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Original Article

Evaluation of Free Radical Scavenging Efficacy of Siddha Formulation *Pirandai uppu* Using *in vitro* Antioxidant Assay

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ABSTRACT

Background: Concentrated forms of the plant extracts such as sathu/uppu possess effective medicinal properties. On concentrating the active principles were also get doubled. These organic substances are valuable resources for contemporary drug research. *Pirandai uppu* is the salt/ash extracted from *Cissus quadrangularis. Pirandai uppu* is used to cure a number of illnesses, including hemorrhoids, peptic ulcers, arthritis and diarrhea. Hence, the present study aims to assess the antioxidant property of *Pirandai uppu* from various antioxidant assays. **Materials and Methods:** The antioxidant properties of *Pirandai uppu* were assessed using DPPH, Nitric Oxide Radical Scavenge Assay, ABTS and Hydrogen Peroxide Radical Scavenge Assay. Percentage of inhibition and IC_{50} values were calculated and compared with that of standards. **Results:** The percentage inhibition of *Pirandai uppu* from DPPH radical scavenging activity ranges from 15.935±4.314 to 78.45±18.05%, The percentage inhibition of *Pirandai uppu* from NO radical scavenging activity ranges from 23.28±6.137 to 70.26±8.898%, The percentage inhibition of *Pirandai uppu* from ABTS radical scavenging activity ranges from 15.72±24.67 to 75.16±22.1% and The percentage inhibition of *Pirandai uppu* from hydrogen peroxide radical scavenging activity ranges from 16.234±0.9669 to 68.87±0.9743%. **Conclusion:** The siddha formulation *Pirandai uppu* has promising free radical scavenging activity in the estimated assays. Thus, *Pirandai uppu* posses a potent antioxidant property. This may be due to rich flavonoid content in *Cissus quadrangularis*.

Keywords: Antioxidants, Pirandai uppu , Siddha, Free Radicals.

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INTRODUCTION

Antioxidants inhibit oxidation of other chemicals. One of the basic meanings of oxidation is the reaction of a substance or element with oxygen, hence the phrase oxidation. It is derived from the French word oxider. Spices and herbs including rosemary, sage and oregano contain significant levels of phenolic chemicals, making them effective antioxidants.[1] There are three primary types of antioxidants: free radical scavengers, which inhibit radical formation by donating hydrogen atoms, oxygen scavengers, which react with oxygen and chelating agents, which trap metal ions that are capable of catalyzing oxidation. Butylated Hydroxy Anisole (BHA) is a type of free radical scavenger.

Ascorbic acid is an oxygen scavenger.[2] Although natural and synthetic antioxidants have similar actions, some synthetic antioxidants, such as BHA and BHT, are now thought to be potentially hazardous to human health.[3] Plant-based diets are thought to minimize the incidence of oxidative stress-induced illnesses.[4] An average diet contains approximately 25000 bioactive phytocompounds and they affect the multiplicity of mechanisms that generate chronic non-communicable diseases.[5]

Using various parts of Plants and plant extracts were considered as the primary treatment methods in ancient days. Siddha system of medicine strictly stays on this stance as it describes the treatment method as ver paaru thazhai paaru minjinakal mela mela parpam chenduram pare. Which means to start with plants and plant-based products are used as a protocol followed by metals and minerals processed with plants. Concentrated forms of the plant extracts such as sathu/uppu possess effective medicinal properties. On concentrating the active principles were also get doubled. These natural chemicals are essential resources for modern medication development. Approximately 59% of newly approved small molecule drugs over the last 20 years have been found to be derived from plants.[6] Our test drug *Pirandai uppu* is an ash/salt extracted from the plant, *Cissus quadrangularis* Linn.

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which is a common plant that grows as a perennial climber. This is a commonly available plant and seen in almost all parts of India. Especially in tropical climates. It belongs to the family Vitaceae and is also known as *Cissus quadrangularis*. The herb is widely referred to as Pirandi-Tamil, Vajravalli-Sanskrit, Hadjod-Hindi, Kandvel-Marathi, Haddjor-Punjabi, Hadbhanga-Oria, Vedhari-Gujrati and Nalleru-Telugu.[7] The phytochemical studies on *Cissus quadrangularis* reveals the presence of many active compounds such as Flavonoids, phenolics, tannins, triterpenoids, glycosides and saponins.[8]

Potential antioxidant qualities of *Cissus quadrangularis* extracts, both methanolic and ethanolic, have been confirmed by a number of free radical scavenging tests. It has been used to treat obesity, inflammation, antibacterial infections, seizures, Indigestion, anorexia nervosa, flatus, intestinal mass, epitasis, Bronchial asthma and dysfunctional menstrual cycles.[9-11]

