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Original article

Comparative proximate analysis, phytochemical screening and antioxidant study of leaf and root extracts of *Decalepis hamiltonii* Wight & Arn.

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Abstract

Decalepis hamiltonii Wight & Arn. (DH, family: Asclepiadaceae) is an endemic and endangered plant in India. The plant is commonly known as Swallow root and rarely located in Bangalore, Karnataka. The present study was revealed to establish proximate analysis, phytochemical screening and antioxidant activity on leaves and root methanolic extract of the domesticated DH plant. Moisture content and ash content was estimated for both leaves and roots and gave higher values for leaves (7.4 % and 6.7 %, respectively) than roots. Thereafter, various elements such as Fe, Cu, Zn, Cd, Cr, Pb, Ni, As, K, P, Ca and Na were estimated and revealed absent of non essential heavy metals (Cd, Cr, Ni, Pb, As) in leaves whereas below detectable limits of the same was detected for root sample. Various chemical tests for leaves and roots were carried out and revealed presence of flavonoids, tannins, glycosides, steroids, terpenoids, carbohydrate and phenols. Furthermore, total phenolic, total flavonoids content was resulted higher for leaves extracts and the same trend followed for antioxidant activity when IC_{50} values were compared with standard ascorbic acid and roots extract. Finally concluded that leaves extract had powerful antioxidant properties than roots extracts and the activity was dose dependent manner.

Key words: *Decalepis hamiltonii* Wight & Arn. antioxidant studies, elemental analysis, methanol extract, phytoconstituents, proximate analysis

1. Introduction

Since ancient time, a vast number of species have gone extinct from natural processes. Today, most plant species become extinct because of habitat destruction due to large population, introduction of non-native organisms and direct cutting. The value of endangered species has increased with the recognition that human activities cause extinction. In general, benefits of plant species are classified as ecological, economic and social with identification of higher therapeutical activities. Many traditional medicines rely mostly on medicinal plants which are further dependent on chemical races. Chemical races with respect to genetic diversity and ecological diversity are both components of biological diversity of endangered plant. Survival ability of a species in environmental change is directly depends on genetical diversity. Decalepis is such an example of climbing shrub with aromatic tuberous roots plant belongs to an endangered and is characterized by its ability to exist under different climatic conditions. The plant has domesticated from the natural habitat and surely there are many changes occurred along with many physical and metabolic changes especially in chemical constituents.

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Looking at that the present plant *Decalepis hamiltonii* Wight & Arn. (DH), belongs to family Asclepiadaceae, was selected which is grown only in the Southern part of India. The plant is commonly known as swallow root plant. It grows in between the rocks. Milky latex is present in the entire plant especially in leaves and roots (Vedavathy, 2004). Traditionally, the root is used as health drink and currently it is used as various food preparations, consumed as pickles and beverages (Harish *et al*., 2005; Reddy *et al*., 2007) and plenty pharmaceutical applications (Wealth of India, 1959). Research evidences revealed the root contain volatile oil, main chemical component as 2-hydroxy-4-methoxybenzaldehyde. Apart from that, root also contains benzaldehyde, salicylaldehyde, methyl salicylate, vanillin (Nagarajan *et al*., 2001), beta amyrin acetate, alpha and beta amyrin (Murti and Seshadri, 1941). The root of this plant is use for treatment of skin diseases, blood purifier, diarrhoea, diuretics, *etc.* (Chopra *et al*., 1956; Dey *et al*., 1999; Arutla *et al*., 2012). It is also used in Parkinson's diseases (Johromi *et al*., 2015). Recent study established antioxidant nature of root extract. 4-hydroxyisophthalic acid (4-HIPA) isolated from aqueous extract of *D. hamiltonii* roots and studied for treatment of neurodegenerative disorders by cellular antioxidant defense system (Haddadi *et al*., 2016). Whereas, very few reports of leaves extracts on therapeutic effects, as antimicrobial effect was revealed recently (Rajani *et al*., 2016). Hence the present study has undertaken to establish detail proximate analysis, phytochemical screening and antioxidant study of leaf and root methanolic extracts of DH and explore their new area of therapeutic applications.

