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Original Research

Authentication of Ethnoveterinary Important Grasses Through Microscopic Techniques: Insights Into the Anatomical and Phytochemical Analysis of Grasses

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Abstract:

Ethnoveterinary medicine is crucial in many rural areas of Pakistan, as residents in remote and marginal areas rely heavily on traditional herbal medicines to cure their domestic animals. Reactive oxygen species (ROS) formed spontaneously as by products of reactions with O_2 molecules, chemically damage the organic elements of the cell such as nucleic acids, proteins, and lipids. In the current research work, the anatomical, phytochemical and antioxidant of some ethnoveterinary grasses were studied. A total of four species were collected from different villages of Hafizabad during the months of April and May 2021. The collected species were identified as *Cenchrus setigerus* Vahl, *Diplachne fusca* (L.) P. Beauv., *Imperata cylinderica* (L.) Raeuschel and *Sporobolus coromandelianus* Kunth. For the anatomical studies, stems and leaves were cut into thin sections with a microtome. The anatomical characteristics observed were a compact epidermal layer, large cortical cells, thickened sclerenchyma, central and scattered vascular bundles, a large metaxylem, a small protoxylem, a pitted phloem, and a centrally located pith. To investigate the phytochemical and antioxidant potential, the crude methanol extract was prepared by maceration and subjected to fractionation with *n*-hexane, petroleum ether, chloroform, methanol, and water. *D. fusca* showed strong scavenging activity i.e. 75.87±0.14 of

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DPPH at 250 µL concentration. The total antioxidant activity evaluated by phosphomolybdenum activity showed the best results in the methanol fraction of *D. fusca*. At a concentration of 125 µL, the methanol extracts showed maximum reducing potential of 1.20±0.06. The methanol extracts of all grasses showed maximum ferric-reducing antioxidant potential except *I. cylinderica*, which showed a maximum potential of 1.77±0.054 TE µM/mL in chloroform extracts. The methanol extract of *I. cylinderica* had the highest TPC value of 118.32±1.27 (GAE) mg/mL. This study provides experimental confirmation that grasses, when consumed, can be used as natural antioxidants and can be used to combat various diseases caused by reactive oxygen species.

Keywords: ethnoveterinary, anatomy, antioxidants, phytochemicals, phosphomolybdenum

Introduction

In pastures, plains, and mountains, wild grasses play an important role in feeding grazing livestock [1, 2]. The domestic economy of Pakistan includes a significant contribution from livestock. Agriculture contributes to 21% of the country GDP, while livestock accounts for 11.9% of it; hence, the livestock industry subsidizes about 55.52% of the agricultural sector [3]. Due to its high dependence on agriculture and livestock, Pakistan is the third largest milk producing country in the world [4]. The source -poor farmers of Pakistan rely heavily on traditional medicine due to limited access to modern preventive health practices and lack of modern health facilities in their areas [5]. The term "ethnoveterinary medicine" is widely used to refer to traditional knowledge, practices, philosophies, and methods of caring for animal health and treating various diseases [6]. Due to the innovation of certain active ethnoveterinary medicine products in recent years, ethnoveterinary medicine practices have increased significantly [7]. According to Aziz et al. [8], the lack of appropriate animal husbandry techniques causes about 30–35% of losses in emerging countries where rural residents are particularly dependent on livestock for their livelihood [9].

According to the studies of Chauhan et al. [10] and Dhama et al. [11], oxidative stress increases the susceptibility of dairy cattle to disease. Cattle diseases are related to alterations in oxidative metabolism that occur mainly during the transition period (during the transition from late pregnancy to the beginning of lactation). Apart from the costs associated with treatment, diseases at this stage have a significant negative economic impact on the livestock industry [12]. This is because affected cows are unable to produce their maximum milk yield. The increased formation of free radicals, typically generated in the mitochondria as a result of cellular respiration during the electron transport chain reaction, leads to oxidative stress [13]. Another effect of over production of reactive oxygen species (ROS) is oxidative stress, which is known to be a factor contributing to dysfunctional inflammatory responses. Antioxidants help to lower the energy level of existing free radicals, reduce the number of free radicals generated in the body, and stop oxidation chain reactions to reduce the extent of free radical-induced damage [14]. They achieve this by oxidizing themselves. The term "antioxidant" has been defined in various ways, such as substances that in small amounts can significantly slow or prevent the oxidation of easily oxidizable substances [15] or that can change reactive species into non-reactive species [16]. According to Rafiq et al. [17], antioxidants are also called reducing agents. In addition to natural antioxidants, various synthetic antioxidants such as butylated hydroxytoulene (BHT) and butylated hydroxyanisole (BHA) have also been identified. However, since these synthetic antioxidants carry a higher risk of adverse effects, research on natural antioxidants is crucial [18]. Natural antioxidants have attracted much attention in preventive medicine in recent years [19]. A considerable number of antioxidants are produced by plants, and they may also be a source of novel molecules with antioxidant capabilities [20].

Poaceae-related plant species are the most important component of agricultural crops and livestock feed, as well as one of the most important sources of employment and income for many rural dwellers throughout the world [21]. Globally, grasses are used by rural people as feed for domestic animals and as a remedy for human and animal diseases [22]. Poaceae, also known as the family of grasses, is the fifth-largest family of flowering plants, with 6,000 genera, 10,000 species, and about 50 tribes [23]. The Poaceae, of which there are 492 species and 158 genera in Pakistan [23, 24], have no conceivable medical uses. Due to the presence of several biologically active elements, grasses play an important role in traditional health care [25]. According to El-Shahir et al. [26], phytochemicals are naturally occurring chemical compounds found in medicinal grasses, especially in the leaves and roots, which act as defense mechanisms and protect animals from various diseases. Certain plants of the Poaceae family are used as medicines for hypertension, diabetes, inflammation, anthelmintics, ulcerative colitis, diuretics, and antioxidants [27]. Ethnoveterinary grasses contain essential elements such as vitamins A, C, and E, flavonoids, lignin, and tannins, which are phenolic compounds, These compounds have antioxidant potential and protect cells from the destructive effects of ROS reactive oxygen species [28].

C. setigerus is a perennial erect grass with a growth height of 25-40 cm, belonging to the tribe Paniceae. The grass can be found almost everywhere. A highly

Table 1. Classification of grasses their tribes and localities.

S. No	Name of species	Tribe	Location	Collection date
1.	*Cenchrus setigerus* Vahl	Paniceae	Village Wingay, Distt. Hafizabad.	4/5/2021
2.	*Diplachne fusca* (L.) P. Beauv.	Eragrosteae	Village Bawray, Distt. Hafizabad	11/4/2021
3.	*Imperata cylinderica* (L.) Raeuschel	Andropogoneae	Village Prem kot, Distt. Hafizabad	5/4/2021
4.	*Sporobolus coromandelianus* (Retz.) Kunth	Sporoboleae	Village Kila ramkor Distt. Hafizabad	15/5/2021

nutritious grass, *C. setigerus* is valued for producing edible fodder and for irregular grazing during dry periods in the tropics [29]. According to Jabeen et al. [30], the grass is reported to increase milk production in cattle and give them a smooth, supple appearance.

S. coromandelianus is an annual grass belonging to the tribe Sporoboleae. However, it is mainly found in India, Pakistan, and South Africa [31]. The grass is important from an ethnobotanical point of view because it is excellent fodder for sheep, goats, and cattle. The considerable antioxidant potential of *S. coromandelianus* was described by Maithani et al. [32].

D. fusca, a halophytic species (tribe Eragrosteae), occurs in Pakistan and shows the greatest adaptations to salinity [33]. In the saline regions of Pakistan, *D. fusca,* sometimes known as saltgrass or kallar grass, is a common plant [34]. It is a perennial grass with height up to 1.5 meters. This grass is used as cow fodder. No ethnopharmacological studies have been conducted, but similar studies have been conducted on various Diplachne species.

