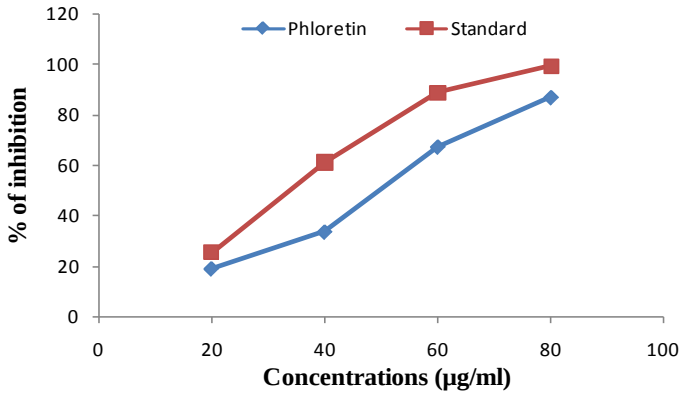

Figure 1. Free radical scavenging activity of phloretin using DPPH.

of test compound phloretin. The IC_{50} value for phloretin and ascorbic acid was 48.56 µg/ml and 34.91 µg/ml, respectively. From the results, ascorbic acid displays more scavenging activity over the phloretin. This might be due to the presence of methoxy group which increases the accessibility of radical center of DPPH to ascorbic acid [24].

Free radical scavenging activity of the phloretin is concentration dependent, and the concentration of phloretin increases, the radical scavenging activity was also increased. The lower IC_{50} value reflects better protective action. From the results, it may be postulated that phlorein was able to reduce the stable free radical DPPH to yellow colored diphenyl picrylhydrazine and which exhibits the better free radical scavenging activity of phlorein. It was reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic condition [25]. Based on the data obtained from this study, phloretin is a powerful free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body.

3.2. Total Antioxidant Capacity

Total antioxidant activity of phloretin was determined by phosphomolybdenum method, which is based on the reduction of Mo(V1) to Mo(V) by the sample and the subsequent formation of green phosphate/Mo(V) compounds in acidic pH with a maximum absorption at 695 nm. **Table 2** shows the total antioxidant activity of phloretin and standard compound ascorbic acid at different concentrations like 20, 40, 60 and 80 µg/ml. Phloretin exhibited effective and powerful antioxidant activity in all concentrations. The percentage of inhibition was found as 22.13%, 42.56%, 69.65% and 89.32% for 20, 40, 60 and 80 µg/ml of phloretin, respectively. **Figure 2** is also revealed that the total antioxidant capacity of different concentrations like 20, 40, 60 and 80 µg/ml of phloretin. The total antioxidant activity of phloretin was increased with increasing the concentration. The IC_{50} value for phloretin and ascorbic acid was 44.83 µg/ml and 42.40 µg/ml, respectively. The results are in agreement with the report of Aliyu *et al*. [26], and they investigated the antioxidant activity of *Ethulia conyzoides*, which indicates that the concentration dependent total antioxidant capacity.

Table 2. Total antioxidant activity of phloretin by phosphomolybdenum assay.

Concentration (µg/ml)	Total antioxidant activity	
	Phloretin (%)	Standard ascorbic acid (%)
20	22.13 ± 1.54	22.35 ± 1.80
40	42.56 ± 3.15	51.23 ± 4.09
60	69.65 ± 4.87	72.54 ± 5.80
80	89.32 ± 6.23	86.35 ± 6.91
	IC_{50} = 44.83 µg/ml	IC_{50} = 42.40 µg/ml

Values are expressed as mean ± SD of triplicates.

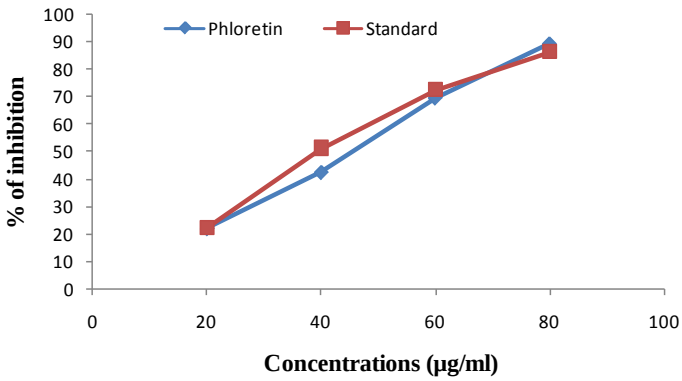

Figure 2. Total antioxidant activity of phloretin by phosphomolybdenum assay.

