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Preliminary phytochemical analysis and assessment of total phenol and total flavonoid content of *Haworthiopsis limifolia* Marloth. and its antioxidant potential

Riti Shah[1], Pooja Sharma[2], Nainesh R Modi[3*]

[1] M.Sc. student, Department of Botany, Bioinformatics, Climate Change Impacts Management, School of Science, Gujarat University, Ahmedabad, Gujarat, India

[2] Research Scholar, Department of Botany, Bioinformatics, Climate Change Impacts Management, School of Science, Gujarat University, Ahmedabad, Gujarat, India

[3] Associate professor[3], Department of Botany, Bioinformatics, Climate Change Impacts Management, School of Science, Gujarat University, Ahmedabad, Gujarat, India

Abstract

Haworthiopsis limifolia Marloth is a member of family Asphodelaceae which is native to South-Africa and is a common house plant. The species is in the vulnerable category of IUCN red list category, which is due to urbanization, overgrazing and illegal trades of the plant. The plant is quite unexplored but holds some traditional medicinal values which show the therapeutic potential of the plant in the future, also having antioxidants can help improve some immune responses. In this study we have screened the phytochemicals present in the plant for example, alkaloids, phenols, flavonoids, terpenoids, carbohydrates and glycosides. The total phenol and total flavonoid contents were studied as a part of quantitative analysis. Total phenolic content was determined using the Folin- Ciocalteu method and it was observed to be 203.33 ± 1.66 mg gallic acid equivalent/g of sample, whereas the total flavonoid content was studied using the Aluminium chloride colorimetric method, which estimated as 8.33 ± 3.33 mg quercetin equivalent/g of sample. In addition to this, the antioxidant property of the plant was also investigated by following the 2, 2- Diphenyl-1-picrylhydrazyl radical scavenging assay, and the IC_{50} value observed for the ascorbic acid was 133.421 ± 0.464 µg/ ml, and for the plant sample was 243.241 ± 1.12 µg/ ml.

Keywords: antioxidant, flavonoids, *Haworthiopsis limifolia* marloth, phenols, phytochemicals

Introduction

Haworthiopsis limifolia Marloth is a houseplant and the member of Asphodelaceae family which is peculiar for its predominant succulent herbs with basal rosette of the leaves. It is native to South-Africa [1]. The family has seven genera which are petaloid monocotyledon succulents [2]. The classification has always been a matter of conflict for *Haworthia* genus, and in 1804, *Aloe* genus was split into the genus *Haworthia* [3]. The genus is quite unexplored and has a few detailed studies in its name, but according to few researches, the traditional healers have been using the plant in treatment of several ailments like constipation, wound healing, mental health problems, and AIDS. Also being close to genus *Aloe*, it does have a lot of morphological and phytochemical similarities which can be beneficial to the mankind if proper studies can prove the applications of the plant. The genus comes under CAM category (crassulacean acid metabolism) which enables the survival of the plant in dry climatic conditions [4]. The members of the family have reported uses as anti-acne, anti-inflammatory, wound healing and anti-ageing [5]. In countries like Algeria, Cyprus, India, Pakistan, Egypt, Libya, Spain, Turkey and Palestine, these plants are used for burns, colds, eczema, microbial infections, jauncide, mephritis, peptic ulcers, skin diseases and rheumatism. Some compounds that have been extracted from the species of family Asphodelaceae are characterized as anti-malarial, antimicrobial, antioxidant, apoptotic, antiparasitic, antiviral, cytotoxicity, anti-melanogenic, and diuretic activities [6]. This proves that even *H. limifolia* also has potent medicinal benefits and this study attempts to understand several vital phytochemicals present in the plant, its quantification and also the antioxidant property. The genus has over 900 recorded species. The species show diverse variation in the color, size, shape and texture of the leaves. It can also be found solitary or in dense clumps or as offsets of stolon (Figure 1) [7].