2. Materials and Methods

2.1 Collection of plant material

The leaves and roots were collected from garden of Krupanidhi College of Pharmacy after authenticated by Dr. Rajasekharan, P.E, Principal Scientist, Indian Institute of Horticultural Research, Hessaraghatta, Bangalore. The plant materials were collected during August, 2017 and also kept for herbarium for future reference (H. No: KCP/PCOG/ *Decalepis hamiltonii-1* /2017) (Figure-1).

(a) Leaves of DH.

(a) Leaves of DH

Figure 1: Leaves and root part of domesticated DH plant.

2.2 Extraction of plant materials

Both leaves and roots were cleaned with running tap water then dried under shade for 6-8 days. After drying, both are powdered separately by hand mixer grinder and kept for proximate analysis by sealed plastic cover to prevent moisturization. Thereafter, methanol (80%) solvent was used separately for both the parts (leaves and roots) extraction using soxhlet apparatus for 8-9 hours. 250 g of each sample was extracted separately with 500 ml of solvent methanol to procure the crude extracts. After completion of extraction, the extracts were filtered and evaporated by rotary flash evaporator at 45°C and further yield was calculated for both separately. The crude extracts were preserved in small glass bottles at refrigeration condition at 4-5°C for further investigation.

2.3 Proximate analysis

Dried leaves and roots were analyzed for moisture content, ash content as the method described by the (AOAC, 2010), various metals and heavy metal (Zn, Fe, Cd, Cu, Pb, Ca, Ni, As, Cr and P) content by Atomic Absorption Spectrophotometer (Model-Aanalyst 100, supplied by Perkin Elmer, USA) using di-acid digestion method (H_2SO_4: $HClO_4$ = 6:4) at various wave length using air acetylene gas mixture. Thereafter, K and Na were determined by using flame photometer (Das *et al*., 2016). Both the samples were triplicate analyzed for the reproducibility of the method and the mean values of concentrations for each element has been reported along with standard error mean (SEM). The risk assessment code (RAC) of metals in leaves and roots was performed following the procedure described by Singh *et al*. (2005) as:

$$RAC\ (\%) = \left(\sum_{n=1}^{n=3} F_n \Big/ \sum_{n=1}^{n=6} F_n \right)$$

where, "Fn" is the concentration of metal in 'n^{th}' fraction.

2.4 Phytochemical studies

Presence of various secondary metabolites such as glycosides, alkaloids, tannins, steroids, lipids, carbohydrates, saponins, flavonoids, phenolic contents, *etc*., were identified preliminary by various chemical tests as per the method described by Kokate (2001).

2.5 Estimation of total phenols

The total phenolic content was determined by Folin-Ciocalteu's method using spectrophotometre (Kim *et al*., 2003) for both roots and leaves of DH. 1 ml of sample (1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of a 7% Na_2CO_3 solution and 13 ml of deionized distilled water was added and mixed thoroughly. The mixture was kept in the dark for 1.30 h. at 23°C for the reaction (blue color form). Gallic acid was used as a standard by prepared dilution of 10, 20, 30, 40, 50 and 60 µg/ml and absorbance was taken at 765 nm. The total phenolic content was determined from extrapolation of calibration curve and was expressed as mg of gallic acid equivalent (GAEs) per g of extract (GA mg/g). The estimation of the phenolic compounds was carried out in triplicate using the formula:

$$T = (C \times V)/M$$

where, T = Total phenolics, mg/g plant extract, in GAE; C = Gallic acid concentration established from the calibration curve (µg/ml); V = volume of extract (ml); M = weight of the extracts (g).

2.6 Estimation of total flavonoids

Total flavonoid content was measured by Aluminium chloride colorimetric assay (Park *et al*., 2008). In a 10 ml test tube, 0.3 ml of extract, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M), 0.15 ml of $AlCl_3.6H_2O$ (0.3 M) and 1 ml of NaOH (1 M) were added and mixed properly. Rutin standard solution was also prepared for standard curve (10, 20, 30, 40, 50 and 60 µg/ml). The solution was mixed well and the absorbance was measured at 506 nm. The total flavonoids were expressed as mg of rutin equivalent (RuE)/g of dried extract.

2.7 Antioxidant study

Oxidation is a chemical reaction that produces free radicals. These free radicals can damage cells which are prevented by antioxidants. An antioxidant agent is inhibits the oxidation of other molecules and protect cells from oxidation. There are many methods for determination of antioxidant activity. In the present study DPPH and hydrogen per oxide scavenging activity were used.

