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Research Article
Standardization and Antioxidant Studies of *Arnebia hispidissima*

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Abstract

Background and Objective: *Arnebia hispidissima* (*A. hispidissima*) a member of the family Boraginaceae, is dye yielding and medicinally important plant. It is widely used in the cosmetic industries. This study has been made for the preliminary standardization of *A. hispidissima* plant. **Methodology:** The standardization evaluation comprises of powder microscopy and fluorescence analysis and TLC profiling. In addition, preliminary phytochemical screening, determination of total phenol and *in vitro* free radical scavenging activity (DPPH radicals scavenging assay and reducing power activity) were preformed utilizing the methanol extract. **Results:** A microscopic study of powder of whole plant of *A. hispidissima* showed different types of trichomes, vessel and fibres. Fluorescence analysis showed different colours under visible light, low UV and high UV. TLC of the hexane extract developed 8, 6, 9 and 10 spots with visible light, low UV, high UV and ninhydrin-H_2SO_4 spray, respectively. The phytochemical analysis of the methanol extract gave a positive indication for the presence of active compounds including alkaloids, carbohydrates, glycosides, steroids, triterpenoids saponins, phenols, tannins, flavonoids and proteins. The quantitative analysis showed the presence of a significant quantity of total phenol and the *in vitro* antioxidant activity clearly showed the terrific antioxidant property. **Conclusion:** The data generated from the present study can be utilized for the identification and quality control of *A. hispidissima* plant.

Key words: *Arnebia hispidissima*, microscopical, phytochemical, chromatography, DPPH, reducing power

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The usage of natural products has grown incredibly in line with the worldwide trend of folks returning to natural remedies[1]. Herbal drugs exert a key impact in health care programs particularly in developing nations. However, hindrance behind the acceptance of herbal medicine in developed nations is the weak or absence of documentation, strict quality control measures. Therefore, documentation and standardization of the crude materials utilized in herbal medicine is vital for the global compliance of this system of medicine[2]. Standardization and quality control of plants are also essential for the worldwide acceptance of herbal products in modern system of medicines[3]. Pharmacognostic standardization and preliminary phytochemical studies were all over accepted in recognizable proof and confirmation of the genuine plant materials[2].

Genus *Arnebia* belongs of the family Boraginaceae. The genus name originates from the Arabic name Sagaret-el-Arneb[4]. A literature review showed that there were some reports on the biological investigation of *Arnebia* genus. In this respect, ethanol extract of *Arnebia nobilis* roots showed bactericidal and fungicidal activities. Acetyl shikonin isolated from *Arnebia euchroma* exhibited significant *in vivo* and *in vitro* anticancer activity[5].

Arnebia hispidissima, is widely distributed from Northern Africa, throughout Egypt, to North parts of India. It is well known in the Arab region as Kaha, Al-Hamra or Arabian primrose. It was common in Qatar and also recorded in Bahrain, Saudi Arabia and UAE[6]. The roots of *A. hispidissima* has been used in treatment of boils, heart ailments, headache and fever, whereas, the flowering aerial parts has been employed to treat tongue, throat as well as fevers and cardiac disorders[7]. *Arnebia hispidissima* also exhibited antibacterial[8] and antitumor activities attributable to naphthoquinones, triterpenoids and pyrrolizidine alkaloids present in the extract. The two isomers alkannin and shikonin are red dyes present in the roots of *A. hispidissima*[9]. Alkannin has anti-inflammatory, antimicrobial and antioxidant activities[10,11]. In addition, it widely used as a food colorant, in cosmetic and textiles industries[12]. The roots of *A. hispidissima* yielded a mixture of naphthoquinones with antibiotic and anticancer activities[13]. The flavonoid glycoside vitexin has been isolated from the fresh flowers of *A. hispidissima*[13].

Since there is no report in literature regarding the standardization parameters of *A. hispidissima*. The present study aimed to standardize the plant via using powder microscopy, fluorescence analysis, TLC analysis and preliminary phytochemical screening.