Fig 1: *H. limifolia* Marloth plant

A few species are observed to be stem-less. The flowers are arranged in wiry erect inflorescences. The floral buds appear erect, but the flowers bloom horizontally. These plants are generally slow growing and perennial, with possibly a life span of a decade. The species *H. limifolia* M. is vulnerable according to IUCN red list category. A lot of the species of this genus are vulnerable and the reason for it is the urbanisation, and over-grazing which has caused the habitat destruction. Unethical collection and trading of these plants has also resulted in the decline of number of species available in the wild. Stable rocky terrains are the preferred habitats for these plants.

The leaves have thick, leathery, hard epidermis in order to survive in the extreme climatic conditions, which even protect it from the fires [8]. The minute flowers are pollinated by bees and proboscis flies. They are well adapted to vegetative propagation and can regenerate it in case of injuries. *Haworthiopsis* plants being good house plants grow well in containers and pots. This particular species is nicely adapted in rockeries and in areas with rainfalls during summer time [9]. Very few studies have suggested that the genus is possibly having a huge diversity of phytochemicals which reflects the medicinal importance of the plant. Phytochemicals like alkaloids, flavonoids, and phenols are produced by the plants as a protective defense mechanism against insects and animals [10]. Studies have depicted that these phytochemicals can lead to various drug discoveries. The phytochemicals are responsible for color, flavour and smell of the plant, along with the beneficiary properties such as antioxidant, antibacterial, antiviral, anti-cancer and many more [11].

Material and Methods

Plant collection

The plant material was collected from the garden area of Tapi district of the Gujarat state. The leaves were separated and washed with distilled water. Further, they were cut into small pieces and oven dried for 48 hrs. After drying, the plant material was made into a coarse powder using a grinder, and stored in air-tight glass bottles at room temperature (Figure 2).

Plant extracts preparation

The extracts were prepared by using cold extraction method (maceration technique). The dried powder of the plant was dissolved in the solvent (methanol and diethyl ether) using the ratio of 1:10 g/ml in the conical flasks and kept in the orbital shaker for 48 hours. All the samples were separately filtered into petri-plates using the Whatman filter paper 1. The solvent was evaporated from the petri-plates and further stored at low temperature. The extraction yield was evaluated by the standard formula-

Yield (%) = (weight of dry extract ÷ weight of plant powder) × 100

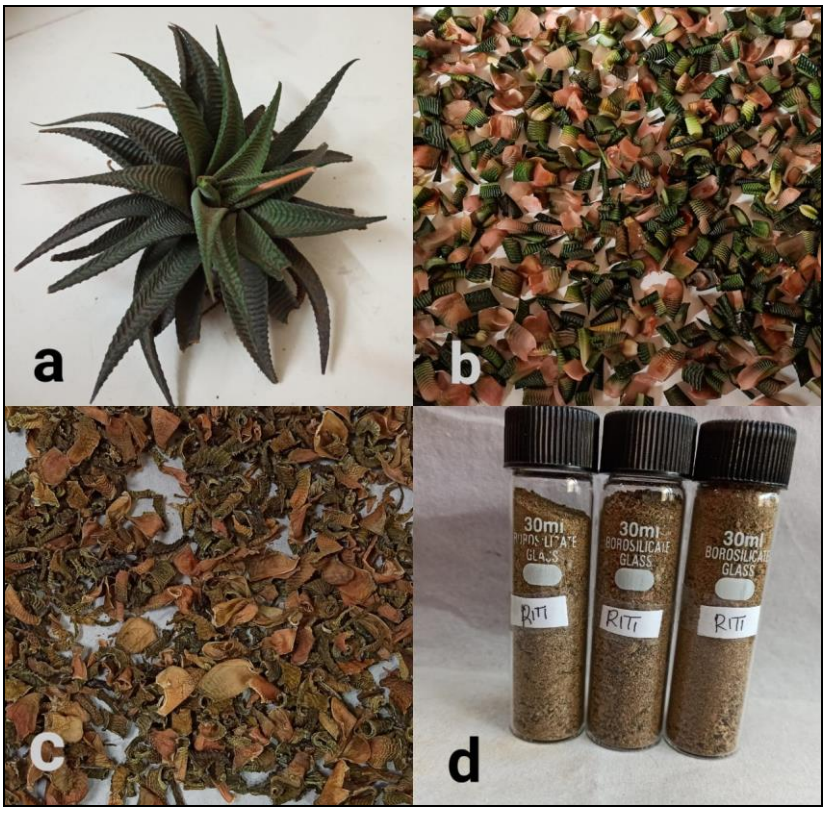

Fig 2: Process of drying the plant (a- whole aerial part b- cleaned cut pieces c- oven dried plant sample d- powdered sample stored in air tight glass bottles)