2.8 DPPH assay

Both the extracts were tested for their free radical scavenging activity against the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and their activity was quantified using spectrophotometer (Brand-Williams *et al.*, 1995) at 517 nm after 30 min. in dark (for reaction). 1ml of 0.1 mM DPPH solution in methanol was mixed with 1ml of plant extract solution of varying concentrations (25, 50, 100, 150 and 200 µg/ml). Corresponding blank samples were prepared using mixed 1ml methanol and 1ml DPPH solution methanol. L-ascorbic acid was used as reference standard (1-100 µg/ml) and the experiment was done in triplicate. The IC_{50} value (µg/ml) of the sample, *i.e.*, the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated by linear regression. The percentage scavenging was calculated using the following formula:

DPPH Scavenging effect (%) = [$(A_{control} - A_{sample} / A_{control}) \times 100$]

2.9 Hydrogen peroxide scavenging activity

Hydrogen peroxide solution (2 mmol/l) was prepared in phosphate buffer (pH 7.4). 0.5 ml of extract at various concentrations (25, 50, 100, 150 and 200 µg/ml) was added to hydrogen peroxide solution. For each concentration, a separate blank sample was prepared. Absorbance of hydrogen peroxide by spectrometer at 230 nm was determined, followed by after 15 min. reading calculated for blank solution containing phosphate buffer without hydrogen peroxide (Nabavi *et al.*, 2009). The percentage inhibition of H_2O_2 scavenging activity was calculated using the below formula:

% scavenging activity = [1- (Absorbance of test / Absorbance of control)] × 100

2.10 Statistical analysis

Data are expressed as mean ± SEM from three replications. For antioxidant assays, One-way ANOVA test followed by post Tukey's test ($p < 0.05$) was used to analyze metallic contents and the differences among IC_{50} of various extracts for different antioxidant assays. The IC_{50} values were determined using the Graph Pad Prism 5 software. *p* values less than 0.05 were considered to be statistically significant.

3. Results

3.1 Yield of the extracts

The yield of the extracts of DH leaves and roots was estimated after methanolic extract and the results revealed that root extract contains more percentage of extractive (8%) than leaves extract (6.8%) and was tabulated in Figure 2.

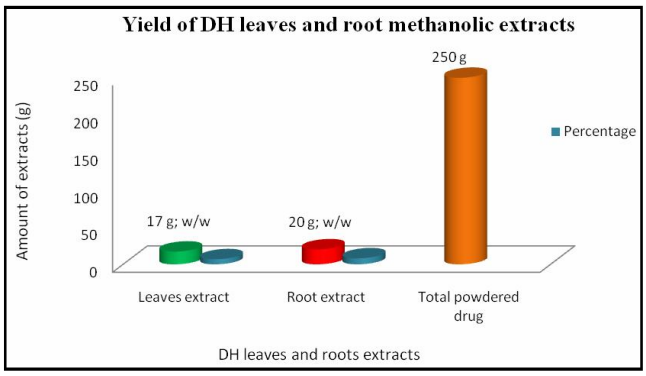

Figure 2: Percentage yield of methanolic DH leaves and root extracts.

3.2 Proximate analysis

3.2.1 Moisture content and total ash content

The powdered leaves and roots were estimated for moisture content and total ash content using moisture pan balance as well as heating at 450°C in muffle furnace, respectively. Results revealed shade dried leaves contain more ash and moisture than roots (Table 1).

Table 1: Determination of physical parameters in DH leaves and roots

DH plant parts	Total amount	Moisture content (%)	Ash content (%)
Leaves	5 g powder	7.4	6.7
Roots	5 g powder	3.6	3.8

3.2.2 Elemental analysis

AAS was used for determination of various metals especially heavy metals in roots and leaves of DH. Results showed below detectable limit of non essential heavy metals such as Cd, Pb, Ni and As whereas Zn, Cu, Fe, Ca, Na, K, and P contents were detected quite significant amount in both leaves and roots. The accuracy and precision of the analyzed report for metals were checked against SRM-2709 (San Joaquin soil from the National Institute of Standards and Technology, Gaithersburg, MD) and SRM-1645 (river sediment from the National Bureau of Standards, Washington, D.C.) and showed all the certified and reference elements were within the expected values (95 to 110% recovery). Leaves showed presence of K, Zn and Fe in higher concentration than that of roots whereas Cu, Ca, Na, and P contents were higher in roots (Table 2 and 3). RAC was determined as per method described by Singh *et al.* (2005) to identify the metal release risk in leaves and root samples as well as their uptake pattern by growing plants (Table 4).