3.3. Superoxide Anion Scavenging Activity

Superoxide is a highly reactive molecule produced through metabolic processes that reacts with various substances. Superoxide dismutase is present in aerobic and anaerobic organisms catalyses the breakdown of superoxide radicals [27]. Percentage scavenging of superoxide anion examined at different concentrations like 20, 40, 60, 80 µg/ml of phloretin was depicted in **Table 3** and graphically represented in **Figure 3**. The percentage of scavenging superoxide radical increased with the enhanced concentration of phloretin. The maximum scavenging activity of phloretin and ascorbic acid was found at 80 µg/ml as 82.42% and 98.51%, respectively. The IC_{50} value of phloretin and ascorbic acid was recorded as 44.16 µg/ml and 31.62 µg/ml, respectively. *In vitro* superoxide radical scavenging activity is measured by riboflavin/light/NBT (Nitro blue tetrazolium) reduction. This method is based on the generation of superoxide radical by auto oxidation of riboflavin in the presence of light. Superoxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of superoxide anion radical contributes to redox imbalance and associated with harmful physiological consequences [28]. From these results, it was found that the phloretin showed potent superoxide anion free radical scavenging activity.

3.4. Metal Chelating Activity

Table 4 shows the metal chelating activity of phloretin and ascorbic acid. **Figure 4** represents the metal chelating activity was increased with increasing concentration of phloretin and ascorbic acid. At 80 µg/ml, phloretin had highest metal chelating activity 89.23% and the reference compound ascorbic acid was showed 98.65%. The IC_{50} value for phloretin and standard ascorbic acid was 44.59 µg/ml and 30.9 µg/ml, respectively. Ferrozine can quantitatively form complexes with Fe^{2+}. The complex formation is disrupted in the presence of chelating agents with the result that the red colour of the complex is decreased. The chelating activity of coexisting chelator was

Table 3. Superoxide radical scavenging activity of phloretin.

Concentration (µg/ml)	Superoxide radical scavenging activity	
	Phloretin (%)	Standard ascorbic acid (%)
20	22.85 ± 6.50	31.25 ± 2.50
40	49.28 ± 6.25	64.23 ± 5.13
60	68.57 ± 5.50	89.54 ± 7.16
80	82.42 ± 5.00	98.51 ± 7.88
	IC_{50} = 44.16 µg/ml	IC_{50} = 31.62 µg/ml

Values are expressed as mean ± SD of triplicates.

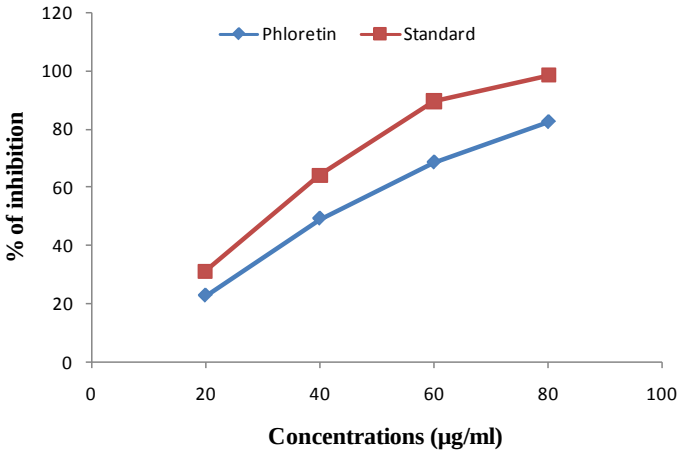

Figure 3. Superoxide radical scavenging activity of phloretin.

Table 4. Metal chelating activity of phloretin.

Concentration (µg/ml)	Metal chelating activity	
	Phloretin (%)	Standard ascorbic acid (%)
20	20.61 ± 5.92	35.23 ± 2.81
40	42.76 ± 5.65	65.21 ± 5.28
60	73.07 ± 5.11	78.51± 6.28
80	89.23 ± 4.85	98.65 ± 7.89
	IC_{50} = 44.59 µg/ml	IC_{50} = 30.9 µg/ml

Values are expressed as mean ± SD of triplicates.