Chemicals and Reagents

The chemicals used during the study were bought locally from India (AR: analytical grade) i.e. methanol, diethyl ether, acetic acid, sulphuric acid, $FeCl_3$, $CuSO_4$, lead acetate, NaOH, $AlCl_3$, Na_2CO_3, and potassium acetate. Reagents like Folin-Ciocalteu phenol reagent, DPPH (Sigma-Aldrich Co., Germany), Gallic acid, quercetin, and ascorbic acid (Himedia, India) were used under different tests.

Other chemical reagents like Benedict's reagent, Fehling's solution A and B, sodium nitroprusside were purchased from Finar chemicals, Ahmedabad.

Qualitative phytochemical screening

30 mg extract was dissolved in 30 ml methanol and diethyl ether solvents separately to prepare 1mg/ml concentration of the stock solution. Both the methanolic and diethyl ether extracts were further subjected for the phytochemical analysis to determine the presence of various secondary metabolites like alkaloids, flavonoids, phenols, carbohydrates, proteins, fats, glycosides, terpenoids, saponins andsteroids. Preliminary phytochemical screening was done by following methods [12].

Alkaloids

- Mayer's test: 1 ml extract was added to 2 ml Mayer's reagent; creamy precipitates suggest the presence of the alkaloids.
- Wagner's test: 1 ml extract was added to 1 ml Wagner's reagent, red brown color indicates that the alkaloids are present.
- Hager's test: 1 ml extract was added to 1 ml Hager's reagent, presence of yellow precipitates reveals the presence of the alkaloids.
- Dragendorff's test: 1 ml extract was added to 2 ml Dragendorff's reagent; orange coloration shows the presence of the alkaloids.

Carbohydrates

- Molisch's test: 1 ml extract was added to 1 ml Molisch's reagent, formation of a violet ring signifies the presence of the carbohydrates.
- Fehling's test: 1 ml extract was treated with 1 ml Fehling's A and 1 ml Fehling's B reagent. It was boiled for the 2 minutes. Red precipitates prove the presence of the carbohydrates.
- Barfoed's test: 1 ml extract was added to 1 ml Barfoed's reagent and boiled for 2 minutes; red precipitates are an indication for the presence of the carbohydrates.
- Benedict's test: 1 ml extract was added to 1 ml Benedict's reagent and boiled for 2 minutes; colored precipitates show the presence of the carbohydrates.

Glycosides

- Acetic acid test: 1 ml extract was added to 2 ml chloroform, with 2 ml acetic acid, and drop-wise conc. H_2SO_4 was added to the test-tube, while simultaneously cooling on ice; violet to blue to green color change depicts the presence of the glycosides.
- Ammonia test: 1 ml extract was treated with 3 ml chloroform, shake well, and 10% ammonia solution was added, pink color reveals the presence of the glycosides.
- Ferric chloride test: 1 ml extract was treated with 1 ml glacial acetic acid and 1 ml 2% $FeCl_3$ solution, this was added to a test-tube containing 1 ml of conc. H_2SO_4, it forms 2 layers, the upper layer having red brown color, and the lower layer having blue green color signifies the presence of the glycosides.

Proteins

- Millon' test: 1 ml extract was treated with 1 ml Millon's reagent; white color suggests the presence of the proteins.
- Copper sulphate test: 1 ml extract was added to 1 ml 2% Cu_2SO_4, 1 ml ethanol, and 1 pellet of KOH, the ethanol layer has pink color which signifies the proteins presence in the plant extract.