I. cylindrica is a perennial rhizomatous grass belonging to the Poaceae family. It is distributed mainly on grasslands and soils. The grass is used as animal feed and has been shown to be useful in treating inflammation and fungal diseases in animals, making it relevant from an ethnobotanical perspective [35]. *I. cylindrica* frequently exhibits beneficial biological properties, according to several studies [36]. The present study was carried out considering importance of the grasses in ethnoveterinary medicine. The grasses were collected from four different villages of Hafizabad Distict in Punjab province and analyzed anatomically and phytochemically. The antioxidant activities and phytochemical potential such as tannins, saponins, and terpenoids, were evaluated.

Materials and Methods

Study Area Details

The study area is located in central Punjab, Pakistan, and is known as the “City of Rice” because of its agricultural rice production. It is located between 32 -20’ north latitude and 73 46’ east longitude. The area is 240 meters (800 feet) above sea level. The northern and northwestern boundaries of the district are formed by the Chenab River. The research area covers 2,367 km², and the total forest area of the district is about 550.4 ha (0.23%) of this area. This region has a semi-arid climate with year-round temperatures ranging from 48°C in summer to 1°C in winter. According to Altaf et al. [37], the annual rainfall ranges from 50 to 70 mm and the relative humidity ranges from 25 to 85%. According to Punjab development statistics 1,156,954 people lived in the study region in 2017, of which 52% were men and 48% were women. The population is divided into rural areas (72.74%) and urban areas (27.26%). Most of the country’s inhabitants raise livestock for subsistence, such as bulls for plowing. Cows, buffaloes, sheep, and goats also provide milk, butter, curd, and meat. Locals in this region treat a variety of animal diseases with different plants.

Collection and Identification of Plant Samples

Fresh plant samples of the species *C. setigerus, D. fusca, I. cylinderica,* and *S. coromandelianus* were collected from four villages of Hafizabad District Punjab Pakistan, during April and May 2021. The local names of the plants were first noted after they were photographed. Preliminary identification and authentication of the specimens were made using the available literature. Specimens were given botanical names and family names, which were then listed alphabetically (Table 1). The correctly identified specimens were deposited in the herbarium of the Department of Botany, Government College University, Lahore, Punjab, Pakistan.

Sectioning and Staining Procedure

The substance was fixed in a formaldehyde-acetic alcohol solution (formaldehyde 10%, acetic acid 5%, ethanol 50%, and distilled water 35%) for 48 h before being transferred to an acetic alcohol solution for long-term storage. Thin pieces of the different plant parts were cut using industrial tools. Methanol blue solution was used to stain the thin pieces. Measurements were made with an ocular micrometer adjusted with an object micrometer under a light microscope (Nikon 104, Japan). A stereomicroscope and a digital camera were used to photograph the stained pieces. A variety of parameters, including leaf thickness, size of dermal

Table 2. Different reactions used to determine the presence of some chemicals in grass species.

Secondary metabolite	Name of test	Reactants	Expected result
Phenols	Ferric chloride test	1 ml of the filtrate + 1 ml of 1% $FeCl_3$	Appearance of blue or green color
Flavonoids	Alkaline reagent test	5 ml of the filtrate + equal volume of 20% NaOH	Appearance of yellow color
Alkaloids	Mayer's test	2 ml of the filtrate + 2 ml of 1% HCl + kept in boiling water bath for 5min + 6-7 drops of Mayer's Wagner's reagent added to the filtrate	Appearance of brown/ orange/ red/ creamish precipitates
Cardiac glycosides	Keller-Killiani test	2 ml of the filtrate + 1 ml of glacial acetic acid + 3-4 drops of 5% $FeCl_3$ + 1m of conc. H_2SO_4 was added carefully to the solution	Development of brown ring at the interface or the appearance of violet color
Carbohydrates	Molisch's test	2 ml of the filtrate + 2 ml of Molisch's reagent and shaken vigorously + 2 ml of conc. H_2SO_4 was added carefully along the wall of the test tube	Appearance of reddish ring at the junction of two liquids
Reducing sugars	Fehling's test	1 ml of the filtrate + 2 ml of Fehling's solution (A: 7% $CuSo_4$ in distilled water containing two drops of dil. H_2SO_4), B: 12 % KOH and 35 % sodium potassium tartrate in distilled water. Mixed A and B in equal amounts) and boiled for 5 mins	Appearance of brick red precipitate
Proteins	Burette test	2 ml of the filtrate + 1 ml of 40% NaOH + 1-2 drops of $CuSO_4$ was added slowly to the solution	Appearance of violet color showed the presence of peptide linkages in the solution
Tannins	Ferric chloride test	2 ml of filtrate+ 1 ml of 5% $Fecl_3$	Yellow brown precipitates
Saponins	Froth test	0.5 ml of the filtrate + 0.5 ml of the distilled water and shaken vigorously for about 30 seconds	Formation and perseverance of froth
Amino acids	Nimhydrin test	5 ml extract+ few drops of Ninhydrin reagent	Formation of purple color

tissue, vascular tissue, parenchyma, and mechanical tissue, were recorded in the data [38-44].

Phytochemical Studies

The collected grasses were thoroughly cleaned to remove soil fragments and debris that had attached to them, and then dried in the shade at room temperature for another fifteen days. To prepare the dried material for future analysis, it was finely ground and pulverized before being placed in air tight containers.

Extraction Procedure

The fine powder was soaked in methanol and kept in a cabinet (at room temperature) for 8 days to prevent or reduce light penetration. At least twice a day, the mixtures were shaken manually to accelerate the process. The mixture was then evaporated to dryness in a rotary evaporator after being filtered through a Whatman filter paper No. 1. A portion of the resulting extract was stored in a sterile container in a cool, dark place for future study. The remaining crude extract was divided among water, chloroform, ethyl acetate, and methanol using the liquid-liquid fractionation method [45] For further test trials, these sub-fractions were also stored individually in sterile vials in a cool, dark place.

Primary Phytochemical Tests

To define the chemical profile of plant extracts, various qualitative tests are performed. To confirm the presence of secondary metabolites, preliminary phytochemical tests of the plant extracts have been performed using various protocols [46-47] (Table 2).

Antioxidant Studies

DPPH Radical Scavenging Activity

The assay was performed according to the method described by Chu et al. [48]. Different amounts of standard or crude extract (60 µL, 80 µL, 100 µL, 150 µL, and 250 µL) were mixed with 3 mL of methanol solution of DPPH (0.1 mM) and 1 mL of distilled water. After thoroughly shaking the reaction mixture, it was allowed to stand at room temperature for an hour.. Then the absorbance was measured at 517 nm using methanol as a blank in the spectrophotometer. The free radical scavenging activity increased as the absorbance of the reaction mixture decreased. The following formula was used to determine the percentage of DPPH discoloration in the samples:

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

Table 3. Length and width of different parameters of stem section of the grasses.