MATERIALS AND METHODS

Chemicals: Chemicals and reagents utilized in this study were of analytical grade. DPPH (1,1-Diphenyl-2-picrylhydrazyl), $K_3[Fe(CN)_6]$ potassium ferricyanide, $FeCl_3$, trichloro acetic acid (TCA), gallic acid (GA) and ascorbic acid (AA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin-ciocalteus's phenol reagent and sodium carbonate were obtained from Merck (Damstadt, Germany) and other chemicals used in the study were obtained from BDH.

Plant material: The herbs of *A. hispidissima* were collected near Al-kharj Region of Saudi Arabia in the month of March, 2015. Using the morphological features the samples were authenticated by Dr. Osman Almekki and the voucher specimen from the plant was deposited in the Herbarium, College of Pharmacy (PSAU-CPH-2-2015) Prince Sattam bin Abdulaziz University, Al-Kharj, KSA. The whole plants were washed, shade dried and grinds to make powder and then it was labelled and stored for further studies.

Powder microscopy: Different types of non-glandular trichomes of *A. hispidissima* powder were observed by using microscope imaging software. Different cell components were observed and photography was done by using digital camera[14].

Fluorescence analysis: The fluorescence analysis of whole powdered *A. hispidissima* plant was carried out according to the method of Sriwastava *et al.*[15] with some modifications. The analysis was done by treating the plant powder with different solvents and reagents. After treatment, the sample solutions were examined under UV at short (254 nm) and long (312 nm) radiations. The colours obtained after treatment with different reagents under various radiations were observed[16].

Thin layer chromatographic analysis: TLC study of hexane extract of *A. hispidissima* was prepared and a small spot was made on a pre-coated silica gel 60 F 254 plates[17]. The solvent system hexane: Ethyl acetate (70: 30), was used for observing spots and calculating the retention factor (Rf) values.

Methanolic extract preparation: Powder of the whole *A. hispidissima* plant was macerated with methanol for 24 h and filtered by using filter paper (Whatman No. 1). The clear filtrate was dried using a rotary evaporator (Equitron, Roteva-8763 RV). Preliminary phytochemical screening, total phenol contents and *in vitro* antioxidant activity were preformed using the dry extract.

Preliminary phytochemical analysis: Screening of the methanol extract of *A. hispidissima* for various phytochemical constituents was conducted using reported methods[18].

Determination of total phenolic content (TPC): TPC of *A. hispidissima* plant was determined following the Folin-Ciocalteu method and gallic acid as standard compound[19]. All experiments were conducted in triplicates.

In vitro free radical scavenging activity

DPPH radicals scavenging assay: The DPPH assay as described by Hsu *et al.*[20] was conducted. Equal volumes of DPPH solution 0.1 mmol L^{-1} and various concentrations of *A. hispidissima* extract (2-10 mg mL^{-1}) were used. DPPH solution and methanol was used as control and solution of ascorbic acid was used as positive controls. The samples were measure spectrophotometrically at 517 nm to determine the reduction in the DPPH free radical. The experiment was conducted in triplicate. The inhibition percentage for scavenging DPPH free radical was calculated from the equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

Ac and As represents the absorbance of control and sample, respectively.

Reducing power activity: The reducing potential of the methanol extract was determined according as outlined by Oyaizu[21]. Ascorbic acid was used as reference standard. The test was performed in triplicate.

Statistical analysis: All experiments in the present study were performed in triplicate and the details of the results were presented as mean±standard error of mean (SEM). Free Graphpad prism version 7.01 and Microsoft Excel, 2007 were used for the graphical and statistical evaluations.

RESULTS

Powder microscopy: Microscopic observation of *A. hispidissima* powder indicated the presence of different types of covering trichomes such as pointed unicellular trichome, non-glandular, multicellular trichome, covering trichome with pitted wall, non-glandular unicellular trichomes with pitted wall, two celled, lance-shaped trichome and different sizes of unicellular, non-glandular trichomes (Fig. 1). Other microscopic characters such as bordered pitted vessel, small reticulately thickened vessel associated with sieve tissue, fibres and prismatic type of ca-oxalate crystal were seen in the powder (Fig. 2).