Siddhars were efficient to use a single herb in various forms.[12] By changing its mode of administration or mode of usage improves the efficacy of many herbs. There is a special technique called uppu prepared from plants. Many siddha literatures like Murugesa Muthaliyarmooligai uppu vaguppu and anuboga vaithiya navaneetham mention the methods of extracting salts from herbal plants. Uppu (Herbal salt/ash) is considered as a unique medicine of Siddha system. It is the concentrated form a single part or the whole plant. Right from the ancient days there are references available in Siddha literature regarding the various methods of extracting salt from ash of many herbal plants. In practice Siddha doctors use some herbal salts for various medications such as Sennaiuruvi uppu having Wound healing and antiulcer activity, Masikai uppu having styptic and astringent activity, Kadukkai sathu used as General tonic and hematinic, Etti uppu having Antipyretic and antiepileptic activity and Seenthil sathu used as Antipyretic and haeminitic.[13] As the herbal salts are concentrated extracts from plants, they might have better potency than the mother herb. Based on this idea *Pirandai uppu* extracted from *Cissus quadrangularis* is chosen as a test drug. *Pirandai uppu* is prepared by 2 various methods. In 1st method the whole plat of Cissus is dried under shadow and burnt to ash. The ash is dissolved in water and heated. Then the sediment is taken as *Pirandai uppu*.[14] In the second method the Juice of *Cissus quadrangularis* is mixed with sodium chloride crystal salt and taken for pudam.[15] Though both the forms of *Pirandai uppu* are in use till date, the second method is used extensively when compared with the first procedure. The scientific validation of these preparations is still lacking.

Various disorders have been treated with *Pirandai uppu* such as arthritis, Diarrhoea, Peptic ulcers, haemorrhoids etc.,[16] In general, a plant or an herb that successfully cures various diseases comes under the category termed as antioxidants. So, an antioxidant can be considered to prevent oxidation and protect the important cell components by neutralizing the detrimental effects of free radicals. Free radicals are the substances that are produced as a result of cellular metabolism.[14,17] These Free radicals have an unpaired electron in the molecule's outer (valance) shell and are formed when metabolism of oxygen takes place within the body. This explains why free radicals can react with proteins, lipids, carbohydrates and DNA and are highly reactive. These free radicals take an electron from the closest stable molecules by attacking them. A chain reaction starts once the attacked fragments lose their electrons, turning it into a free radical and ultimately that leads to the description of a living cell. Free radicals can originate from nitrogen (RNS, Reactive Nitrogen Species) or oxygen (ROS, Reactive Oxygen Species).

Among all O_2 [Superoxide], HO [Hydroxyl], HO_2 [Hydroperoxyl], ROO [Peroxyl], RO [Alkoxyl] are oxygen derived free radical and H_2O_2 oxygen is a non-radical. Nitrogen derived oxidant species are mainly NO [Nitric Oxide], ONOO [Peroxyl nitrate], NO_2 [Nitrogen Dioxide] and N_2O_3[Dinitrogen trioxide].[18,19] A proper antioxidant: antioxidant balance exists in a normal cell. However, as production species increase or antioxidant levels decrease, this balance may be upset. We refer to this phase as oxidative stress. Biopolymers, such as proteins, nucleic acids, Polyunsaturated Fatty Acids (PUFAs) and carbohydrates, are harmed by oxidative stress. Lipid peroxidation, which involves transition metal ions and Reactive Oxygen Species (ROS), is the oxidative degradation of polyunsaturated lipids. A variety of cytotoxic chemicals are produced as a result of a molecular process of cell injury, of which Malondialdehyde (MDA), 4-Hydroxynonrnal (HNE), are the aldehydes. A multitude of human disorders are caused by oxidative stress, which seriously damages cells.[20] such as Alzheimer's disease, Parkinsonism, atherosclerosis, carcinoma, osteoarthritis, immunological disorders and neurodegenerative diseases, etc. since the past decades the interest in search of natural antioxidants has been increasing.[21] In addition *Pirandai uppu* can be used as a daily supplement in daily routine instead or along with common salt as it is considered as a good antioxidant curing many diseases. Hence, we aimed to evaluate the antioxidant property of *Pirandai uppu* using various assays.