Grass	Parameters	Epidermal cells	Cortical cells (large)	Cortical cells (small)	Vascular bundles	Metaxylem	Protoxylem	Phloem
Cenchrus setigerus	Length	0.11±0.01	1.1±0.05	0.6±0.05	1.18±0.16	0.66±0.16	0.25±0.02	0.12±0.02
	Width	0.17±0.03	0.96±0.12	0.7±0.07	1.16±0.03	0.4±0.02	0.17±0.01	0.09±0.006
Diplachne fusca	Length	0.26±0.03	1.06±0.08	0.8±0.05	1.35±0.08	0.35±0.02	0.28±0.01	0.41±0.04
	Width	0.33±0.003	1.05±0.05	1.01±0.12	1.23±0.03	0.36±0.03	0.33±0.01	0.36±0.03
Imperata cylinderica	Length	0.15±0.05	0.13±0.03	0.9±0.11	1.29±0.04	0.55±0.02	0.22±0.03	0.45±0.03
	Width	0.18±0.03	1.20±0.1	0.60±0.04	1.43±0.01	0.42±0.06	0.30±0.03	0.44±0.05
Sporobolus coromendelianus	Length	0.15±0.05	1.13±0.03	0.9±0.11	1.29±0.04	0.55±0.02	0.22±0.03	0.45±0.03
	Width	0.18±0.03	1.20±0.1	0.60±0.04	1.43±0.01	0.42±0.06	0.30±0.03	0.44±0.05

Where, A control = DPPH solution absorbance A sample = Absorbance of sample

Total Antioxidant Activity by Phosphomolybdenum Method

The phosphomolybdenum complexation method of Prieto et al. [49] was used to evaluate, the total antioxidant activity of the different fractions. When MO (VI) is reduced to MO (V), a complex is formed, which is noticeable by the appearance of a green color. Briefly, 125, 250, and 500 µg mL^{-1} of each crude extract were added to 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). 4 mL of the reagent solution formed the blank solution. The vials were capped, and heated to 95°C in a water bath for 90 minutes. After cooling to room temperature, the absorbance of the mixture at 695 nm was calculated and compared with the control. The antioxidant activity is expressed as a ratio to that of BHT.

Determination of Total Phenolic Contents

The total soluble phenolic content of each extract was calculated using a slightly modified version of the spectrophotometric test previously described by [50]. Briefly, 1 mL of each grass extract was mixed with 9 mL of distilled water, combined with 1 mL of the Folin-Ciocalteu phenol reagent, and then 10 mL of Na_2CO_3 was added. The mixture was allowed to rest at room temperature. A UV-visible spectrophotometer was used to determine the absorbance at 725 nm after 40 min. Using a standard calibration curve prepared for various amounts of gallic acid, total phenolics are presented as micrograms of gallic acid equivalents (GAE) per gram of material.

Ferric Reducing Antioxidant Power (FRAP) Assay

The instructions of Benzie and Strain [51] for the FRAP assay were followed with some minor adjustments. Stock solutions contained 20 mM ferric chloride hexahydrate solution and 300 mM acetate buffer (pH 3.6) and 10 mM TPTZ solution in 40 mM hydrochloric acid. Acetate buffer, TPTZ solution and ferric chloride hexahydrate solution were combined to prepare the fresh working solution, which was then warmed to 37 °C before use. Trolox and grass sample solutions were both prepared in methanol (250 µg mL^{-1}). Separate test tubes containing 10 mL of each crude extract were filled with 2990 mL of the FRAP solution to give a final volume of 3 mL. The solution FRAP had time to react with the crude extracts of the plants. The absorbance of the colored product (Ferrous Tripyridyltriazine complex) was checked at 593 nm. The results are expressed in µmol TE mL^{-1}.

Statistical Analysis

The results of each experiment were reported as mean values, and each experiment was performed in triplicate. Data were collected and subjected to analysis of variance, and means were related using the Duncan's multiple range test.

Results

Anatomical Studies

Environmental changes have little effect on the anatomical characteristics of a plant. Plant species, genera, and families have been identified using anatomical information. It is often used in systematic identification because it provides a better classification position for aberrant groupings and reveals relationship patterns that may not have been fully conveyed by morphological characters [52-55]. Four species of

Table 4. Length and width of different parameters of leaf section of the grasses.

Grass	Parameters	Epidermal cells	Sclerenchyma cells	Bundle sheath cells	Mesophyll cells	Bulliform cells	Xylem	Phloem
Cenchrus setigerus	Length	0.53±0.21	0.35±0.07	0.75±0.05	1.31±0.38	1.23±0.28	0.35±0.02	0.09±0.00
	Width	0.58±0.10	0.18±0.01	0.56±0.03	1.9±0.11	0.76±0.03	0.31±0.01	0.11±0.02
Diplachne fusca	Length	0.43±0.03	0.30±0.05	0.46±0.03	1.33±0.17	1.00±0.01	0.18±0.01	0.33±0.03
	Width	0.33±0.02	0.26±0.03	0.46±0.06	1.23±1.17	0.76±0.03	0.28±0.01	0.20±0.03
Imperata cylinderica	Length	0.48±0.04	0.25±0.03	0.57±0.03	1.25±0.17	0.77±0.01	0.19±0.05	0.23±0.06
	Width	0.36±0.05	0.69±0.43	0.50±0.15	1.13±0.07	0.66±0.04	0.38±0.21	0.23±0.05
Sporobolus coromendelianus	Length	0.48±0.04	0.25±0.03	0.57±0.03	1.25±0.17	0.77±0.01	0.19±0.05	0.28±0.06
	Width	0.36±0.05	0.69±0.43	0.50±0.15	1.13±0.07	0.66±0.04	0.38±0.21	0.23±0.05

Table 5. Detection of phytochemical compounds in Methanol extracts of the grasses

Phytochemicals	Plant Taxa			
	Cenchrus setigerus	Diplachne fusca	Imperata cylinderica	*Sporobolus coromendelianus*
Alkaloids	-	-	-	-
Carbohydrates	-	-	+	-
Cardiac Glycosides	+	-	-	-
Flavonoids	+	-	-	-
Phenols	+	++	++	+
Proteins	+	-	+	+
Reducing Sugars	+	+	+	+
Saponins	+	+	+	+
Tannins	-	+	-	+

ethnoveterinary grasses were examined for their anatomical characteristics. Epidermis and basal tissues accounted for most of the transverse section of the stems of four grasses. Because the vascular bundles were scattered, the ground tissue was not divided into cortex and pith. The primary ground tissue was parenchymatous, while the central sections had larger cells. The ground tissue consisted of many elliptical to radially elongated vascular bundles that formed the vascular tissue. The stem sections of all grasses are shown (Fig. 1). The peripheral vascular bundles in *C. setigerus* and *I. cylinderica* were tiny in size but numerous. The core vascular bundles were limited in number, relatively thinly dispersed, and of large size. The vascular bundles of all grasses had xylem and phloem in the same line, no cambium and phloem extending just outside the xylem. Each vascular bundle was surrounded by a single bundle sheath of parenchymatous character. Larger metaxylem vessels were seen in the xylem, surrounded by smaller protoxylem. The phloem was visible above the metaxylem vessels. Thick walled sclerenchyma cells could be seen above the phloem. Table 3 compares the dimensions of the length and width of the stem. The epidermis, mesophyll cells, vascular bundles, and sclerenchyma tissue were present in the T.S of the leaves of all four grass species (Fig. 2). All four species had bulliform cells in their intercostal areas. With the exception of *S. coromandelianus*, in which the conducting bundles were of equal size, the leaves generally had vascular bundles of different diameters. Larger and smaller vascular bundles were observed in *C. setigerus* and *I. cylinderica*, respectively. Protoxylem and metaxylem arteries were found in vascular bundles. Well-developed phloem and sclerenchyma cells were seen around the vascular bundles. The mesophyll tissue shows no cellular differentiation between palisade and spongy tissues and consists of parenchymatous cells with extensive intercellular gaps. Table 4 compares the dimensions of length and width of the stem.