Fluorescence analysis: Results of the fluorescence analysis of *A. hispidissima* are given in Table 1. The powdered samples were treated with various solvents or reagents as distilled water, 1N NaOH in water, methanol, 1N NaOH in methanol, 1N HCl, 50% HNO_3, conc. HNO_3, 50% H_2SO_4, conc. H_2SO_4, 50% KOH and 0.01 M iodine solution. The fluorescence analysis of these mixtures was observed under visible light as well as UV light (254 and 312 nm). The powdered samples showed light green, light golden and light brown colors under visible light in treatment with water, 1N NaOH in water and methanol, respectively. Blue color was observed in 50% KOH and 0.01 M iodine solution under UV light of short wavelength. The powder showed dark brown color under UV light of short wavelength in 50% HNO_3 and conc. HNO_3 treatments. The fluorescence analysis of *A. hispidissima* powder showed dark brown colour under UV light of long wavelength when treated with 50% HNO_3, conc. HNO_3 and 50% H_2SO_4. Various colours like yellow, pink, violet and silver were also observed under different light conditions (Table 1).

Table 1: Fluorescence analysis of *A. hispidissima* powder

	Observation		
Solvents/reagents	Visible/day light	UV 254 nm (Short wavelength)	UV 312 nm (Long wavelength)
Powder	Green	Light blue	Light blue
Water	Light green	Sage green	Light sage green
1N NaOH in water	Light golden	Slate gray	Sky blue
Methanol	Light brown	Light pink	Pink
1N NaOH in methanol	Yellow	Pink	Brown
1N HCl	Amber	Violet	Navy
50% HNO_3	Pink	Dark brown	Dark brown
Conc. HNO_3	Pink rose	Dark brown	Dark brown
50% H_2SO_4	Creamy white	Light brown	Dark brown
Conc. H_2SO_4	Golden	White	White cream
50% KOH	Emerald	Blue	Silver
0.01 M iodine solution	Golden	Blue	Royal silver