Table 2: Concentration of elements (mg/kg) in methanolic leaves and roots extract of DH. Same letter(s) in a particulars column between two samples represent non-significant difference in particular element

Sample	Extractants	P	K	Na	Ca	Zn	Cu	Fe
Leaves	Methanol	$13.42^{a} \pm 0.12$	$42.02^{b} \pm 0.24$	$44.23^{b,c} \pm 0.10$	$28.01^{a} \pm 0.02$	$5.42^{a,f} \pm 0.23$	$5.43^{a} \pm 0.02$	$18.32^{a} \pm 0.22$
Roots	Methanol	$16.03^{a} \pm 0.42$	$28.87^{a} \pm 0.31$	$57.20^{b,d} \pm 0.11$	$47.11^{b,e} \pm 0.03$	$4.22^{a,f} \pm 0.40$	$8.30^{a} \pm 0.04$	$8.14^{a,g} \pm 0.11$

Mean ± SEM; One-way ANOVA followed by post Tukey's comparison with all pair of columns; ($p < 0.05$ = significant);

Table 3: Concentration of non essential heavy metals (mg/kg) in methanolic leaves and roots extract of DH; Same letter(s) in a particulars column between two samples represent non-significant difference in particular element

Sample	Extractants	Cd	Pb	Ni	As	Cr
Leaves	Methanol	ND	ND	ND	0.02 ± 0.01	ND
Roots	Methanol	0.34 ± 0.02	ND	0.06 ± 0.10	0.24 ± 0.01	ND

ND: Not detectable (detectable limits of Cd, Cr, Ni, Pb and As are 0.6 mg/l, 0.3 mg/l, 0.1 mg/l, 0.3 mg/l and 0.3 mg/l, respectively).

Table 4: RAC determination for leaves and root samples of DH

Heavy metals	RAC*		Category of sample with respect to RAC	
	Leaves	Roots	Leaves	Roots
Cd	0.0	0.34 ± 0.02	①	①
Pb	0.0	0.0	①	①
Ni	0.0	0.06 ± 0.10	①	①
As	0.02 ± 0.01	0.24 ± 0.01	①	①
Cr	0.0	0.0	①	①

*RAC: <1 (category 1, no risk); 1-10 (category 2, low risk); >10-30 (category 3, medium risk); >30-50 (category 4 , high risk) and >50 (category 5, very high risk)

3.2.3 Phytochemical studies

Preliminary phytochemical details was identified by performed detailed chemical tests for individual secondary metabolites and revealed the presence of various group of constituents that was tabulated in Table 5. The study indicated that leaves of DH contains prominently tannins, flavonoids, glycosides whereas traces of carbohydrate, phenolics, steroids and terpenoids. All these constituents are present in root prominently.

Table 5: Detail chemical tests for methanolic leaves and roots extracts of DH

Chemical tests	Methanol extract of leaves	Methanol extract of roots
Carbohydrate	+	++
Proteins	--	--
Lipids	--	--
Alkaloids	--	--
Glycosides	++	++
Flavonoids	++	++
Tannins	++	++
Phenolics	+	++
Terpenoids	+	++
Steroids	+	++

(++) = present prominently; (+) = present; (—) = absent

3.2.4 Estimation of total phenolics and flavonoids

Based on the preliminary chemical tests, further total phenolics and flavonoids were estimated for both leaves and roots separately and the results were tabulated in Table 6.

Table 6: Total phenolic and flavonoid content in leaves and roots extracts of DH

Plant parts	Total phenolics (GA mg/g) ± SEM	Total flavonoids (RuE mg/ g) ± SEM
Methanolic root extract	53.22± 0.022**	28.20 ± 0.110**
Methanolic leaves extract	56.12± 0.011**	36.32± 0.030**

Mean ± SEM (n =3); One-way ANOVA study followed by Tukey's post test was carried out at significant level, $p < 0.05$.GA= Gallic acid; RuE= Rutin equivalent

3.3 Antioxidant study

Based on the constituent present, further antioxidant study was carried out separately for leaves and root extracts of DH plant. There are various methods to determine antioxidant activities among them. DPPH and hydrogen peroxide methods were used to determine antioxidant property of the said plant and established comparative study by calculated IC_{50} values (Table 7). Ascorbic acid showed IC_{50} value at 50 µg/ml concentration whereas methanolic leaves and roots extracts showed IC_{50} value at concentration 100 µg/ml and 150 µg/ml, respectively. Significant results were obtained when antioxidant study was compared with both leaves and roots extracts of DH (Figures 3 and 4).