Phytochemical Assessment

Phytochemistry, sometimes known as plant chemistry, deals with the wide range of organic compounds that plants acquire [46, 47]. According to the findings of phytochemical study of grasses,

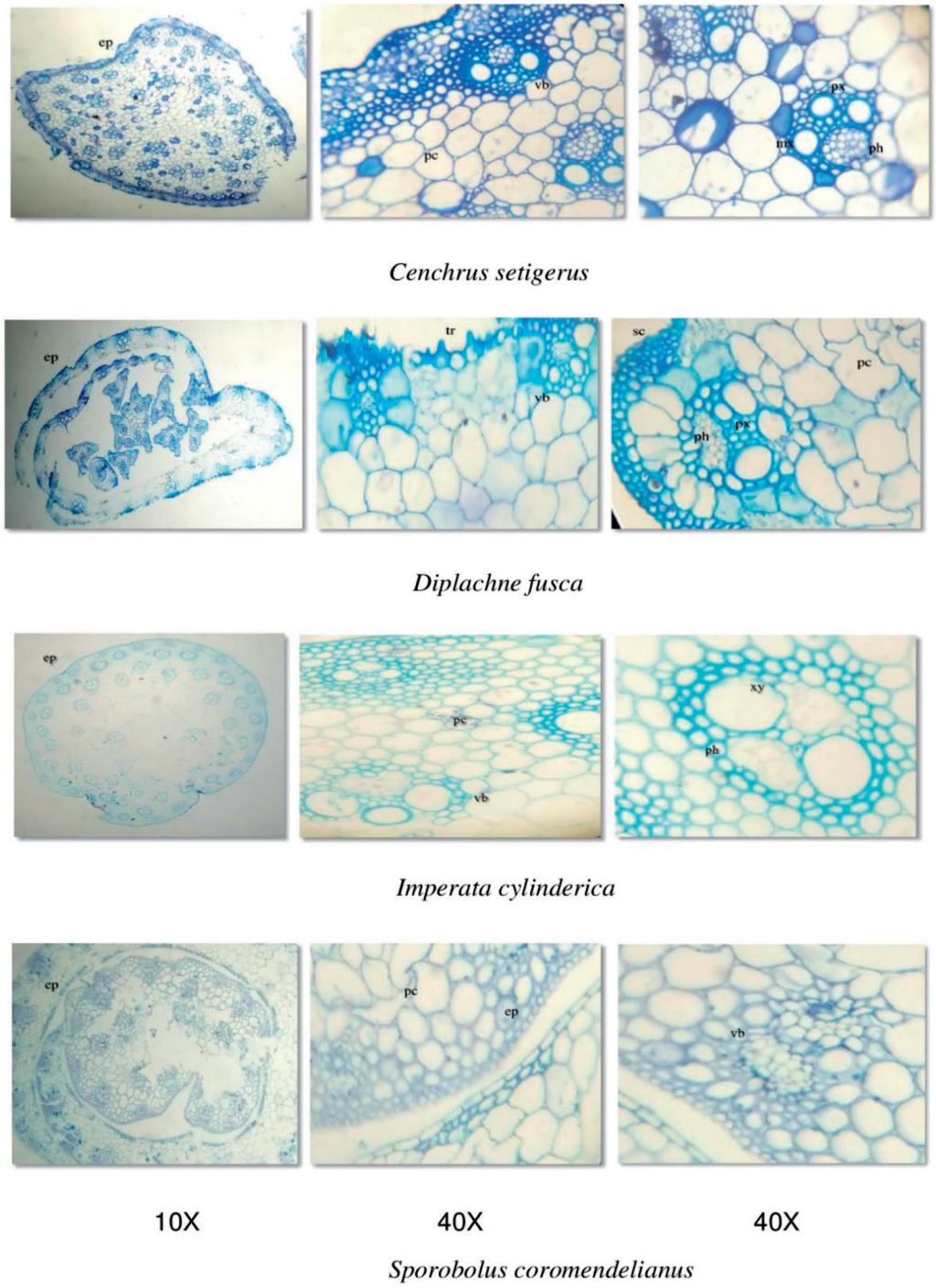

Fig. 1. T.S of Stem sections of all the grasses. The first image of all the grasses show the stem sections at 10X, the scattered vascular bundles are seen in the section. The second image shows the cortical cells and hypodermal vascular bundles and parenchyma cells at 40X. In the third image, vascular bundles are seen; Bundle sheath cells, large sized metaxylem surrounded by small sized protoxylem and sieved phloem.

carbohydrates, phenols, reducing sugars, saponins, tannins and cardiac glycosides were found. Phenols and saponins were found in all four grasses. With the exception of *D. fusca,* the Burette test to determine protein content was positive. The Molisch test to determine carbohydrates was positive only in *I. cylinderica*, *while C. setigerus, S. coromandelianus*, and *D. fusca* had no significant carbohydrate value. The content of cardiac glycosides was detected only in *C. setigerus*, while the other three grasses from the study area did not have significant content of cardiac glycosides. The results of the for alkaline reagent test performed to determine flavonoids were the same as the content of cardiac glycosides. The Mayer test performed several times to detect alkaloids showed that all grasses had no alkaloid content. The results of the phytochemical analysis are shown (Table 5).