MATERIALS AND METHODS

Preparation of *Pirandai uppu*

Ingredients

Pirandai saru (juice of *Cissus quadrangularis*), Kariuppu (common crystal salt i.e. sodium chloride).[22]

Procedure

After harvesting about 650 mL of well-grown *Cissus quadrangularis* stem juice (Pirandai saru), 300 g of sodium chloride (Kariuppu) is added. In a mud pot (agal), it was heated until dry and then kept for the calcinations process. The antioxidant assays are conducted using appropriate traditional

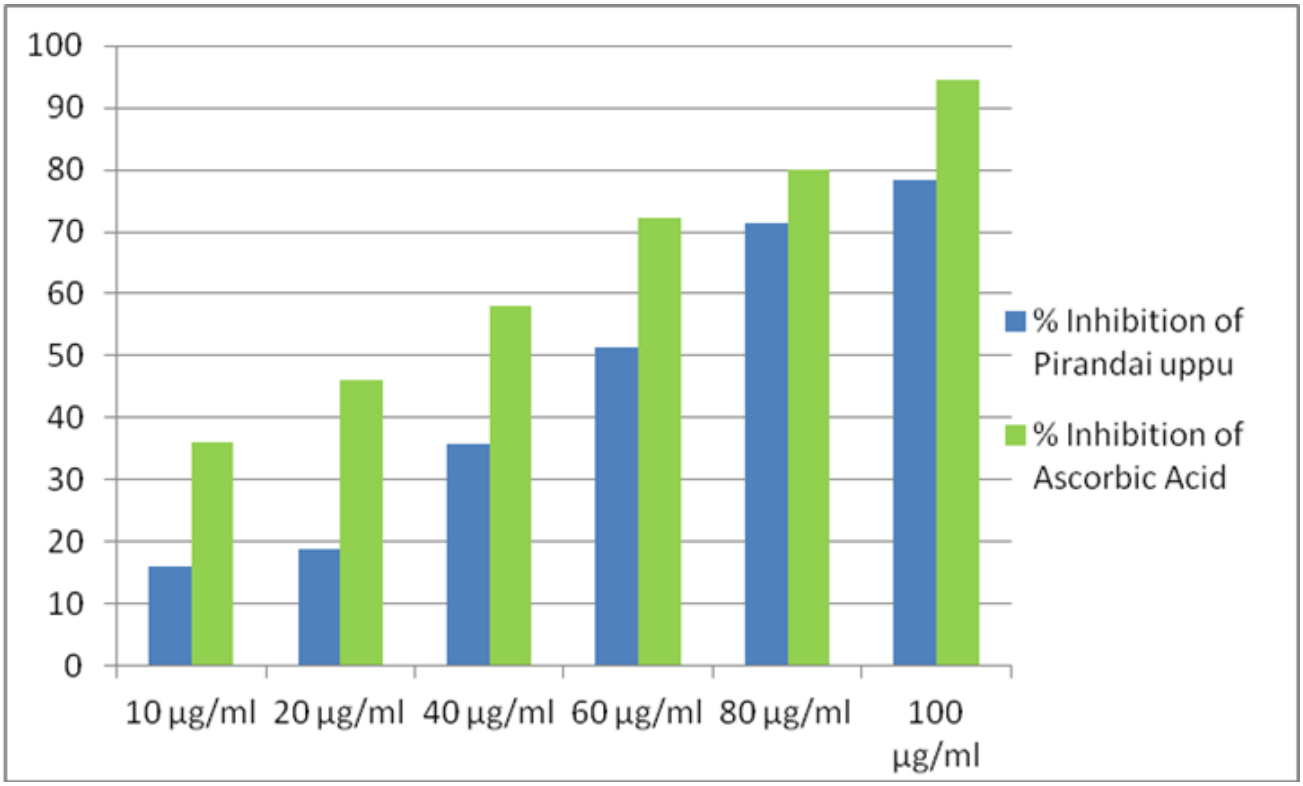

Figure 1: IC_{50} values of *Pirandai uppu* and Ascorbic acid calculated from DPPH radical scavenging assay.

methodologies. Primarily the date are collected and processed in terms of percentage inhibition. The efficacy obtained is compared with a standard. Following four assays were implemented. The readings are calculated based on the change in colour and the absorbance is measured in certain wavelength.

DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) Assay

The antioxidant activity of *Pirandai uppu* was assessed using the DPPH radical scavenging study using the following principle.[23] Ascorbic acid was used as the control in this study. To start with various dilutions of *Pirandai uppu* and ascorbic acid were prepared in different test tubes with the following chronological concentrations namely 10-100 µg/mL (10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL). To a test tube containing 2.5 mL of *Pirandai uppu*, at every concentration, 1 mL of 0.3% DPPH mixed in methanol was added and left aside at room temperature for some time. Followed by a keen incubation of about 15 min the mixture was taken to a temperature of 37°C. The same procedure was repeated with the control. Then the antioxidant property was measured based on the absorbance of *Pirandai uppu* in all the mentioned concentrations and compared with the control. Generally, methanol is used as blank in these kinds of studies. The readings were noted as level of absorbance using double-beam UV Spectrophotometer Absorbance at a fixed wavelength of at 517 nm. To calculate the proportion of DPPH free radical scavenging activity of the *Pirandai uppu* and Ascorbic acid following formula was applied,

$$\% \text{ of DPPH radical scavenging capacity} = [(Ab)_{control} - (Ab)_{PU}] \times 100$$

$Ab_{control}$ -Absorbance of Control,

Ab_{PU} -Absorbance of *Pirandai uppu*,

From the obtained values the amount of *Pirandai uppu* necessary to scavenge DPPH radical by 50% (IC_{50} value) was also identified.

Nitric Oxide Radical Scavenging Assay

The antioxidant activity of *Pirandai uppu* was assessed using the nitric oxide radical scavenging study using the following principle.[24] Gallic acid was used as the control in this study. To start with various dilutions of *Pirandai uppu* and Gallic acid were prepared in different test tubes with the following chronological concentrations namely 10-100 µg/mL (10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL). To a test tube containing 1 mL of *Pirandai uppu*, at every concentration, 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was added and left aside at room temperature for some time. Followed by a keen incubation of about 180 min the mixture was taken to a temperature of 25°C. Then an equivalent amount of recently made Griess reagent was introduced into the mixture. (Griess reagent has equal parts of 0.1% naphthyl ethylene diamine dihydrochloride and 1% sulphanilamide in 2.5% phosphoric acid). The same procedure was repeated with the control. Then the antioxidant property was measured based on the absorbance of *Pirandai uppu* in all the mentioned concentrations and compared