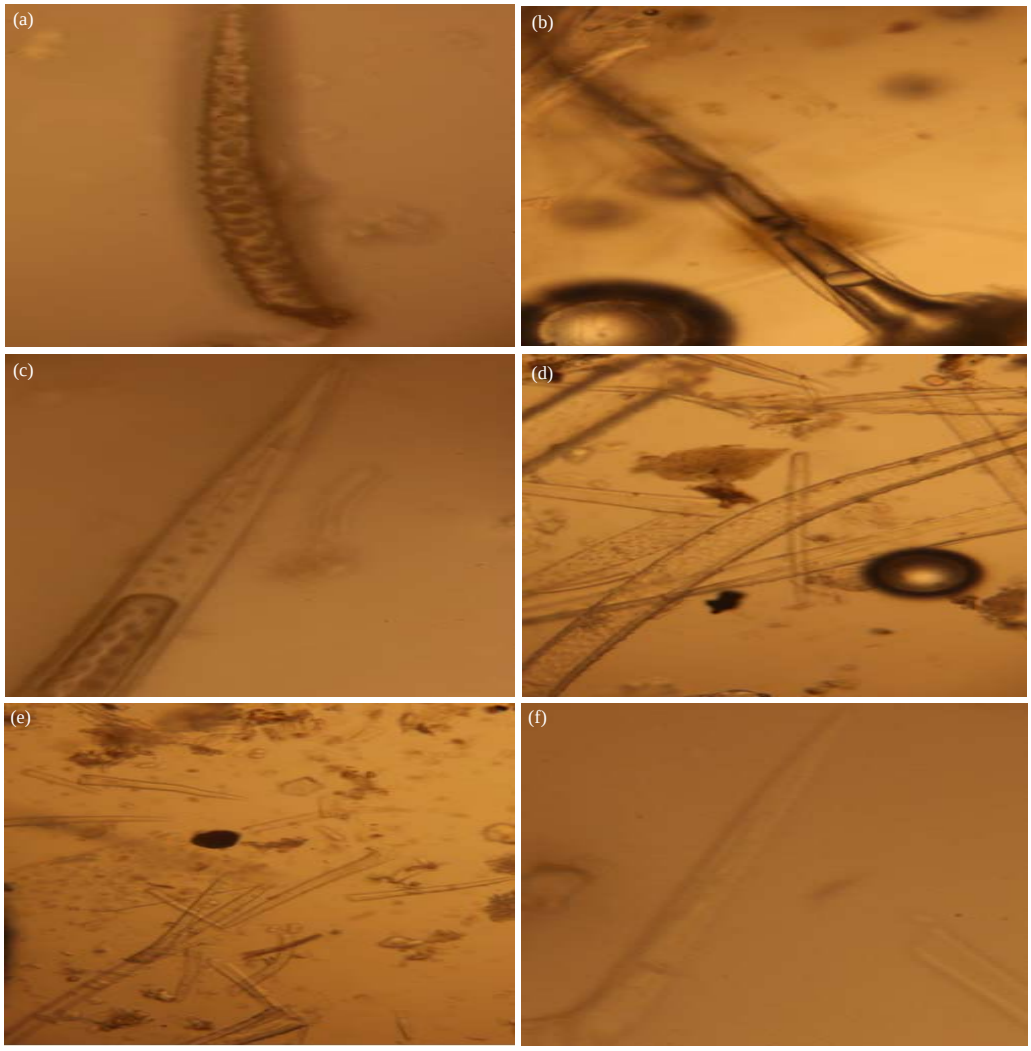

Fig. 1(a-f): Microscopic view of the different types of non-glandular trichomes of *A. hispidissima* plant aerial powder at ×20, (a) Pointed, unicellular trichome, (b) Non-glandular, multicellular trichome, (c) Covering trichome with pitted wall with pointed end, (d) Non-glandular, unicellular trichomes, with the pitted wall, (e) Different size of unicellular, non-glandular trichomes and (f) F: Two celled, lance-shaped trichome

Table 2: Thin layer chromatography analysis of hexane extract of *A. hispidissima* herb powder on Silica gel 60 F_{254} pre-coated sheets

Conditions	Number of spots	Hexane extract R_f value
Visual	8	0.16, 0.37, 0.47, 0.53, 0.63, 0.66, 0.79, 0.91
Short UV (254 nm)	6	0.09, 0.16, 0.37, 0.47, 0.53, 0.57
Long UV (312 nm)	8	0.09, 0.16, 0.25, 0.37, 0.47, 0.53, 0.57, 0.91
Ninhydrin-H_2SO_4	10	0.09, 0.16, 0.25, 0.47, 0.53, 0.57, 0.66, 0.79, 0.91,0.96

Solvent system Hexane: Ethyl acetate (7:3), average of n = 3

Thin layer chromatographic study: Several solvent systems, such as Ethyl acetate: Hexane (7:3), Methanol: Chloroform (1:9) and Methanol: Chloroform (1:1) was tested but TLC analysis of the hexane extract of *A. hispidissima* using Hexane: Ethyl acetate (70: 30) revealed the presence of several promising spots (Fig. 3). The hexane extract of *A. hispidissima* showed 8, 6, 8 and 10 distinct spots with visual, short UV, long UV and Ninhydrin-H_2SO_4, respectively. The R_f values of these spots were presented in Table 2.

Preliminary phytochemical screening: The results obtained from the phytochemical analysis of *A. hispidissima* methanol extract indicated the presence of alkaloids, carbohydrates, glycosides, saponins, steroids and