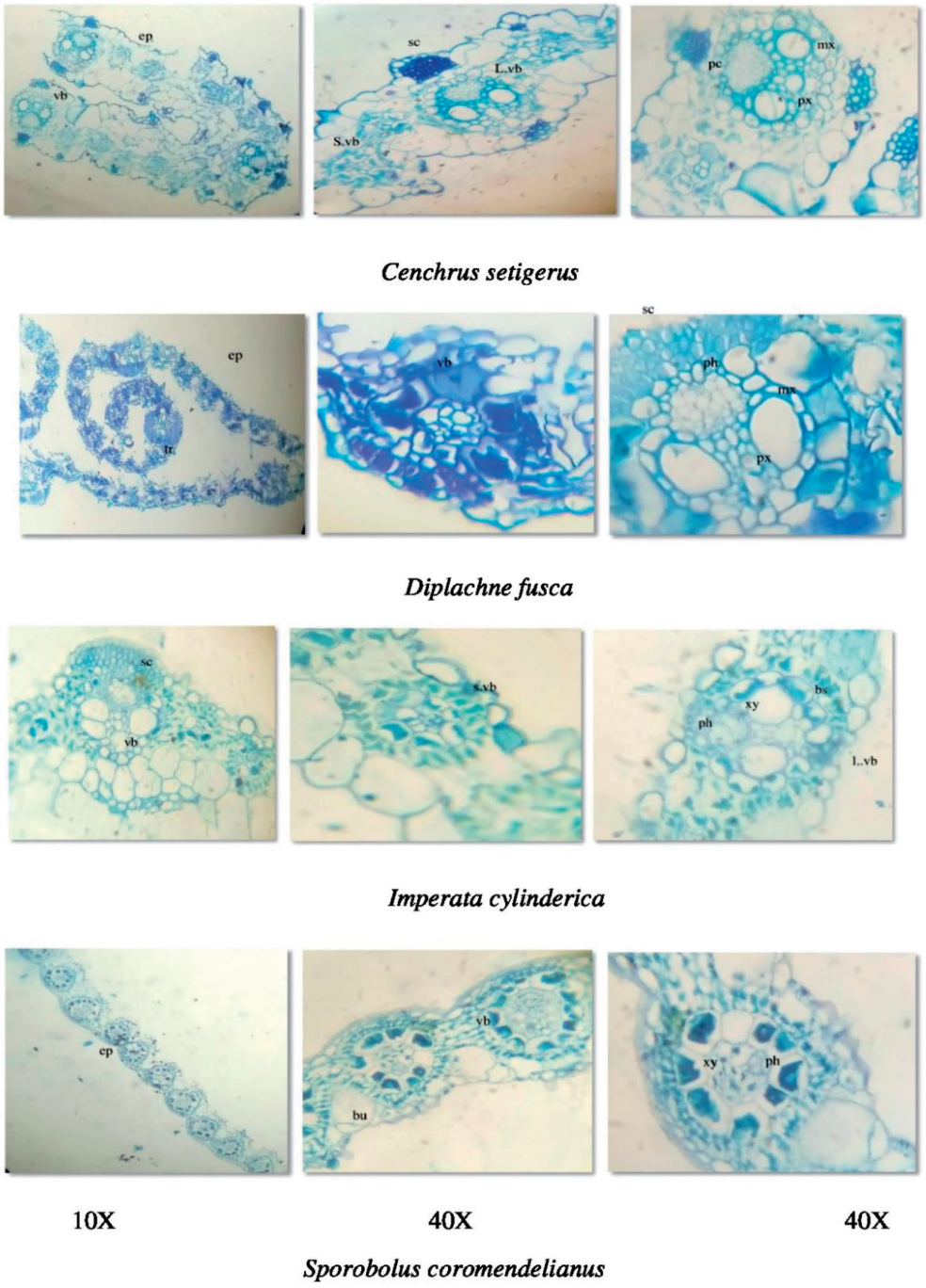

Fig. 2. T.S of Leaf sections of all the grasses. The first image of all the grasses shows the leaf sections at 10X, in which the epidermal cells and trichomes are seen. The second image at 40X shows the Bulliform cells and sclerenchyma cells in C. setigerus, D. fusca and S. coromendelianus and smaller vascular bundles in I. cylinderica and third image displays the clear view of vascular bundles showing bundle sheath cells surrounding the large sized metaxylem cells and small sized protoxylem and sieved phloem.

Antioxidant Activities

Scavenging of DPPH Radicals

Antioxidant properties of plants have recently attracted great interest worldwide. Plant antioxidants serve as a natural reservoir of for bioactive chemicals. They are essential for plant acclimation and adaptation to environmental obstacles, but are also beneficial for human health [56, 57]. The most absorbent free radical at 515-517 nm is DPPH, a stable hydrophilic radical. DPPH transforms into the colorless chemical hydrazine after receiving electrons from reducing substances such as phenols. Absorbance is reduced by this structural modification. According to Kim et al. (2019), antioxidants are substances that possess this ability. After the reduction of DPPH, a decrease in absorbance at 517 nm was observed. Table 6 shows the percentage free radical scavenging of the different fractions at different concentrations of the four grasses *C. setigerus, D. fusca,*

Table 6. Percentage % DPPH free radical scavenging activity of different solvent extracts of the grasses.

Sr. No.	Solvent	Conc. (µg/ml)	% Scavenging *(C. setigerus)*	% Scavenging *(D. fusca)*	% Scavenging *(I. cylinderica)*	% Scavenging *(S. coromandelianus)*
1.	Methanol	20	32.28±0.39	42.38±0.17	52.78±0.42	41.42±0.53
		60	41.96±0.50	55.62±0.24	44.68±0.27	53.4±0.76
		80	53.51±0.31	63.93±0.73	50.93±1.22	61.43±0.31
		100	51.76±0.54	60.44±0.67	53.17±0.08	58.09±0.86
		150	52.12±0.14	61.35±0.21	62.39±0.67	69.37±0.01
		250	51.17±0.24	75.87±0.14	63.22±0.07	65.58±0.57
2.	N-hexane	20	29.15±0.18	39.05±0.07	42.22±0.75	51.49±0.47
		60	32.96±0.18	42.18±0.20	39.21±0.48	45.89±0.02
		80	35.10±0.14	45.32±0.39	52.55±1.24	47.37±0.86
		100	37.85±0.11	44.61±0.16	53.12±0.49	52.51±0.04
		150	31.40±0.69	47.31±0.20	60.97±0.96	44.18±0.21
		250	37.01±0.09	49.30±0.33	63.47±0.56	60.53±0.53
3.	Chloroform	20	25.82±0.09	34.05±0.61	47.91±0.76	56.02±0.05
		60	22.55±0.18	47.12±0.60	52.88±0.07	65.76±0.88
		80	37.91±0.07	54.06±0.76	50.44±0.13	63.92±0.62
		100	28.60±0.19	66.35±0.63	40.85±0.09	64.98±1.22
		150	40.40±0.06	65.44±0.66	46.48±0.16	72.49±0.23
		250	54.14±0.28	72.96±0.07	50.83±0.13	74.49±0.14
4.	Aqueous Solution	20	18.40±0.04	39.36±0.04	63.69±0.24	56.50±0.43
		60	20.41±0.07	38.84±0.22	70.60±0.30	58.76±0.18
		80	30.96±0.15	52.91±0.51	58.11±0.22	54.04±0.08
		100	32.66±0.55	42.35±0.69	63.94±0.19	49.54±0.77
		150	32.66±3.56	47.87±0.39	65.31±0.06	54.38±0.28
		250	29.32±0.18	55.86±1.38	67.02±0.15	60.58±0.85
BHT	Standard		77.3±0.7	77.3±0.7	77.3±0.7	77.3±0.7

I. cylinderica, and *S. coromandelianus*. The methanol extract of stem and leaves of *D. fusca* and leaves showed the highest scavenging activity at a concentration of 250 µL, i.e. 75.87±0.14 while the aqueous solution extract of *C. setigerus* showed the lowest scavenging activity at a concentration of 20 µL, i.e. 18.40±0.04. The percentage of DPPH was found to be concentration-dependent, i.e. the higher the concentration, the greater the percentage of inhibition (Fig. 3).

Total Antioxidant Activity

Antioxidant activity has been demonstrated in a variety of herbs, including grasses and various Indian and Chinese plants. The flavones, isoflavones, flavonoids, anthocyanins, coumarin lignans, catechins, and isocatechins are responsible for most of the antioxidant activity [58]. The technique is based on the fact that antioxidants reduce molybdenum (VI) to molybdenum (V), which leads to the formation of a green phosphate Mo (V) complex at acidic pH. This assay involves electron transfer, which depends on the structure of the antioxidant [59]. The results of the comparison of TAA with the reference antioxidant BHT are shown in Table 7. The results show that the methanol fraction of *D. fusca* has the best activity at a concentration of 125 µL i.e. 1.20±0.06, and the *n*-hexane extract has the lowest potential at a concentration of 125 µL, i.e., 0.27±0.04. BHT, a standard with total antioxidant activity of 11.22±0.09, was used to compare the results. Fig. 4 shows the antioxidant activity of the different fractions.