Phenols

- Ferric chloride test: 1 ml extract was added to 1 ml 5% $FeCl_3$; dark green color depicts the presence of the phenols.
- Lead acetate test: 1 ml extract was added to 0.5 ml lead acetate; white precipitation reveals the presence of the phenols.
- Folin Ciocalteu test: 1 ml extract was added to 1 ml Folin Ciocalteu reagent; blue green color indicates the presence of the phenols.

Flavonoids

- Sodium hydroxide test: 1 ml extract was added to 3 ml 2% NaOH, turns yellow, 1 ml dil. H_2SO_4 is added; yellow color will disappear signifying the presence of the flavonoids.
- Lead acetate test: 1 ml extract was treated with a few drops of 10% lead acetate solution; yellow precipitates show flavonoids presence.

Saponins

- Foaming test: 1 ml extract was added to 20 ml distilled water, and it was shaken vigorously; if foam appears, it shows the presence of the saponins.

Fixed oils and fats

- Oil stain check: extract was poured drop-wise on a filter paper, if oil stains are observed; it signifies the presence of the fixed oils.

Terpenoids

- Copper acetate test: 1 ml extract was treated with 1-2 drops of copper acetate solution; emerald green precipitation defines the occurrence of the terpenoids.
- Chloroform test: 1 ml extract was treated with 2 ml chloroform and 3 ml of conc. H_2SO_4, it forms a layer, and if there is a formation of red brown color ring it proves the presence of the terpenoids.

Cardiac glycosides

- Sodium nitroprusside test: 2 ml extract was added to 1 ml pyridine and 1 ml 20% sodium nitroprusside; pink or red color depicts the presence of the cardiac glycosides.

Steroids

- Salkowaski's test: 2 ml extract was shaked with 1 ml chloroform, and conc. H_2SO_4 was added side by side, red color shows the occurrence of the steroids.
- Liebermann Burchard's test: 1 ml extract was treated with 2 ml acetic anhydride solution and 2 ml H_2SO_4; violet or green color signifies the presence of the steroids.

Quantitative analysis

The stock solution was prepared with the concentration of 0.2 mg/ml in the methanol solvent. In case of sample, 6 mg of crude extract was dissolved in 30 ml of solvent (methanol) to make the stock solution. The concentration gradient series of 20µg/ml to 200µg/ml was prepared by adding the stock solution into the solvent.

The standard solutions (e.g. Gallic acid for TPC, quercetin for TFC and ascorbic acid for DPPH assay) were prepared similarly with the same concentration of 0.2 mg of standard in 1mL of the methanol solvent.

Total phenolic content

Total phenolic content was estimated using Folin-Ciocalteu method where in 1 ml of Folin-Ciocalteu reagent, 10 ml distilled water, 4 ml 20% Na_2CO_3 were added to the concentration gradient series (standard- Gallic acid, and sample) and incubated in dark for 30 minutes after which the absorbance was measured using a UV spectrophotometer at λ=765nm. The standard gives a calibration curve, from which a regression equation can be derived to calculate the total phenolic content as Gallic acid equivalent (GAE/g) of sample [13]. The procedure was followed twice, one using the standard, and another with the sample as the stock solutions (0.2mg/ml) in the concentration gradient series of 20µg/ml to 200µg/ml. The whole experiment was done in triplicates in order to minimize the errors. The total phenol content was evaluated as millligrams of gallic acid equivalent/gram of sample (mg GAE/g of sample) using the following formula:

GAE= C x V/M

Where, C = concentration of gallic acid obtained from the calibration curve in mg/ml