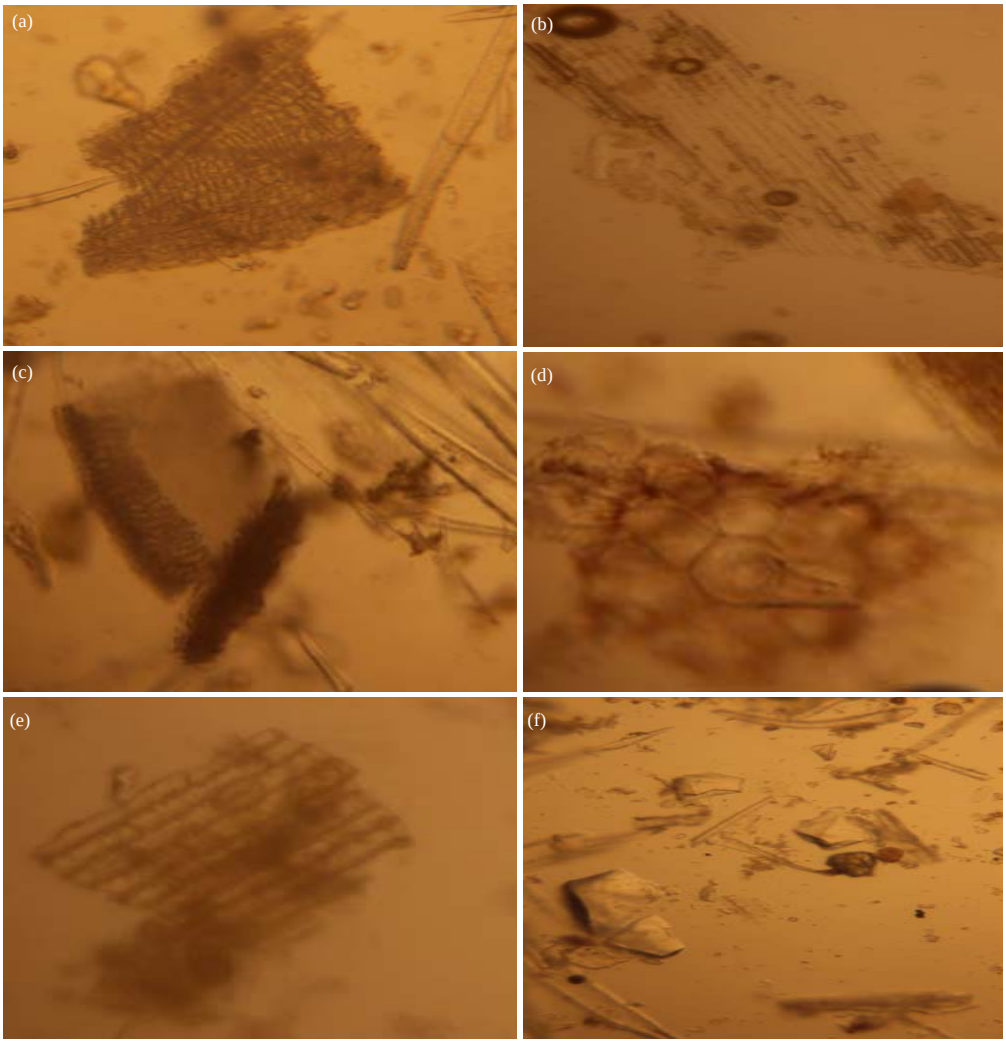

Fig. 2(a-f): Microscopic view of the different tissues of *A. hispidissima* plant aerial powder at ×20, (a) Part of a bordered pitted vessel, (b) Part of a group of fibres associated with underline parenchymatous cells, (c) A small reticulately thickened vessel associated with sieve tissue, (d) Fibres of leaf, (e) Part of a group of fibres and sclereids and (f) Ca-oxalate crystal prismatics

triterpenoids, phenols, phenolic compounds, tannins, flavonoids, proteins and amino acids (Table 3).

Determination of total phenolic content (TPC): The TPC of the methanol extract of *A. hispidissima* was found to be 65.62 ± 0.21 mg/100 g in term of gallic acid equivalent (Fig. 4). The linearity of the curve was good as expressed from the R^2 value of 0.9834.

In vitro free radical scavenging activity

DPPH radicals scavenging assay: DPPH free radical scavenging activity of the methanol extract of *A. hispidissima* is showed concentration dependent increase in the

Table 3: Preliminary phytochemical investigation of *A. hispidissima* methanolic extract

Test (Phytochemicals)	Results
Mayer's test (Alkaloids)	-
Dragendorff's test (Alkaloids)	+
Molisch's test (Carbohydrates)	+++
Fehling's test (Carbohydrates)	+
Modified borntrager's test (Glycosides)	+
Modified fehling's test (Glycosides)	++
Froth test (Saponins)	++
Salkowski's test (Steroids and Triterpenoids)	+
Ferric-chloride test (10%) (Phenols)	++
Folin-ciocalteu test (Phenolic compounds)	+++
Ferric-chloride test (1%) (Tannins)	+
Alkaline reagent test (Flavonoids)	+
Lead acetate test (Proteins and Aminoacids)	+
Ninhydrin test (Proteins and Aminoacids)	++