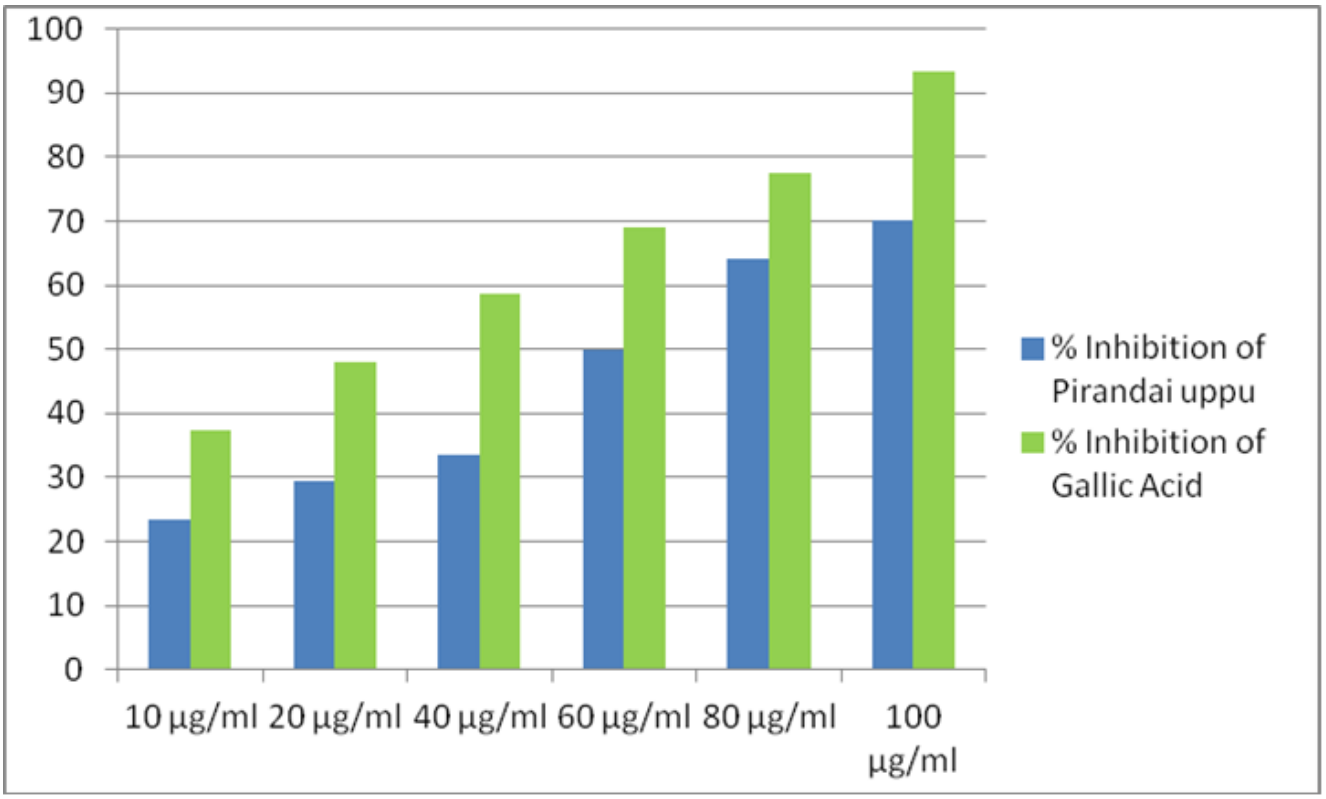

Figure 2: IC_{50} values of *Pirandai uppu* and Gallic acid, calculated from nitric oxide radical scavenging assay.

with the control. The readings were noted as level of absorbance using double-beam UV Spectrophotometer Absorbance at a fixed wavelength of at 546 nm. To calculate the proportion of Nitric oxide free radical scavenging activity of the *Pirandai uppu* and Gallic acid following formula was applied:

% of Nitric oxide free radical scavenging activity=[(Ab) control-(Ab) PU]x100

$Ab_{control}$ -Absorbance of Control,

Ab_{PU}-Absorbance of *Pirandai uppu*,

From the obtained values the amount of *Pirandai uppu* necessary to scavenge Nitric oxide radical by 50% (IC_{50} value) was also identified.

ABTS Assay

The antioxidant activity of *Pirandai uppu* was assessed using the ABTS radical scavenging study using the following principle.[25] Gallic acid was used as the control in this study. To start with various dilutions of *Pirandai uppu* and Gallic acid were prepared in different test tubes with the following chronological concentrations namely 10-100 µg/mL (10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL). Then it was mixed with freshly prepared ABTS reagent. (ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate). The reagent was then kept in the dark at room temperature for 16 hr to allow free radical generation and was then diluted with water (1:44, v/v). To a test tube containing 100 µL of *Pirandai uppu*, at every concentration, 100 µL ABTS reagent in distilled water was added and left aside at room temperature for some time. Followed by a sharp incubation of about 6 min, the antioxidant property was measured based on the absorbance of *Pirandai uppu* in all the mentioned concentrations and compared with the control. The same procedure was repeated with the control. The readings were noted as level of absorbance using double-beam UV Spectrophotometer Absorbance at a fixed wavelength of at 734 nm. To calculate the proportion of Nitric oxide free radical scavenging activity of the *Pirandai uppu* and Gallic acid following formula was applied:

% of ABTS radical scavenging activity=[(Ab)control-(Ab) PU]x100

$Ab_{control}$ -Absorbance of Control,

Ab_{PU}-Absorbance of *Pirandai uppu*,

From the obtained values the amount of *Pirandai uppu* necessary to scavenge ABTS radical by 50% (IC_{50} value) was also identified.

Hydrogen Peroxide Radical Scavenging Assay

The antioxidant activity of *Pirandai uppu* was assessed using the Hydrogen peroxide radical scavenging study using the following principle.[26] BHA acid was used as the control in this study. To

Table 1: IC_{50} values of *Pirandai uppu* and Ascorbic acid, calculated from DPPH radical scavenging assay.

Sample	IC_{50} (µg/mL)
Pirandai uppu	31.7±84.88
Ascorbic acid	27.75±4.967

Data are given as Mean±SD (*n*=3).

Table 2: IC_{50} values of *Pirandai uppu* and Gallic acid, calculated from Nitric Oxide radical scavenging assay.

Sample	IC_{50} (µg/mL)
Pirandai uppu	37.8±36.7
Gallic Acid	25.76±10.9

Data are given as Mean±SD (*n*=3).

start with various dilutions of *Pirandai uppu* and Gallic acid were prepared in different test tubes with the following chronological concentrations namely 10-100 µg/mL (10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. Then it was mixed with freshly prepared Hydrogen peroxide solution. (Hydrogen peroxide solution was prepared by mixing 2 mM of Hydrogen peroxide in 50 mM phosphate buffer at pH 7.4). To a test tube containing 0.1 µL of *Pirandai uppu*, at every concentration, 0.3 µL of 50 mM phosphate buffer (pH 7.4) was added and left aside at room temperature for some time. Then 0.6 mL of hydrogen peroxide solution was added. Followed by a sharp incubation of about 10 min, the antioxidant property was measured based on the absorbance of *Pirandai uppu* in all the mentioned concentrations and compared with the control. The same procedure was repeated with the control. The readings were noted as level of absorbance using double-beam UV Spectrophotometer Absorbance at a fixed wavelength of at 230 nm. To calculate the proportion of Hydrogen peroxide radical scavenging activity of the *Pirandai uppu* and BHA following formula was applied:

% of Hydrogen Peroxide Radical Scavenging=[(Ab)control-(Ab)PU]x100

$Ab_{control}$ -Absorbance of Control,

Ab_{PU} -Absorbance of *Pirandai uppu*,

From the obtained values the amount of *Pirandai uppu* necessary to scavenge Hydrogen peroxide radical by 50% (IC_{50} value) was also identified.

RESULTS

The percentage inhibition of *Pirandai uppu* from DPPH radical scavenging activity ranges from 15.935±4.314 to 78.45±18.05% when compared with percentage inhibition of standard ascorbic acid which ranges from 36.16±7.903 to 94.4±2.43%. The Half maximal inhibitory concentration value of the *Pirandai uppu* was found to be 31.7±84.88 µg/mL and for ascorbic acid it was about 27.75±4.967 µg/mL (Table 1). IC_{50} values of *Pirandai uppu* from DPPH radical scavenging assay is presented in Figure 1.

The percentage inhibition of *Pirandai uppu* from nitric oxide radical scavenging activity ranges from 15.935±4.314 to 78.45±18.05% when compared with percentage inhibition of standard ascorbic acid which ranges from 36.16±7.903 to 94.4±2.43%. The Half maximal inhibitory concentration value of the *Pirandai uppu* was found to be 37.8±36.7 (µg/mL) and for Gallic acid it was about 25.76±10.9 µg/mL (Table 2). IC_{50} values of *Pirandai uppu* from nitric oxide radical scavenging assay is presented in Figure 2.

The percentage inhibition of *Pirandai uppu* from ABTS radical scavenging activity ranges from 15.72±24.67 to 75.16±22.1% when compared with percentage inhibition of standard gallic acid which ranges from 22.82±14.73 to 89.91±2.812%. The corresponding Half maximal inhibitory concentration value of *Pirandai uppu* was found to be 51.11±24.67 µg/mL and for Gallic acid it was about 37.12±8.77 µg/mL (Table 3). IC_{50} values of *Pirandai uppu* from ABTS radical scavenging assay is presented in Figure 3.

The percentage inhibition of *Pirandai uppu* from hydrogen peroxide radical scavenging activity ranges from 16.234±0.9669 to 68.87±0.9743% when compared to the inhibition percentage of standard BHA which ranges from 25.61±2.451 to 86.07±7.006%. The corresponding Half maximal inhibitory concentration value of *Pirandai uppu* was found to be 65.2±8.745 µg/mL and for BHA it was about 43.13±2.219 µg/mL (Table 4). IC_{50} values of *Pirandai uppu* from hydrogen peroxide radical scavenging assay is presented in Figure 4.

DISCUSSION

Considering the findings of our current study it is evident that *Pirandai uppu* is having potential free radical scavenging action. Plants generate phenolics as secondary metabolites and these metabolites possess an extensive sort of medicinal properties including anticarcinogenic, antioxidant, antimutagenic, free radical-scavenging activities etc.,[27] Understanding the roles of different antioxidants and their activity is challenging. *In vitro* antioxidant assays provide a measure of a compound's efficacy and have advantages over quantitative antioxidant components.

