Pharmacogn. Res., 2024; 16(1):123-131.
https://www.phcogres.com

Original Article

Pharmacognostic Standardization, Heavy Metal Analysis and Antioxidant Potential of *Iris kashmiriana* Baker Rhizome

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ABSTRACT

Background: *Iris kashmiriana* Baker, often known as Mazarmond, is a plant species classified under the Iridaceae family. This specific botanical specimen has notable therapeutic properties and has been traditionally used for the treatment of many diseases. The rhizomes are considered to be a substantial repository of secondary metabolites and have been extensively investigated for their potential in many activities, including immunomodulation, anticancer capabilities, antibacterial effects, and anthelmintic qualities. Currently, there is a lack of study about the establishment of standardization methods for the plant. **Aim:** the purpose of this research was to conduct standardization, phytochemical analysis, heavy metal analysis, and assessment of the antioxidant capacity of the rhizomes of *Iris kashmiriana* Baker, a native plant found in the Kashmir valley. **Materials and Methods:** The botanical specimens were gathered, subjected to a process of air drying in a shaded environment, and afterwards ground into a fine powder. The techniques used for conducting pharmacognostic investigations and phytochemical screening were in compliance with the guidelines set out by the World Health Organization (WHO) and the Indian Pharmacopoeia. **Results:** Several distinct features were observed after the implementation of organoleptic, microscopic examination, standardization, and phytochemical analysis. The quantities of all heavy metals were found to be under the acceptable range set by the World Health Organization (WHO). Furthermore, the plant extracts demonstrated significant antioxidant activity. **Conclusion:** This research serves as a first endeavour in the process of standardizing *Iris kashmiriana* rhizomes, with the objective of creating a comprehensive profile of their attributes. The results obtained from this inquiry may be used as a valuable point of reference for taxonomic verification and quality control of the perennial plant. Furthermore, the use of standardized rhizomes may be investigated as a potential alternative in the field of medicine for addressing various ailments. This alternative approach presents a higher level of safety and a decreased likelihood of unpleasant responses.

Keywords: *Iris kashmiriana* Baker, Standardization, Phytochemical analysis, Antioxidant activity.

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Received: 06-08-2023;
Revised: 12-09-2023;
Accepted: 12-09-2023.

INTRODUCTION

The genus Iris, belonging to the family Iridaceae, is widely recognized as one of the most intricate genera within this family.[1] The taxonomic group under consideration consists of around 70-80 genera, with a total of approximately 1800 species.[2] Iris species have a global distribution, spanning many climatic zones from tropical to temperate locations. In the Kashmir Himalayas, iris species may be found inhabiting several ecological niches, including cemeteries, alpine and sub-alpine meadows, gardens, roadsides, graveyards, saffron fields, and streams.[3] Various types of Irises have been used in traditional folk medicine to address diverse diseases. *Iris kashmiriana* is an example of a medicinal species of iris that is indigenous to the Kashmir region. In the Indian region, this particular species is often referred to as the Kashmiri iris, but in the specific area of Kashmir, it is known by its local names, namely 'Mazamond', 'Mazarmund', or 'safed mazarmond'. The term "mazarmond" is derived from the combination of two Kashmiri words. In Kashmiri language, the term "mazar" refers to a cemetery, while "mond" denotes a swollen component, namely a rhizome. The plant has a height ranging from 50 to 70 cm, with occasional instances where it may extend up to a maximum length of 150 cm. The stem of the plant exhibits

DOI: 10.5530/pres.16.1.15

green spathes, which are around 7-11 cm in length. These spathes correspond to the leaves of the flower bud. The plant has ensiform leaves with scarious ribs and edges, reaching a length of 40-60 cm. During the months of April to June, this particular plant exhibits the simultaneous blooming of 4-6 flowers. These flowers display a variety of hues, ranging from pure white to white with a subtle blue hue. The white blooms have a resemblance to other iris species, namely *Iris albicans*. The flower consists of three big outer petals, which are rounded or obovate in shape and referred to as falls, as well as three interior little petals that are elliptic or obovate in shape and known as standards. The branch of the style measures 5 cm in length and has a complete stigma. The filaments have a cream or white coloration and possess a maximum length of 2 cm. The object has a green-colored ovary characterised by distinct grooves and ridges. Following the blooming stage, there is an infrequent occurrence of capsule formation, which encloses seeds that exhibit colours ranging from red brown to dark red brown.[4,5] Several pharmacologically active compounds have been extracted from this particular species, including iridin, irisolidone, isonigricin, iriskahmirianin, irigenin, and tectorigenin. Historically, this substance has been used for the management of eczema and respiratory ailments, as well as for pain reduction and the alleviation of inflammatory conditions.[6]