$^{-}$Absent, $^{+}$Trace, $^{++}$Moderate amount, $^{+++}$Significant amount

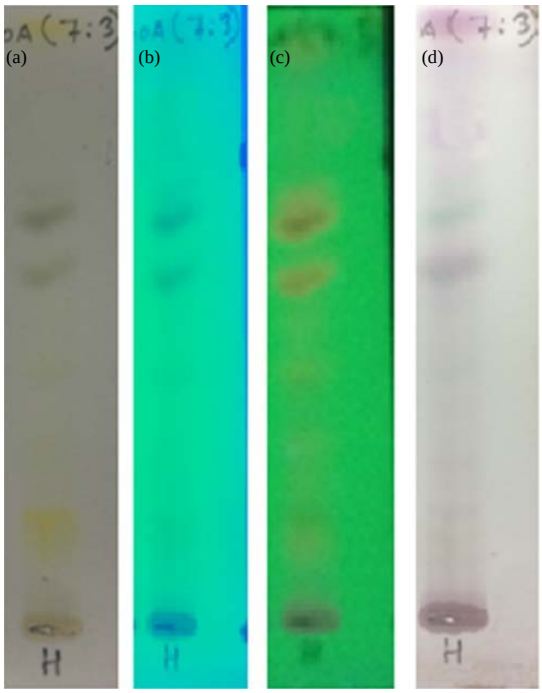

Fig. 3(a-d): Hexane extract chromatograms (a) Day light, (b) Short UV, (c) Long UV (d) After spraying ninhydrin in H_2SO_4

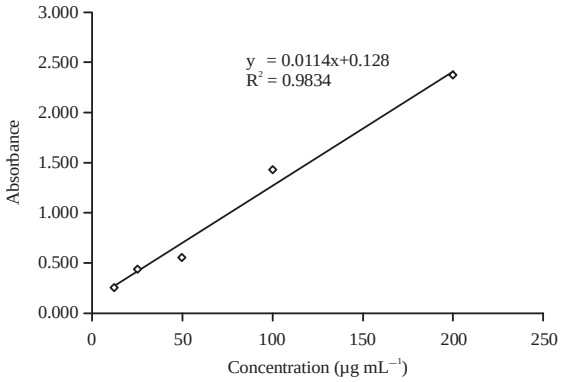

Fig. 4: Standard calibration curve of gallic acid, concentration y = 0.0114x-0.128, R^2 = 0.9834. Each point represents the mean of 3 experiments

percentage antioxidant activity at the used range (10-1000 g mL^{-1}) of the plant extract were tested. The percent inhibition was 26.33, 45, 62.33, 77 and 86%, for the concentrations 10, 50, 100, 500 and 1000 µg mL^{-1}, respectively (Fig. 5). The used standard ascorbic acid at concentrations of 10, 50, 100, 500, 1000 µg mL^{-1} exhibited a percentage inhibition of 60.18, 72.96, 85.97, 93.91 and 97.36, respectively.

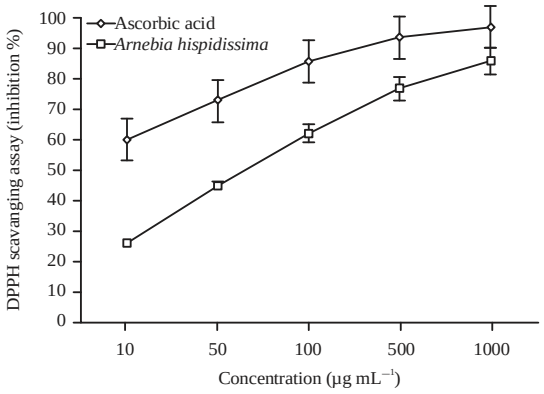

Fig. 5: *In vitro* free radical scavenging activity of *A. hispidissima* extract by DPPH radicals scavenging assay at different concentrations (µg mL^{-1})
Values were expressed as mean±standard deviation (n = 3)