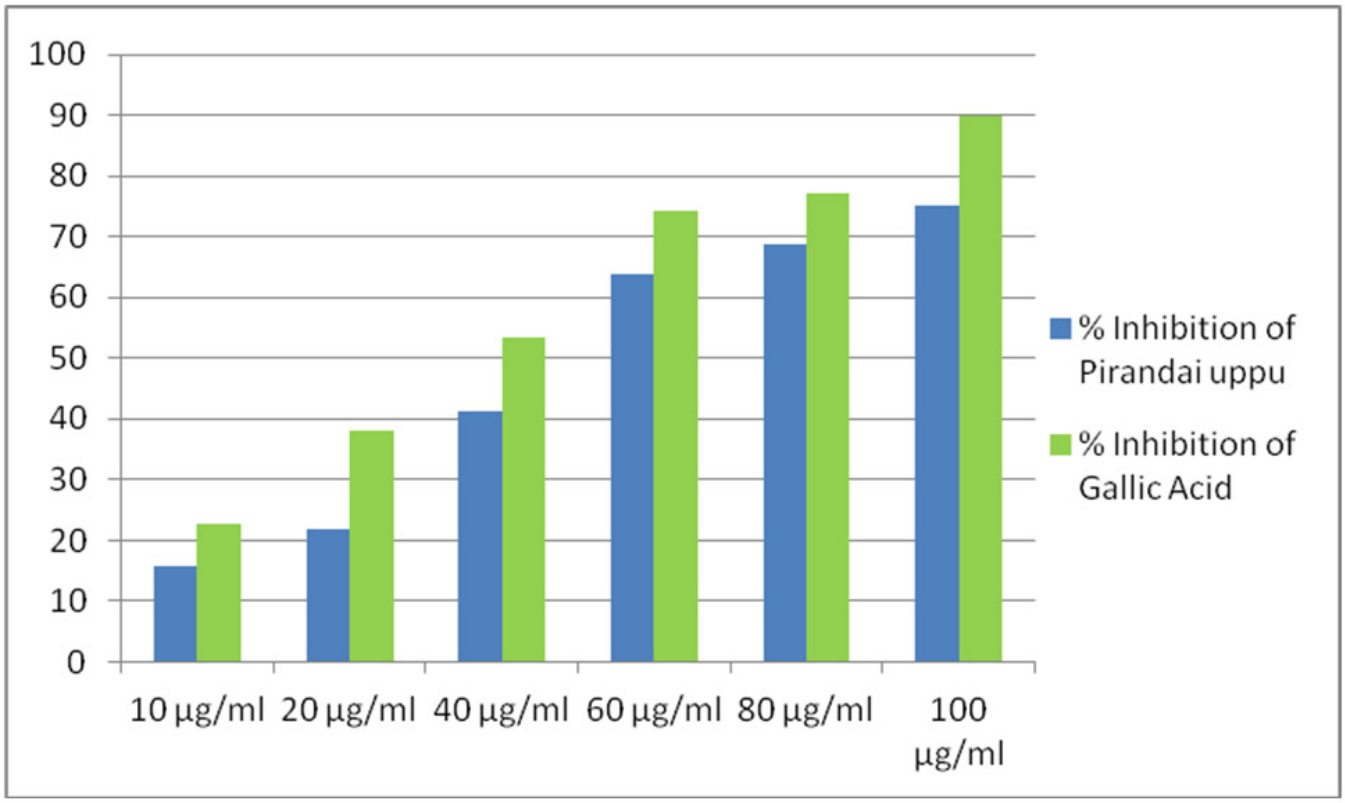

Figure 3: IC_{50} values of *Pirandai uppu* and Gallic acid, calculated from ABTS radical scavenging assay.