MATERIALS AND METHODS

Fresh underground part of *Iris kashmiriana* were collected from Rangrate area of district Budgam in Kashmir in May 2017 at an altitude of 1655m and Prof. Akhtar H. Malik. Taxonomist at Centre for Biodiversity and Taxonomy (CBT), Department of Botany, then identified and authenticated the plant material. The reference numbers allotted to the species *Iris kashmiriana* 2229-KASH and for the future references a sample specimen of collected plant materials was deposited in the herbarium of the Centre for Biodiversity and Taxonomy, Department of Botany, the University of Kashmir.

After collection and authentication of this species, using a pulverizer rhizome of plant after drying in air under shade were powdered. The powder was stored in an airtight container for future use after passing the powder via sieve number 40.

Chemicals and Reagents

All chemicals and reagents, utilized were of analytical grade (AR grade) and were procured from CDH, Sigma Aldrich, Himedia and Ranbaxy Fine Chemicals Ltd.

Methodology

Standardization of raw plant materials

Standardization and pharmacognostical evaluation of the raw plant material was done according to the standardization parameters described in Indian Pharmacopoeia and Ayurvedic Pharmacopoeia of India and by following WHO guidelines.[7,8]

Macroscopic and Microscopic evaluation of Plant Material

In order to assess the macroscopic properties of *Iris kashmiriana* rhizomes, an examination was conducted on freshly harvested rhizomes which included a visual assessment of many attributes such as size, shape, colour, texture, and surface characteristics.[9]

Physicochemical Constants Determination

Physicochemical variables such as extractive values,[10] Loss on drying, Ash values, swelling index,[11] pH values and foaming index[12] were evaluated as per WHO guidelines and IP 1996.[13]

Heavy metal analysis

The wet digesting technique, as outlined by Okelobo in 2002, was used to assess the heavy metal content within the plant material. In the digestion flask, a mass of 1 g of the powdered material was subjected to digestion using a mixture of 5 mL of nitric acid and perchloric acid. The combination was subjected to heating at a temperature of 110°C until the solution achieved clarity, signifying the full digestion of the organic matter. Subsequently, the digested solution was let to undergo cooling at ambient temperature, followed by the addition of 100 mL of deionized water for the purpose of dilution. The solution was subjected to analysis using an Atomic Absorption Spectrophotometer (AAS) to determine the concentrations of lead, copper, chromium, and cadmium.[14]

Extract preparation

The rhizomes of *Iris kashmiriana* were subjected to sieving using a mesh size of 40, followed by weighing, and afterwards employed for extraction. The powder was weighed and afterwards subjected to extraction using a macerator with 70% ethanol. Aqueous extracts were produced using the decoction technique. Subsequently, the hydroalcoholic and aqueous extracts were concentrated at decreased pressure. Sequential Liquid-Liquid Extraction (LLE) of hydroethanolic extracts from both plants was performed using several solvents, including hexane, Dichloromethane (DCM), ethyl acetate, and n-butanol. The drying process for all the extracts was conducted at a temperature range of 35-40°C using a rotavapor at decreased pressure. The dried extracts were then weighed and placed in a dessicator for a period of time. Subsequently, they were stored in an air tight container at a temperature of 4°C for future experimental purposes.[15,16]

Preliminary Phytochemical screening for qualitative chemical analysis

The preliminary phytochemical screening of extracts was done to determine the presence of various secondary metabolites such as alkaloids, saponins, proteins and amino acids, carbohydrates, tannins, flavonoids, phenolics, glycosides, terpenoids, and sterols by using various chemicals tests according to the standard methods.[17,18]

Quantitative estimation of Phytoconstituents

Determination of Total Phenolic Content (TPC)