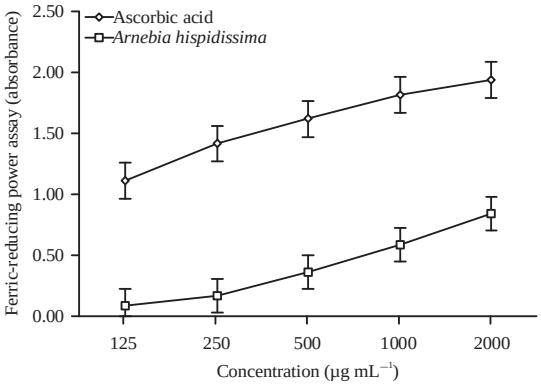

Fig. 6: *In vitro* free radical scavenging activity of *A. hispidissima* extract by reducing power activity at different concentrations (µg mL^{-1})
Values were expressed as mean±standard deviation (n = 3)

Reducing power activity: Results in Fig. 6 showed the reducing power of the methanolic extract of *A. hispidissima*. Both the methanol extract of *A. hispidissima* and standard exhibited a dose dependent reducing power. At 125, 250, 500, 1000 and 2000 µg mL^{-1} the absorbance obtained with the methanol extract of was 0.081, 0.162, 0.357, 0.581 and 0.836, respectively. At the same concentrations, ascorbic acid showed absorbances of 1.105, 1.412, 1.616, 1.811 and 1.936, respectively. Thus, the extract exhibited a lower reducing ability compared with the standard.

DISCUSSION

Standardization of the herbal material helps to ensure the identity, quality, purity and safety of drug for the human use.

Various parameters were studied such as powder microscopy, fluorescence analysis, thin layer chromatographic analysis, phytochemical analysis, total phenolic determination and *in vitro* free radical scavenging activity.

Powder microscopy: Microscopic analysis is one of the cheapest methods to correctly identify the particular drug and the surety of raw material. Microscopical studies of stem, leaf and root was helpful in the identification of these parts of *A. hispidissima*. Echinate hair walls granulate or even smooth hair wall, either needle-shaped or granulated wax deposits on their surfaces considerate as a common characteristic of the Boraginaceae family[22]. The present results showed that powder of *A. hispidissima* consists of pointed unicellular trichome, non-glandular, multicellular trichome, covering trichome with pitted wall and different sizes of unicellular, non-glandular trichomes. These features may be used as identification characters. The obtained powder microscopy results will provide a tool for the identity of *A. hispidissima* to maintain its quality and purity. Microscopic tool also enable the detection of any adulteration with other herbs.

Fluorescence analysis: Different chemical constituents present in the plant material are responsible for the fluorescence phenomenon. Fluorescence analysis is an important parameter of pharmacognostical evaluation[16]. Some secondary metabolites of the plants exhibit fluorescence in the visible region while others produce fluorescence when exposed to ultraviolet radiations. Some of the non-fluorescent materials are converted into fluorescent derivatives or decomposition products using different reagents. The results of fluorescent analysis of *A. hispidissima* powder showed characteristic coloration in treatment with various chemical reagents. These data were helpful for identifying and ascertaining the quality of the collected crude drug.

Thin layer chromatographic study: To support phytochemical screening, the methanol extract of *A. hispidissima* was subjected to TLC. Thin layer chromatography is one of the most common and important analytical tool for both qualitative and quantitative analyses of different classes of natural products[23]. It gives a preliminary view of secondary metabolite contents of the plants. In the present study, TLC analysis suggested the presence of different kinds of phytochemicals in *A. hispidissima* extract. TLC of the hexane extract of *A. hispidissima* revealed 6 and 8 spots under short and long UV, respectively after using of Hexane: Ethyl acetate (70: 30) as a solvent system. The reported spots were separated with enough space and having various R_f values showing the presence of 6-8 phytochemicals. Most of the compounds were visualized after derivatization on spraying with ninhydrin-sulphuric acid reagent (10 spots).