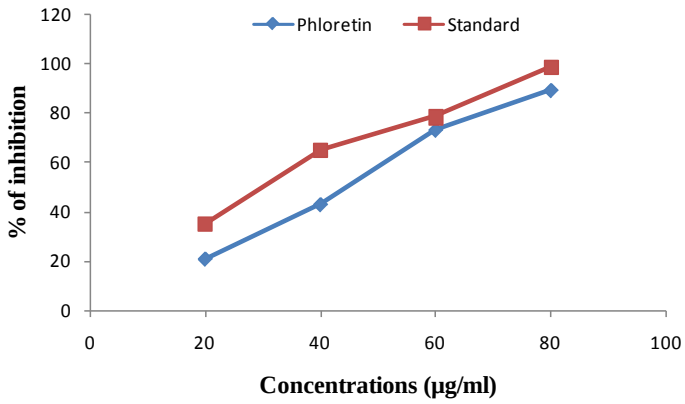

Figure 4. Metal chelating activity of phloretin.

estimated by the measurement of colour reduction. In this assay phloretin and standard ascorbic acid interfered with the formation of ferrous and ferrozine complex. It indicates that they have chelating activity and capture ferrous ion before ferrozine. Iron can stimulate lipid peroxidation by Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [29] [30]. The formation of Fe^{2+} ferrozine complex is not complete in the presence of phloretin, which demonstrate that phloretin chelates iron. The absorbance of Fe^{2+} ferrozine complex was linearly decreased in a dose dependent manner (20 - 80 µg/ml). Similar report was observed in morphine is a flavonoid compound. Metal chelating capacity of morphine is significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents, who form sigma bonds with a metal are effective as secondary antioxidant, because it reduce the redox potential thereby stabilizing the oxidized form of the metal ion [31]. The results obtained from this study revealed that the phloretin have a marked capacity for iron binding, which suggests that the compound phloretin act as a peroxidation protector may be related to its iron binding capacity.

3.5. Reducing Power

Table 5 and **Figure 5** shows that the reductive capability of phloretin and standard ascorbic acid. For the measurements of the reductive ability, we investigated that the Fe^{3+}-Fe^{2+} transformation in the presence of phloretin and ascorbic acid. The reducing power increased steadily with increasing concentration of phloretin (20 - 80 µg/ml). Increased absorbance with the increased concentrations of the reaction mixture indicated that the increased reducing power. However, the highest scavenging activity was observed at 80 µg/ml of phloretin and standard ascorbic acid. Reducing power indicates that the compounds are electron donors, which can act as primary and secondary antioxidants [32]. Different studies have been indicated that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [33]. Similar results for reducing power activity

Table 5. Reducing power activity of phloretin.

Concentration (µg/ml)	Reductive capability (absorbance at 700nm)	
	Phloretin (%)	Standard ascorbic acid (%)
20	0.28 ± 0.03	0.31 ± 0.03
40	0.54 ± 0.05	0.65 ± 0.05
60	0.72 ± 0.06	0.79 ± 0.07
80	0.85 ± 0.07	0.92 ± 0.08

Values are expressed as mean ± SD of triplicates.

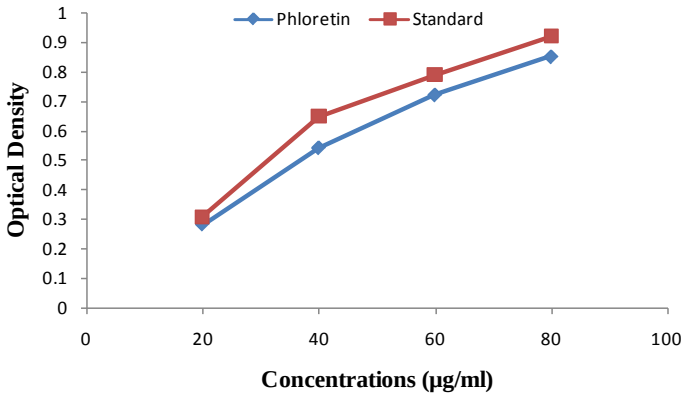

Figure 5. Reducing power activity of phloretin.

have been observed in the flavonoid isolated from *Tartarian buckwheat* and the results exhibited that the total flavonoid has significant reductive ability and radical scavenging effect *in vitro* [34]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound have been attributed to various mechanisms, which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity.

4. Conclusion

In this study, the *in vitro* antioxidant activity of the phytocompound phloretin was confirmed by DPPH free radical scavenging method, superoxide radical scavenging method, reducing power assay, total antioxidant activity by phosphomolybdenum method and metal chelating activity method. The IC_{50} value was determined for both phloretin and standard ascorbic acid. The results of this study clearly indicate that the phloretin has powerful antioxidant capacity against various reactive oxygen species by *in vitro* assays. It is suggested that the phloretin compound could have great importance as a therapeutic agent in the treatment of diseases and preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases.

Author Disclosure Statement

All the authors report no conflicts of interest.

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