The total phenolic content in various fractions were quantified using the Folin-Ciocalteu technique with certain modifications. In this investigation, all the samples were prepared using extracts at a concentration of 1 mg/mL in methanol. A volume of 0.5 mL of the methanolic solution containing the extract was combined with 2.5 mL of a Folin-Ciocalteu reagent solution with a concentration of 10%, as well as 2.5 mL of a sodium bicarbonate solution with a concentration of 7.5%. Subsequently, the samples were subjected to incubation at a temperature of 40°C for a duration of 45 min, while being kept in a light-restricted environment. The absorbance at 765 nm was ultimately determined in relation to the blank, which refers to the reaction mixture devoid of any extract. The samples were prepared in triplicate for each assay, and the average absorbance value was determined. The gallic acid standard was used in this methodology, and the results were quantified and reported in terms of gallic acid equivalent.[19,20]

Determination of Total Flavonoid Content (TFC)

The determination of the total flavonoid content in different fractions were conducted using the spectroscopic technique. A solution of methanol was used to make fractions with a concentration of 1mg/mL for all the samples. Next, 1 mL of a 2% solution of Aluminium Chloride ($AlCl_3$) was introduced to 500 µL of each fraction. The samples were then incubated at room temperature for 1 hr. The absorbance at a wavelength of 415 nm was measured using a spectrophotometer. Three replicate samples were generated for each assay, and the average absorbance value was determined. The experimental procedure used rutin as the standard in the protocol, and the outcomes were quantified and reported as the rutin equivalent in mL/g of fraction.[21,22]

In vitro Antioxidant activity determination

Reducing power

The approach described in Alam *et al.*, was used to assess the reduction power of the several plant extracts under investigation, with some modifications.[23] The experiment included adding a mixture of 1 mL extract with concentrations ranging from 100-500 µg/mL to a solution containing 2.5 mL of 0.2M phosphate buffer with a pH of 6.6, as well as 2.5 mL of potassium ferricyanide with a strength of 1%. The standard substance used in this procedure was ascorbic acid, and an equivalent concentration of ascorbic acid was utilised. The solution was subjected to incubation at a temperature of 25°C for a duration of 20 min. A volume of 2.5 mL of a 10% solution of Trichloroacetic Acid (TCA) was introduced into the mixture in order to halt the ongoing chemical reaction. The centrifugation process was conducted for a duration of 10 min at a rotational speed of 1000 revolutions per minute (rpm). Subsequently, a volume of 2.5 mL was extracted from the top layer of the centrifuged mixture. 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ were combined. Subsequently, absorbance at a wavelength of 700 nm was determined using a spectrophotometer in opposition to the blank sample. The augmented absorbance of the reaction mixture indicated a greater reducing potential or reducing power.

DPPH free radical scavenging activity

The technique used to assess the free radical scavenging activity of fractions of hydroalcoholic extract of *Iris kashmiriana* included modifications to the DPPH test.[24] The concentration of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) used in this experimental protocol was 1 mg/mL. A solution of ascorbic acid was prepared at a concentration of 1 mg/mL, whereas the test sample was prepared at a concentration of 10 mg/mL. The provided procedure was adhered to in order to prepare 1 mL aliquots of the test sample at desired concentrations ranging from 5 to 100 µg/mL using methanol and DPPH. Subsequently, the combination was incubated under light-deprived conditions at ambient temperature for a duration of 15 min. The DPPH assay demonstrated a change in colour, which served as an indicator of the presence of antioxidant activity. The optical density was measured at a wavelength of 517 nm. The samples were examined in triplicate, and the % DPPH radical scavenging activity was determined using the following formula.

$$\%\,\text{Scavenging activity} = \times 100$$

Where, Ac=Control absorbance (DPPH without sample), As=Sample absorbance.

RESULTS AND DISCUSSION

Pharmacognostic profile of *Iris kashmiriana* Baker

Macroscopical Evaluation

Colour: Dark brown (Externally), Yellowish (internally).

Odor: Characteristic.

Shape: Irregular.

Size: 7-8 cm long, 1.5-2.5 cm wide, 0.5-0.7 cm thick approximately and it varies with size.

Texture: Fibrous and smooth (Figures 1 and 2).