V = volume of the extract solution in ml

M = Weight of the extract in g

Total flavonoid content

Total flavonoid content was determined using aluminium chloride colorimetric method. The method uses 0.1ml 10% aluminium chloride, 0.1ml 1M potassium acetate solution in the concentration gradient series where the stock solution used was prepared with the concentration of 0.2mg/ml and was incubated for 30 minutes. The absorbance was measured using a UV spectrophotometer at λ= 415nm. The standard flavonoid used was Quercetin, and from the calibration curve, the regression equation was derived that determines the total flavonoid content as Quercetin equivalent (QE/g) of sample [14]. The procedure was followed twice, one using the standard, and another with the sample as the stock solutions (0.2mg/ml) in the concentration gradient series of 20µg/ml to 200µg/ml. The entire experiment was repeated in triplicates to minimize errors. The total flavonoid content was estimated as millligrams of Quercetin equivalent/gram of sample (mg QE/g of sample) using the following formula:

QE= C x V/M

Where, C = concentration of quercetin acid obtained from the calibration curve in mg/ml

V = volume of the extract solution in ml

M = Weight of the extract in g

Antioxidant activity

DPPH radical scavenging assay

Antioxidant activity was studied using the DPPH radical scavenging assay. The DPPH (2, 2- Diphenyl-1-picrylhydrazyl) reagent was freshly prepared by dissolving 4mg DPPH in 100 ml of methanol to make 0.004% DPPH solution. 2 ml of prepared DPPH was added to the concentration gradient series of samples and incubated in complete darkness. When the absorbance was measured using a spectrophotometer at λ= 517nm, a gradual decrease was determined. Ascorbic acid was used in the same concentration (0.2mg/ml) as a positive control. The test was repeated in triplicates to avoid any errors. The observation was expressed as IC_{50} value, obtained by plotting the graph of percentage inhibition (% I) against concentration. The formula for calculating percentage inhibition (% I) [15] is as follows:

$$I\% = [(A_{control} - A_{sample}) \div A_{control}] \times 100$$

$A_{control}$ is the absorbance of control= absorbance of solution with all reagents except sample,

A_{sample} is the absorbance of sample= absorbance of sample or the standard.

Statistical analysis

Every value obtained is the accumulation of the three replicates so as to avoid any errors while performing all the experiments. All the results are symbolized as mean ± standard error (S. E.). The calculation for IC_{50} value and all other statistical analysis were performed using the latest version of GraphPad Prism 7.0 Windows Software.

Results

Yield value

The yield value is used in the quantification of the phyto-constituents with respect to the crude extract. The methanolic extract had a yield of 8.36% while the yield observed for diethyl ether extract was 1.36%. The formula used was;

Yield (%) = (weight of dry extract ÷ weight of plant powder) × 100

Calculations:

% yield for methanolic extract = (0.836g ÷ 10g) × 100 = 8.36%

% yield for diethyl ether extract = (0.136g ÷ 10g) × 100 = 1.36%

Results for phytochemical screening

Methanolic extract of leaf sample of *Haworthiopsis limifolia* M. evinced the presence of alkaloids, carbohydrates, proteins, phenols, flavonoids, terpenoids, and cardiac glycosides. Whereas, the diethyl ether extract revealed the occurrence of phenols, flavonoids, terpenoids and cardiac glycosides only. This result proves that the polar methanol solvent tends to extract more secondary metabolites from the plant sample as compared to the non-polar diethyl ether solvent (TABLE 1).

Table 1: Preliminary Phytochemical Tests

Chemical tests	Methanolic extract	Diethyl ether extract
Alkaloids		
Mayer's test	+	-
Wagner's test	+	-
Hager's test	+	-
Dragendorff's test	+	-
Carbohydrates		
Molisch's test	-	-
Fehling's test	+	-
Barfoed's test	-	-
Benedict's test	-	-
Glycosides		
Acetic acid test	-	-
Ammonia test	-	-
Ferric chloride test	-	-
Proteins		
Millon's test	+	-
Copper sulphate test	-	-
Phenols		
Ferric chloride test	-	-
Lead acetate test	+	+
Folin-Ciocalteu test	-	-
Flavonoids		
Sodium hydroxide test	+	-
Lead acetate test	+	+
Saponins		
Foaming test	-	-
Fats and fixed oils		
Oil stain test	-	-
Terpenoids		
Copper acetate test	+	+
Chloroform test	+	+
Cardiac glycosides		
Sodium nitroprusside test	+	+
Steroids		
Salkowaski's test	-	-
Liebermann Burchard's test	-	-