Preliminary phytochemical screening: The importance of plants as vital source of drug depends on their secondary metabolites[24]. Preliminary phytochemical screening gives information about the presence of plant metabolites with therapeutic activity.

The present results indicated that methanolic extract of *A. hispidissima* contained all analyzed phytochemicals. These natural compounds are of known biological activities in medicinal plants and most likely they were responsible for the therapeutic potential of the plants. The qualitative phytochemical screening revealed that alkaloids, carbohydrates, glycosides, saponins, steroids and triterpenoids, phenols, phenolic compounds, tannins, flavonoids, proteins and amino acids were present in the methanolic extract of *A. hispidissima*. In this respect, Liu *et al*.[25] mentioned that the roots of *A. hispidissima* contain naphthoquinones including derivatives of alkannin and shikonin. In addition, the flavonoid glycoside vitexin was also obtained from the fresh flowers of *A. hispidissima*[13].

The classes of phytoconstituents detected in the present study were known to have great value in human health. Alkaloids were of well-known medicinal importance including anticancer, antimalarial activities. They were also used to treat pain, inflammation, Parkinsonism, hypertension and other central nervous system disorders[26]. Plant phenols were reported to have the ability to quench oxygen-derived free radicals via donation of hydrogen atom or an electron to the free radicals. Due to the ability of tannins to cause protein precipitation they are used as astringent and possess anti-bacterial, anti-inflammatory and anti-oxidant activities[27]. The flavonoids are the most distributed class of plant phenols with potential antioxidant and anti-inflammatory activity in addition their use in treatment of various cardiovascular diseases due to capillary protective effect[28].

Total phenolics content: Due to the polyhydroxyl groups present in phenols they possess antioxidant and radical scavenging ability[29]. The current results indicated a considerable level of the TPC in the plant extract equivalent to 65.62 mg/100 g in gallic acid, which was lower than that

reported by Amarowicz *et al.*[30]. The polyphenol variation can be explained due to genetic, environmental factors and the extraction procedure[31].

***In vitro* free radical scavenging activity:** Several *in vitro* model systems like DPPH and reducing power activity have been used for assessing the scavenging activity of *A. hispidissima* extract.

DPPH radicals scavenging assay: DPPH radical scavenging assay is simple, rapid, sensitive and reproducible so it is commonly applied[32]. More reduction for DPPH reflects higher antioxidant activity. *A. hispidissima* extract showed a promising concentration-dependent DPPH scavenging effect. The percentage reduction ranged from 26.33 at 10 to 86.00 at 1000 μg mL^{-1}. *Arnebia* like *A. densiflora* and *A. benthamii* are reported to have antioxidant activity in support to the obtained results[33,34]. Phytoconstituents with antioxidant activity can neutralization free radicals (DPPH), either via transfer of hydrogen atom or by transfer of an electron[35]. *A. hispidissima* as a member of the family Boraginaceae known to produce anthraquinones, shikonins and alkannins is expected to have effective scavenging or chelating of superoxide radicals[34,36].

Reducing power activity: Compounds with reducing capacity will have potential antioxidant properties. Reducing power based on the reduction of potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) that in turn reacts with ferric-chloride to form the coloured product ferric-ferrous complex that has an absorption maximum at 700 nm. *A. hispidissima* extract showed marked reducing power ability in a concentration dependent manner. Reducing power of plants is dependent on their hydrogen-donating ability. A strong direct correlation is expected to excite between antioxidant activities and reducing power of plant extracts. Shikonin and its derivatives obtained from tissue cultures of *A. hispidissima* showed a significant antioxidant activity[37].

CONCLUSION

The present investigation including pharmacognostical and preliminary phytochemical studies of *A. hispidissima* provide pharmaco-botanical parameters that can be used as a diagnostic tool for the identification and determination of the quality and purity of the plant material. The obtained results could be helpful in further isolation and purification of medicinally important compounds.

SIGNIFICANCE STATEMENT

This study discovers the botanical and phytochemical characters of *A. hispidissima* that can be beneficial for the quality control of such important herb. This study will help the researcher in drug industry to assure the quality and purity of the entitled plant before utilization. It also correlates the traditional uses of the plant to the measured protective activities.

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