Table 7: IC_{50} values of methanolic leaves and roots extract of DH

Extracts	IC_{50} µg/ml	
	DPPH scavenging assay	Hydrogen peroxide - scavenging activity
Methanol leaves	28.68 ± 0.014	33.72 ± 0.014
Methanol roots	44.21 ± 0.011	49.38 ± 0.031
Ascorbic Acid (Standard)	18.04 ± 0.032	19.59 ± 0.020

Mean ± SEM (n =3); One way ANOVA study followed by Tukey's post test was carried out at significant level, $p < 0.05$

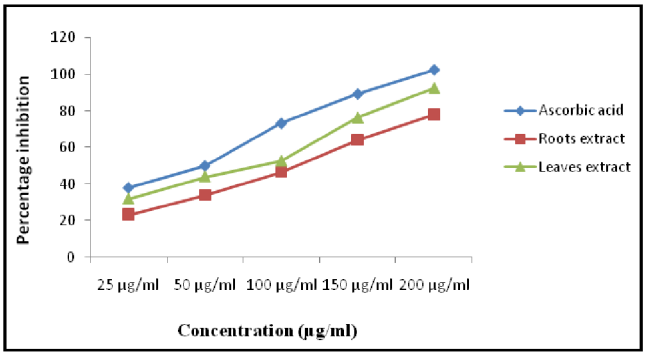

Figure 3: Percentage inhibition of methanolic leaves and roots extracts of DH by DPPH method.

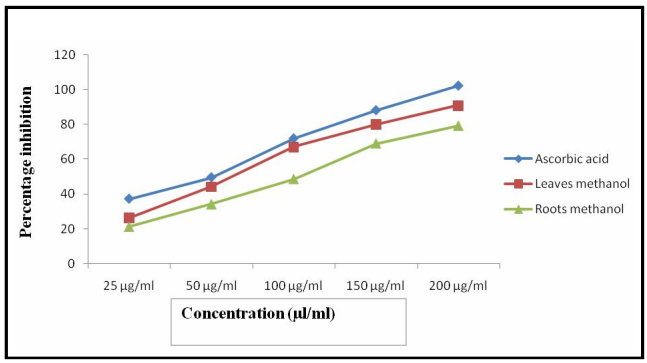

Figure 4: Percentage inhibition of methanolic leaves and roots extracts of DH by H_2O_2 scavenging method.

4. Discussion

4.1 Yield of extracts

Methanol extract of roots provided more percentage of extractive (8%) than methanolic leaves extract (6.8%). This increased percentage of root extract is due to less ash content than leaves. Earlier report was also resulted that methanol root extract gave more yield than other solvent extracted and also that of leaves extracts (Samydurai and Thangapandian, 2012). Our present investigation also followed the same trend as the higher yield showed by the root extract from DH.

4.2 Proximate analysis

4.2.1 Moisture content and ash content

Moisture content and total ash content was estimated for powdered crude drugs (leaves and roots) of DH. Results revealed that both the moisture and ash contents were higher in leaves than roots. This is due to more fibres, veins and vein islets are present in the leaves which enhanced these contents. The earlier research evidences are also proved the same (Borkataky *et al*., 2013; Puthalath *et al*., 2015) where leaves powder showed more moisture and ash contents than any other plant parts.