The table represents the results of the phytochemical tests performed. '+' sign indicates the presence and '-'sign indicates the absence of the respective phytochemicals

Result for total phenolic content

Folin-Ciocalteu method was used to evaluate the total phenolic content of the sample. The phenolic compounds present in the plant depict the redox properties, as well as they show the antioxidant activity. The standards were run using the Gallic acid to obtain the calibration curve, from which the following equation was obtained (Figure 3);

$y = 0.0049x - 0.0971$

$R^2 = 0.9892$

Using the equation, Gallic acid equivalent (mg)/gram of sample was calculated. The result shows that the sample has 203.33± 1.66 mg GAE/g of sample. The higher content of phenols in the plant sample increases the potential of plant having different bioactivities; hence, the extract can show the positive results for the antioxidant activity also.

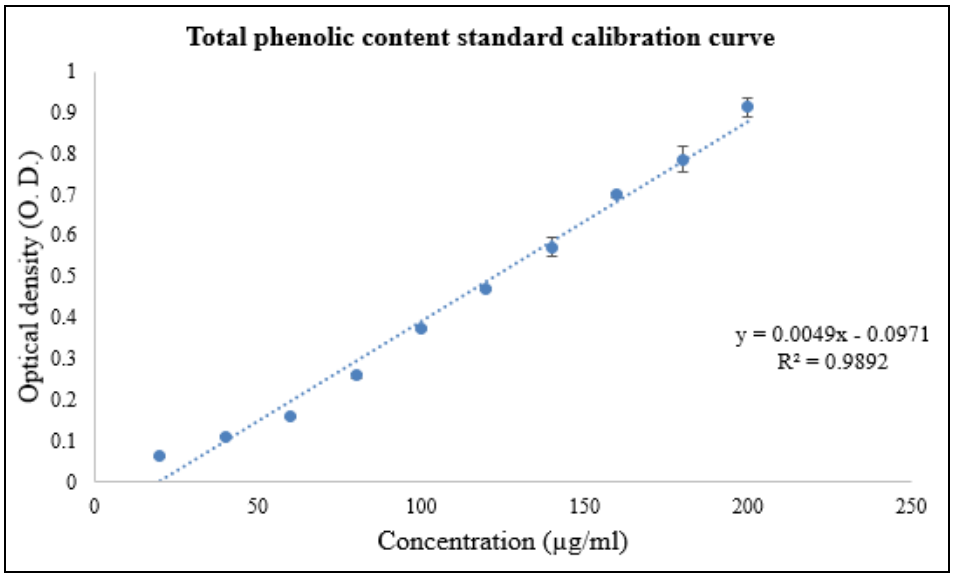

Fig 3: graph showing the standard curve of gallic acid for total phenolic content

Result for total flavonoid content

Aluminium chloride colorimetric method is run by the principle of $AlCl_3$ forming acid stable complex with C4 keto group or C3/ C5 hydroxyl group of flavonoids or flavones thus it is used to evaluate the total flavonoid content. The calibration curve was obtained by running the standard, Quercetin. The curve provides the following equation (Figure 4);

$y = 0.0128x + 0.1165$

$R^2 = 0.9915$

From the above equation, the total flavonoid content of the plant sample was calculated and showed to possess 8.33± 3.33 mg QE/g of sample.