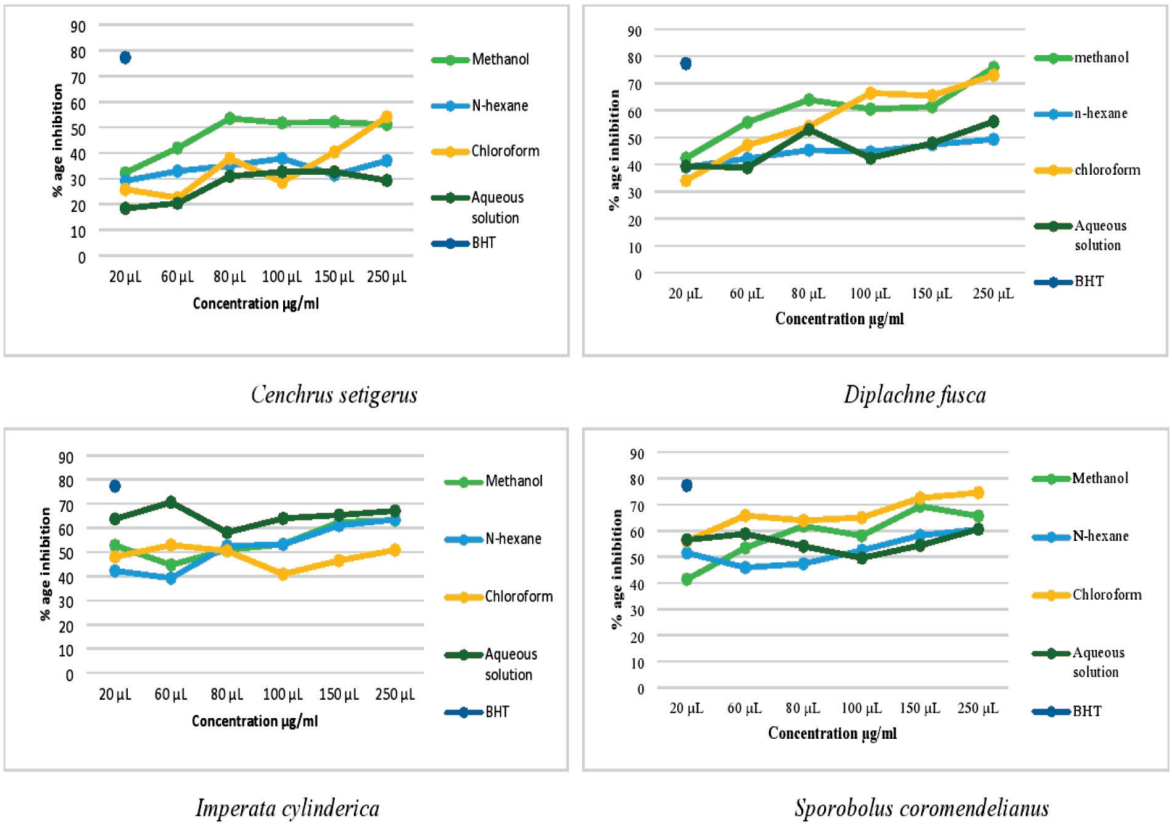

Fig. 3. % DPPH free radical scavenging activity of different solvent extracts of the grasses at various concentrations.

Ferric Reducing Antioxidant Power (FRAP)

This approach was modified by Saura-Calixto [60] to quantify the ferric reducing antioxidant capacity of plant extracts. The ferric reducing antioxidant assay (FRAP) is a common ET-based approach that evaluates the reduction of ferric ion (Fe^{3+})-ligand complex by antioxidants in an acidic solution to the strongly blue-colored ferrous (Fe^{2+}) complex [61]. This assay quantifies the oxidative protection provided by antioxidants against reactive oxygen species. Redox reactions are the inactivation of oxidants by reducing agents, which can be considered electron-donating antioxidants. According to Benzie and Strain (1996), this assay is based on the ability of antioxidants to convert Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ), resulting in the formation of a bright blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. An increase in absorbance corresponds to an increase in reduction capacity, as shown in Table 8. The methanol fraction of *I. cylinderica* had the highest FRAP value of all fractions, 1.77 0.054 mol TE mL^{-1}, whereas the aqueous solution part had a very modest reducing capacity 0.67±0.061 µmol TE mL^{-1} (Fig. 5).

Total Phenolic Contents

Phenolic compounds act as reducing agents, hydrogen donors and are able to scavenge free radicals. The antioxidant activity of phenolic compounds varies greatly, depending on their chemical structure. Table 9 shows that the methanol extract of *I. cylinderica* had the highest concentration of 118.32±1.27 (GAE) mg/mL among the studied extracts, while the aqueous solution portion of *S. coromandelianus* had the lowest concentration, of 0.77±0.05 (GAE) mg/mL. The total phenolic content in the methanol fractions of *C. setigerus*, *D. fusca, I. cylinderica*, and *S. coromandelianus* were 66.93±0.65 GAE mg/mL, 96.93±0.26 GAE mg/mL, 118.32±1.27 GAE mg/mL and 53.15±0.51 GAE mg/mL respectively. The values for total phenolic content of methanol fraction, chloroform, and aqueous solution were considered significant ($p<0.05$), while the values for *n*-hexane was considered non-significant ($p>0.05$) in contrast to the blank value. To calculate the results and plot those in terms of GAE mg/mL, the gallic acid standard curve was used (Fig. 6).

Discussion

According to Martin et al. [62], ethnoveterinary medicine (EVM) is based on folk beliefs, traditional knowledge, abilities, and practices to treat diseases and maintain animal health. Grass is commonly used for ethnotherapeutic purposes by local people in rural Punjab. Four ethnoveterinary grasses, *C. setigerus, D. fusca, I. cylinderica,* and *S. coromandelianus* are

Table 7. Total antioxidant activity of different fractions of solvents of the grasses.

Sr. No.	Solvent	Concentration (µg/ml)	Abs. at 695 nm±SE *(C. setigerus)*	Abs. at 695 nm±SE *(D. fusca)*	Abs. at 695 nm±SE *(I. cylinderica)*	Abs. at 695 nm±SE *(S. coromandelianus)*
1.	Methanol	125	0.80±0.04^{b}	1.26±0.06^{a}	1.19±0.01^{a}	0.47±0.07^{a}
		250	0.68±0.02^{c}	0.98±0.07^{b}	0.82±0.06^{a}	0.56±0.13^{a}
		500	0.96±0.02^{a}	1.16±0.03^{a}	1.13±0.02^{a}	0.64±0.05^{a}
2.	N-hexane	125	0.85±0.03^{b}	0.27±0.04^{b}	0.49±0.04^{c}	0.40±0.01^{b}
		250	0.4±0.01^{c}	0.32±0.02^{b}	1.08±0.06^{a}	0.41±0.09^{b}
		500	1.02±0.03^{a}	1.09±0.06^{a}	0.59±0.05^{b}	0.60±0.04^{a}
3.	Chloroform	125	0.76±0.09^{b}	0.97±0.04^{a}	0.54±0.03^{c}	0.63±0.05^{b}
		250	0.82±0.02^{b}	0.44±0.03^{b}	0.94±0.03^{b}	0.64±0.06^{b}
		500	1.16±0.03^{a}	1.06±0.04^{a}	1.20±0.00^{a}	0.97±0.04^{a}
4.	Aqueous solution	125	0.81±0.02^{a}	0.69±0.10^{ab}	0.58±0.04^{a}	0.33±0.04^{a}
		250	0.81±0.05^{a}	0.76±0.17^{a}	0.39±0.05^{b}	0.17±0.02b
		500	0.61±0.01^{b}	0.97±0.04^{a}	0.32±0.06^{b}	0.31±0.02^{a}
BHT	Standard		1.22±0.09	1.22±0.09	1.22±0.09	1.22±0.09