4.2.2 Elemental analysis

Present investigation revealed the presence of essential elements in leaves and roots of DH, estimated by AAS and UV spectrophotometric determination. Leaves showed higher amount of Fe, Zn and K content than roots and thereafter Cu, Ca, Na, and P contents were higher in roots than leaves. Earlier many literatures reported that accumulation and uptake of elements are depends on the soil nature and soil pH. Acidic soil enhanced accumulation of Fe, Zn in leaves whereas Ca content of roots is higher than leaves due to soil pH (Thompson *et al*., 1997; Aref, 2012; Das and Tribedi, 2015). The same trend followed in the present investigation. Further, non essential heavy metals were detected for presence in leaves and roots. Results revealed that only arsenic content in leaves was very negligible and other metals such as cadmium, lead, nickel and chromium were not detectable which proved safety use of plant parts. The same was detected very negligible quantities of roots which was solely dependent on the soil composition. WHO (1998) prescribed limit of Pb contents of herbal medicine is 10 ppm and the dietary intake limit of Pb is 3 mg/week. Thereafter, it was reported that the lowest level of Cd of 5- 30 ppm, can reduce the yield of the plants (Neil, 1993). Singh *et al*. (2005) reported scale of risk assessment code of metals in leaves and roots and categories limit for various heavy metals. As per that report, our present experiment revealed negligible content of non essential metals which were safe for human use. Furthermore, Das *et al*. (2011) reported that very negligible amount of non essential heavy metals present in Stevia leaves on applied biofertilizers in cultivation field. The same trend also reported in this experiment.

4.2.3 Phytochemical studies

The study indicated that leaves of DH contain prominently tannins, flavonoids, glycosides whereas traces of carbohydrate, phenolics, steroids and terpenoids but all these constituents are present in root as a result roots are having various therapeutic activities. It was reported that any medicinal or therapeutic activities are mainly depends on the active constituents present in the plant part (Borkataky *et al*., 2013). Earlier research reported that solvent extracts also effects on phytochemicals present in plants as well as on various therapeutic activities (Naima *et al*., 2015; Felhi *et al*., 2017). Our experiment also showed maximum constituents in methanol solvent for both roots and leaves of DH.

4.2.4 Estimation of total phenolics and flavonoids

Total phenol content as well as total flavonoid content was estimated higher in roots than leaves extract. Methanol solvent was used for the extraction which increased the solubility of phenols. Earlier reports revealed high solubility of phenols in polar solvents provides high concentration of these compounds in the extracts **(**Zhou and Yu, 2004; Mohsen and Ammar, 2008). Flavonoids are also responsible for the bioactivity of the crude extracts and due to their hydroxyl groups, flavonoids show antioxidant activity and have radical scavenging effect in the plants (Younes, 1981; Tomsone *et al*., 2012). In the present experiment, flavonoids was estimated higher in leaves than roots which showed similar trend to the previous reports (Calabrone *et al*., 2015; Baba and Malik, 2015).

4.3 Antioxidant activity

Antioxidant studies have relation with the phenolic content of the plants. In the present study, leaves extract showed more phenolic and flavonoid contents, hence, leaf extract of DH showed higher scavenging property that may be due to the present of hydroxyl groups existing in the phenolic and flavonoid compounds chemical configuration that can provide the essential constituents as a radical scavenger with redox properties, which allow them to act as antioxidants (Soobrattee *et al*., 2005). As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Plant flavonoids also have antioxidant activity in both *in vitro* and *in vivo* methods (Shimoi *et al.*, 1996; Geetha *et al.*, 2003). Furthermore, flavonoids are also highly effective antioxidant agents for most oxidizing molecules and various other free radicals implicated in various diseases (Bravo, 1998). They suppress reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species and protect oxidations (Agati *et al*.,2012). It was also reported that antioxidant activity inversely dependent on IC_{50} value. IC_{50} value is more indicated less antioxidant activity and vice versa (Siddique *et al*., 2010). The present study also showed the same trend where leaves extract showed higher phenolic and flavonoid content and as

well as higher antioxidant activity. DPPH and hydrogen peroxide scavenging methods gave additional benefits with phenolics and flavonoids for significant results.

5. Conclusion

The present investigation revealed that the methanol extract of DH leaves and roots are the effective sources of antioxidant and many other therapeutic activities in the conventional drug framework. The studies were also obliged to identify the presence of moisture, ash, various secondary metabolites, and potent antioxidant for domesticated DH plant. The discoveries provided much lauded impact of dose dependent antioxidant activity with content of total phenolics and total flavonoids especially in leaves methanolic extract of DH plant. Finally result showed the path to the future researchers to find out more therapeutic activities on leaves extracts and to discover novel phytoconstituents for effective treatments.

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Conflict of interest

We declare that we have no conflict of interest.

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