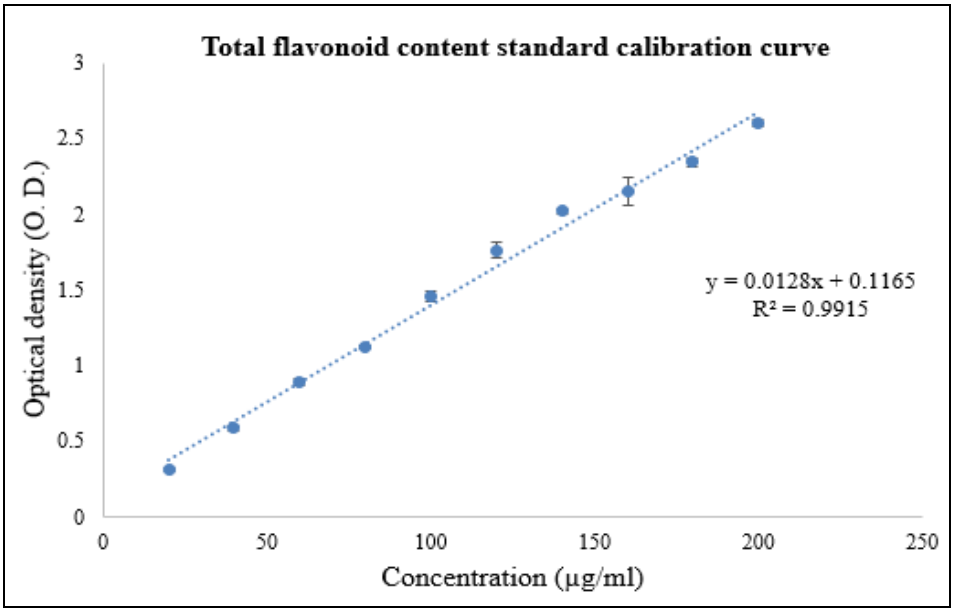

Fig 4: graph showing the standard curve of quercetin for total flavonoid content

Result for antioxidant activity

The antioxidant potential of the plant was assessed by DPPH radical scavenging assay. DPPH is the most commonly used chemical for testing the antioxidant activity of plant samples. It is a rapid effective method for colorimetric analysis. The antioxidant agent reduces DPPH which is signified by the color change from purple to yellow with a sequential decline in the absorbance at 517nm which is measured using a spectrophotometer. To study the antioxidant activity of *H. limifolia* leaves, ascorbic acid was used as the standard antioxidant agent for the comparison. The readings were expressed in terms of 50% inhibition concentration (IC_{50}), which was calculated from the regression equation that was obtained by plotting a graph of concentration against the % inhibition (Figure 5). The values of IC_{50} are inversely proportional to the antioxidant activity, i.e. the lower the value of IC_{50}, the stronger is the antioxidant activity. From this study, the value of IC_{50} for the standard, ascorbic acid was evaluated to be 133.421± 0.464 µg/ml and that of the plant sample was found to be 243.241± 1.12µg/ml.

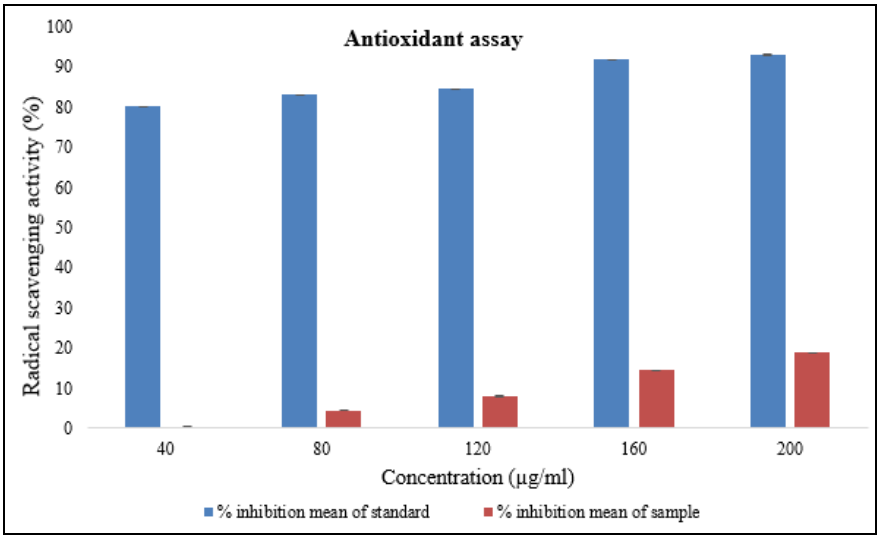

Fig 5: graph showing the comparison of the percentage of radical scavenging activity between the standard (ascorbic acid) and the plant sample (*H. limifolia*).