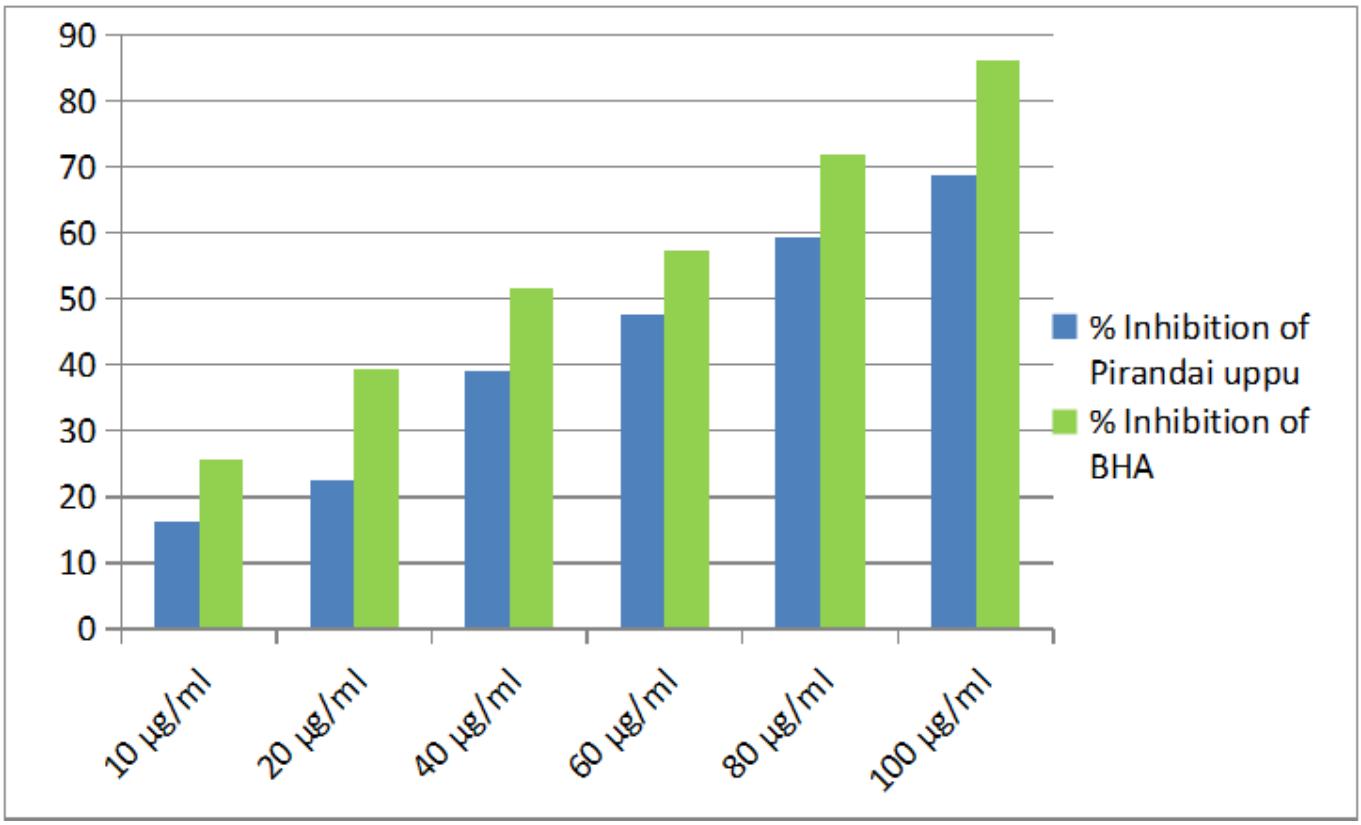

Figure 4: IC_{50} values of *Pirandai uppu* and BHA, calculated from hydrogen peroxide radical scavenging assay.

Table 3: IC_{50} **values of *Pirandai uppu* and Gallic acid, calculated from ABTS radical scavenging assay.**

Sample	IC_{50} (µg/mL)
Pirandai uppu	51.11±24.67
Gallic Acid	37.12±8.77

Data are given as Mean±SD (*n*=3).

Table 4: IC_{50} **values of *Pirandai uppu* and BHA, calculated from Hydrogen peroxide radical scavenging assay.**

Sample	IC_{50} (µg/mL)
Pirandai uppu	65.2±8.745
BHA	43.13±2.219

Data are given as Mean±SD (*n*=3).

Antioxidants work through various processes, including offering H^+ ions to the radicals, dipping the power, foraging the oxygen free radicals, metal sequestering ability, inhibiting the peroxidation of β-carotene and reduces the formation of single unresolvable oxygen molecules.

In all assays the percentage of inhibition increases with increasing concentrations the maximum being in the highest concentration. Further IC_{50} values were calculated. The Half maximal Inhibitory Concentration (IC_{50}) is defined as the concentration of any test substance required to scavenge 50% of the radicals formed at the initial phases. The more effective a material is at searching free radicals, as indicated by a lower IC_{50} value, this suggests the presence of higher level of better antioxidant property.[28] This activity of *Pirandai uppu* may account for its application in treating various kinds of ailments especially those that are associated with free radical generation. The characteristic feature of any compound is recognized to its hydrogen offering acapacity.[29] Formation of free radicals result in auto-oxidation of unsaturated lipids that are present in food.[30] Antioxidants are believed to end the free radical sequential chain of oxidation. These antioxidants in addition donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product that neither commence nor disseminate extra oxidation of lipids.[31] Flavanoids such as quercetin and rutin present in *Cissus quadrangularis* are accountable for its antioxidant properties.[32-34] The same flavonoid portions might be present in *Pirandai uppu* which is responsible for its antioxidant property.

CONCLUSION

As evidenced from the outcomes of the study of *In vitro* anti-oxidant assay on *Pirandai uppu*, it is concluded that this siddha formulation *Pirandai uppu* has better free radical scavenging activity. Thus, *Pirandai uppu* possess a potent antioxidant property. This may be due to rich flavonoid content in *Cissus quadrangularis*. *Pirandai uppu* can be incorporated as daily supplement for preventive measure against several challenging diseases involving oxidative stress as the key mechanism such as cancer. In addition, *in vivo* studies establishing its role as antioxidant should be carried out to further validate the results obtained in this study.

STUDY LIMITATIONS

The study has some reduced specificity as there are many other causes that influence oxidative stress.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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