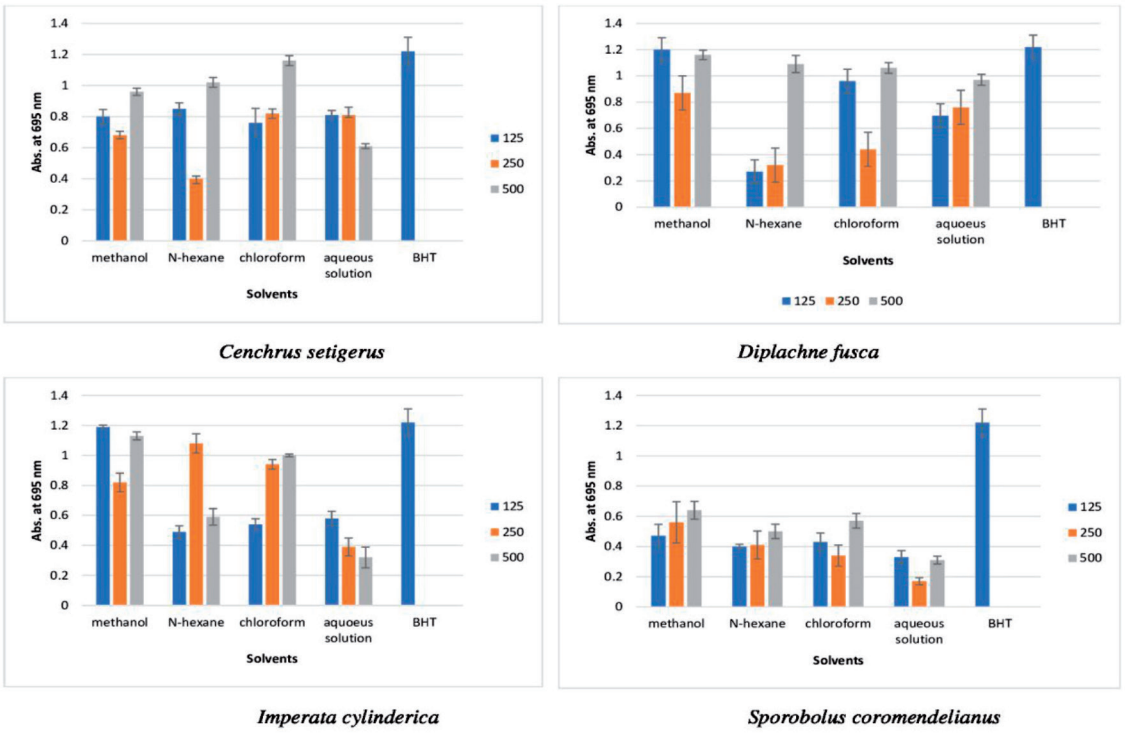

Fig. 4. Total Antioxidant activity of different solvent extracts of the grasses at various concentrations.

investigated in the present study. The ethnomedicinal importance of grasses has been well studied [63-67]. However, the anatomical characteristics and antioxidant capacity of ethnotherapeutic grasses in the study area have not yet been documented. Four species of ethnoveterinary grasses were studied for their anatomical characteristics. The stems of the grasses were cut transversely to reveal a layer of epidermal cells with almost spherical shape, cuticle, cutinized walls, and thick cells. The cortex and the pith of the ground tissue were not differentiated. In the outer part, where they were close together and collateral, there were several

Table 8. FRAP values of different solvent extracts of the grasses.

Sr. No.	Solvent	TE mg/ml ± SE *(C. setigerus)*	TE mg/ml±SE *(D. fusca)*	TE mg/ml±SE *(I. cylinderica)*	TE mg/ml±SE *(S. coromandelianus)*
1.	Methanol	1.40±0.006^{ab}	1.44±0.012^{a}	1.77±0.054^{a} 1.50±0.016^{b}	1.46±0.025^{b}
2.	N-hexane	1.22±0.094^{b}	1.21±0.015^{b}	1.19±0.009^{c}	1.10±0.007^{c}
3.	Chloroform	1.42±0.027^{a}	1.18±0.011^{b}	1.50±0.016^{b}	1.59±0.006^{a}
4.	Aqueous solution	1.10±0.005^{c}	0.67±0.061^{c}	1.22±0.065^{c}	1.46±0.083^{b}

Table 9. Total phenolic contents of different solvent extracts of grasses.

Sr. No.	Solvent	GAE mg/ml±SE (*C. setigerus*)	GAE mg/ml ±SE (*D. fusca*)	GAE mg/ml±SE *(I. cylinderica*)	GAE mg/ml±SE (*S. coromandelianus*)
1.	Methanol	66.93±0.65^{a}	96.93±0.26^{b}	118.32±1.27^{b}	53.15±0.51^{b}
2.	N-hexane	0.96±0.081^{d}	14.16±0.63^{c}	21.82±0.48^{c}	9.23±0.15^{c}
3.	Chloroform	50.20±0.10^{b}	93.42±1.23^{a}	81.36±1.25^{a}	72.57±0.45^{a}
4.	Aqueous solution	7.79±0.54^{c}	1.24±0.08^{d}	20.66±0.75^{d}	0.77±0.05^{d}

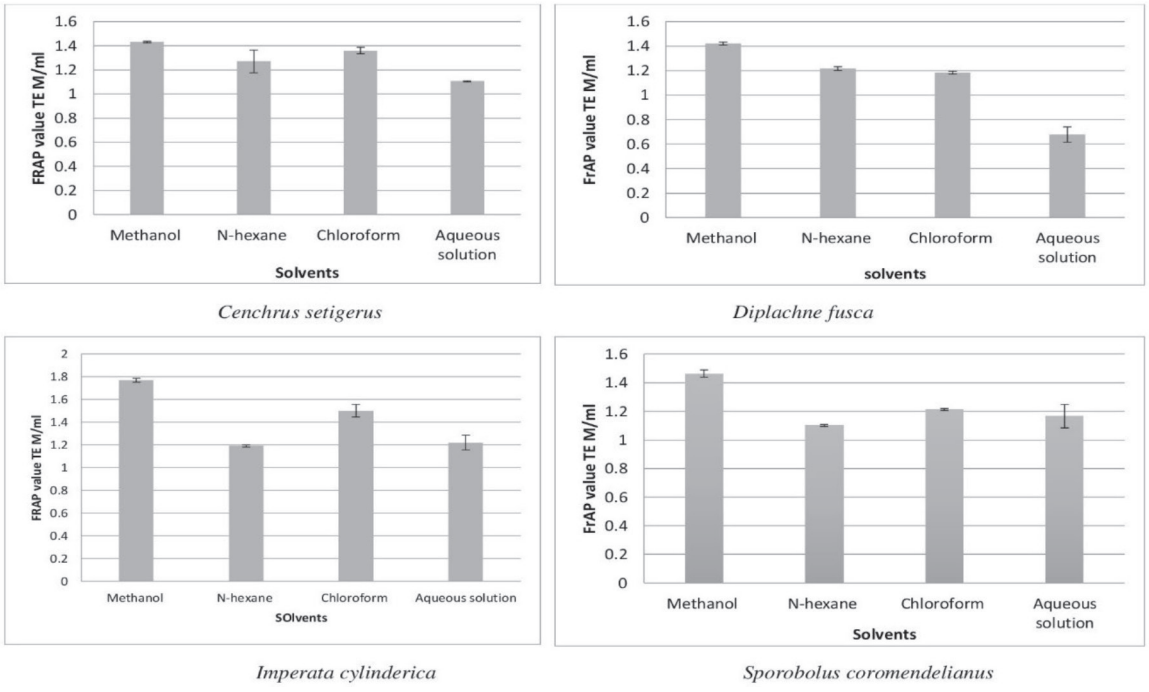

Fig. 5. Different solvent extracts used for the estimation of the FRAP value in the grasses.

vascular bundles irregularly distributed and more or less circular. In Napier and Kas [66] the vascular bundles are scattered over the entire hypodermis. In the grasses, there are two different types of vascular bundles. The peripheral vascular bundles are smaller in several monocotyledonous species as reported by Emamverdian et al. [68]. The size of central vascular bundles was larger. Different bamboo species have four different types of vascular bundles as reported by Javadian et al. [69]. Protoxylem and two large metaxylem were seen. Cells called bundle sheaths surround the vascular bundles. Arora and Kumar, [70], Gandhi and Albert, [71], and Ahmed et al. [72] found similar findings in the stem of *Saccharum spontaneum* L., stem section of *C. setigerus* and stem section of *I. cylinderica*. The leaves of all four grasses showed upper and lower epidermis, mesophyll cells, vascular bundles, and sclerenchyma cells in their transverse sections. All grasses exhibited