Shows the percentage inhibition for the standard, Shows the percentage inhibition for the plant sample.

Discussion

As the technological interventions increase, the traditional knowledge and the crude drugs from the plants become more and more significant. The phytochemical screening and various bioactivities show the potential use of the plants in curing several diseases and ailments. *Haworthiopsis limifolia* is a plant on which there aren't enough studies that shows its importance. The current study attempts to explore the plants secondary metabolites, their quantitative assessment and also the antioxidant property present in it. The antioxidant property is essentially helpful in protecting the cell against the damage caused by the free radicals, which can protect the human body from various life threatening diseases such as cancer. The study of the plant collected from various agro-climatic zones can help evaluate the overall properties and chemical composition of the plant and making optimum use from it in order to combat several diseases [16]. The aerial parts *H. limifolia* have shown presence of metabolites like alkaloids, saponins, carbohydrates, glycosides, flavonoids, sterols, anthraquinones, polyphenols, tannins, fatty acids, and properties like antibacterial, antifungal, anti-oxidant, anti-inflammatory, wound-healing and cytotoxic activities in the study performed earlier [17]. In other studies, the IC_{50} value observed for *Haworthiopsis limifolia* was 143.6 mg/ml, while from our work we concluded that the IC_{50} value is 243.241±1.12 [18]. *Bulbine* genus is close to *Haworthiopsis* genus in the family Asphodelaceae, and this genus has also reported antioxidant, anti-inflammatory, antimicrobial, wound-healing activities, along with other activities like antiviral, anticancer, anti-plasmodial and antitrypanosomal [19]. Total flavonoid content of *Bulbine frutescens* was found to be 71.60 ± 0.05 mg QE/g extract [20] whereas *Haworthiopsis limifolia* has a total flavonoid content of 8.33 ± 3.33 mg QE/g extract. *Gasteria bicolor* which also belongs to family Asphodelaceae reported that the total phenolic content (588.91mg/g), total flavonoid content (42.27 mg/g) and antioxidant activity (DPPH assay) in terms IC_{50} as 0.27µ/ml [21]. The methanolic extract of *Aloe barbadensis* showed the occurrence of proteins, carbohydrates, phenols, glycosides, tannins and steroids in the preliminary study, and the total flavonoid content (14.29 ± 0.44mg QE/g of sample), total phenol content (30.53 ± 0.30mg GAE/g of sample) and the anti-oxidant property with the IC_{50} value as 81.91 ± 0.04% [22]. This result shows a comparable study of the bioactivity of various plants which are closely associated and may serve beneficial in the future in several pharmaceuticals and other industries.

Conclusion

The core objective of the study was to screen the presence of various secondary metabolites, their quantitative assessment and antioxidant property of *Haworthiopsis limifolia* leaves. The plant *Haworthiopsis limifolia* is selected in order to explore the potentials of the plant as there aren't enough studies based on the plant with an in-depth knowledge on the metabolites, compounds and activities present in the plant. The presence of several important phyto-constituents shows that the plant possesses some significant pharmaceutical property that can have medicinal applications in the future. The DPPH radical scavenging assay proves the antioxidant potential of the plant. The phenol content of the plant and its antioxidant activity hold a significant correlation. The phytochemicals present in the plant are alkaloids, carbohydrates, proteins, phenols, flavonoids, terpenoids, and cardiac glycosides.

The further study of the phytochemicals can help detect the potent capabilities of the plant *Haworthiopsis limifolia* like anticancer, antibacterial, anti-inflammatory, antifungal, and anti-diabetic. The older studies show traditional medicinal uses which can be applied today by exploring all the properties of the plant *Haworthiopsis limifolia.* With varying agro-climatic zones, the constituents and its activities also vary, making it essential to study the array of activities that the plant acquires in order to enhance the applications and utilisations of the plant in diverse industries.

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