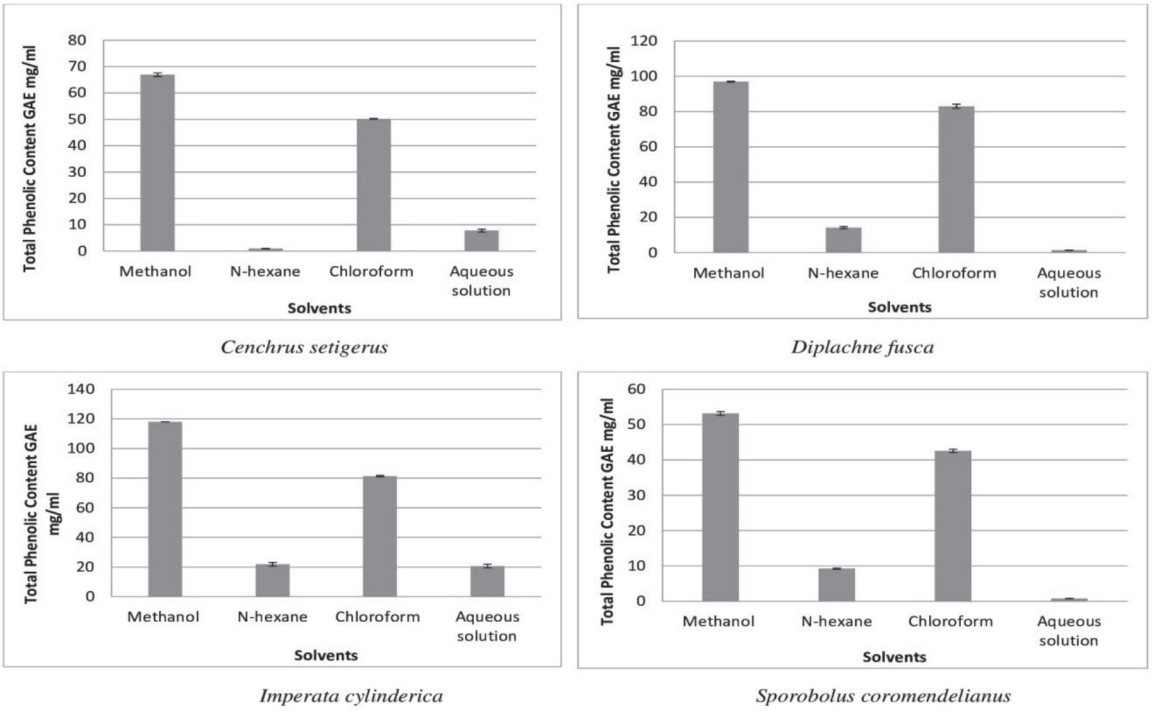

Fig. 6. TPC evaluation of various solvent extracts of the grasses.

the microhairs and trichomes noted by Freire et al. [73] in *Cynodon dactylon* (L.) Pers. According to the study of [74] in the scenario of *I. cylinderica*, the mesophyll tissue consisted of parenchymatous cells with extensive intercellular spaces while the spongy and palisade tissues were undifferentiated. According to [75], all leaf species have bulliform cells in the upper epidermis. This is also true for the leaf of the plant *Elytrigia intermedia* (Host) Nevski. Antioxidants are crucial for the prevention of oxidative stress, which is associated with the development of many chronic diseases and disorders [76]. For the past thirty years, there has been a great interest in natural antioxidants because synthetic antioxidants such as BHT and BHA, which are commonly sold in the market, are toxic and hazardous to health. To determine the natural antioxidant capacity of ethnoveterinary grasses, tests are conducted to determine the reducing power and free radical scavenging activity. To determine the antioxidant activity of ethnoveterinary grasses collected from different zones of Hafizabad District, four different antioxidant tests were performed: DPPH, FRAP, TPC, and phosphomolybdate. According to the results of DPPH experiment, *D. fusca* showed the highest capacity to scavenge free radicals. At a concentration of 250 µL, the chloroform extracts showed the highest value of 75.87±0.14. The reference standard substance BHT showed the highest performance of, 77.3±0.7. The maximum scavenging activity was shown by the chloroform fractions of *setigerus, I. cylinderica* in aqueous solution, and *S. coromandelianus* in chloroform with values of 54.14±0.28, 70.60±0.30, and 74.49±0.14, respectively. As Fatima et al. [77] found in the ethanol concentration of *I. cylinderica* leaves, the radical scavenging activity increased with the concentration of the extract in the solution. The results of DPPH assay on *Sporobolus pyramidalis* P.Beauv. and *Sporobolus africanus* (Poir.) were reported by Gebashe et al. [78] in 2020. The phosphomolybdenum technique was used to evaluate the total antioxidant activity. Among all grasses, *D. fusca* showed the highest reduction potential with 1.20±0.06. The results were evaluated in comparison with the standard BHT value, which was 1.22±0.09. At a concentration of 500 µL, the chloroform fraction of *C. setigerus* showed maximum reduction potential, i.e., 1.16±0.03. In different concentrations of *I. cylinderica*, the methanol extract had the strongest scavenging potential, i.e., 0.64±0.05 whereas in *S. coromandelianus*, the highest potential i.e. 0.64±0.05, occurred at a concentration of 500 µL of the chloroform extract. *I. cylinderica* showed the highest FRAP value, 1.77±0.054 TE mg/mL. The highest reduction potential for the methanol fractions of *C. setigerus* and *D. fusca* was 1.40±0.006 TE mg/mL and 1.44±0.012 TE mg/mL, respectively. *S. coromandelianus* showed the highest reduction potential, i.e. 1.59±0.006 in chloroform extract. Results of FRAP assays were shown in TEM/mL.*S. coromandelianus* has previously shown high antioxidant potential. *S. coromandelianus* was the subject of the studies conducted by Ajaib et al. [79]. The methanol component of the grass showed the greatest potential for reduction. The FC reagent method was used to calculate the total phenolic content. The total phenols in the methanol fraction of *I. cylinderica* were 118.32±1.27 (GAE) mg/mL. The highest TPC values were found in the methanol fraction of all four grasses, followed by the chloroform, *n*-hexane, and aqueous solution fractions.

[80] reported similar results in *C. setigerus*. Both *in vivo* and *in vitro*, the grass had the highest concentration of phenols in the methanol fractions. The total phenolic content of the methanol fractions from the leaves of *I. cylinderica* was also measured by [78].

Conclusions

Rural populations in Hafizabad, many villages rely on medicinal plants for ethnoveterinary purposes. The grass species for treating diseases and maintaining animal health are ethnoveterinary. The biological and antioxidant capacity of ethnoveterinary grasses of Hafizabad region has not been determined yet. In the current study the morphological, phytochemical, and antioxidant properties of the stems and leaves of four ethnoveterinary grasses were investigated. *C. setigerus* and *S. coromandelianus* did not show significant antioxidant potential, while *D.* and *I. cylinderica* did. Since them ethanol extract dissolves polar molecules better than other nonpolar fractions, the methanol fraction had the highest activity. The results of the various assays showed that these grasses are natural antioxidant sources when consumed by animals. This study provides experimental evidence that extracts of these species can be used as natural antioxidants and to treat a variety of diseases caused by reactive oxygen species. As a result, these species are used by locals as animal feed and have significant therapeutic value. Numerous secondary metabolites identified in some species may play an important role in their antibacterial, antifungal, and antioxidant activities.

Author Contributions

Conceptualization: S.A, & U.H.; Methodology: S.A., A.R. & M. Naseem Khan.; Data Curation: S.A., A.R.B.A, & M.N.K.; Writing-original draft preparation: S.A., B.A. & M.N.K.; Writing-Review and Editing: B.A.R.A, & S.W.; Supervision: U.H.; Funding Acquisition: R.A

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Conflict of Interest

The authors declare no conflict of interest.

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