Microscopy

The use of microscopy is paramount in plant identification due to many factors. Microscopic analysis enables a comprehensive investigation of plant structures, facilitating the identification of crucial diagnostic characteristics that aid in differentiating various plant species. The Transverse section of Iris kashmiriana Baker showed the presence of Cork cells, vascular bundles scattered in Ground tissue, phelloderm and Starch granules (Figure 3). Similarly, Powder microscopy of rhizomes revealed the presence of Starch Granules; Prismatic Calcium oxalate Crystals; Cork cells; and Spiral xylem vessels (Figure 4).

Physicochemical Constants

Table 1 presents the assessment of many physicochemical characteristics, including total ash value, sulphated ash

Table 1: Proximate analysis and extractive values of *Iris kashmiriana*.

Physicochemical parameters	Results	
Total ash value (% *w/w*)	9.085	
Acid insoluble ash value (% *w/w*)	2.355	
Sulphated ash value (% *w/w*)	1.007	
Water soluble ash value (% *w/w*)	1.82	
Loss on drying (% *w/w*)	19.468	
Foaming index (% *w/w*)	Less than 100	
Swelling index (% *w/w*)	3	
Foreign matter (% *w/w*)	0.20	
pH of 1% solution	6.1	
pH of 10% solution	5.7	
Extractive values	**Cold extractive value**	**Hot extractive value**
Ethanolic	10.71	12.743
Aqueous	6.35	7.567
Hydroalcoholic	15.78	24.56

Table 2: Fluorescence analysis of powdered drug of *Iris kashmiriana* with various chemical reagents under visible light, short and long wave length*.

Drug Treatment	Visible light	Short UV (254 nm)	Long UV (360)
Drug untreated	Light Brown	Green	Brown
Drug + Dist. Water	Brown	Brown	Dark Green
Drug + Conc. HCl	Brown	Brown	Green
Drug +Dil. HCl	Brown	Brown	Green
Drug + H_2SO_4	Black	Green	Black
Drug+ Dil. H_2SO_4 (10%)	Black	Green	Black
Drug +HNO_3	Brown	Black	Green
Drug + Dil. HNO_3 (10%)	Orange	Ming Red	Dark Green
Drug + 10% NaOH	Yellow	Vivid Green	Green
Drug + picric acid	Yellow	Black	Green
Drug + Iodine	Ming Red	Black	Dark Green
Drug + Methanol	Brown	Brown	Green
Drug + Ethanol	Brown	Brown	Green
Drug + Acetic acid	Light Brown	Woody Brown	Green
Drug + Chloroform	Brown	Vivid Green	Green
Drug + Pet. ether	Brown	Green	Dark Green
Drug + Ferric chloride	Black	Black	Black
Drug + Ammonia solution	Brown	Vivid Green	Green

value, acid-insoluble ash value, pH, loss on drying, foaming index, foreign matter, swelling index, and extractive values. Fluorescence analysis is of considerable importance in the field of plant analysis, as it provides useful insights into several facets of plant physiology, biochemistry, and ecology. Fluorescence is a phenomenon that arises when a material undergoes absorption of light with a certain wavelength, afterwards emitting light at a longer wavelength. The phenomenon of light emission is often referred to as fluorescence. The powdered material of *Iris kashmiriana* rhizomes revealed a good fluorescence on treating with different chemicals (Table 2). Plants serve as a valuable reservoir of vital minerals that are important for maintaining human well-being. Plants assimilate these nutrients from the soil, subsequently becoming part of the human diet. For this reason, *Iris kashmiriana* was also analyzed for the mineral content. The results were given in Table 3. The analysis of heavy metals in plants for the purpose of standardization is crucial in ensuring the safety, purity, and efficacy of herbal medicines, botanical supplements, and other plant-derived products. The issue of heavy metal contamination in medicinal plants is a subject of concern owing to the inherent risks it poses to human health when these plants are used in traditional medicine or herbal remedies. The wet digestion method was used to analyze heavy metal content in the medication. The investigation showed that the levels of heavy metals in the medicine were below the permitted limits outlined by the World Health Organization (WHO) in their 1998 recommendations (Table 4). The hydroalcoholic extract was taken into the separating funnel and was partitioned with different solvents to yield different fractions. The results are given in Table 5.

Figure 1: *Iris kashmiriana* Baker in graveyard.

Table 3: Mineral content of rhizomes of *Iris kashmiriana*.

Mineral	Mineral content in root (ppm)	Permissible limit NMT
Cobalt (Co)	1.82	No regulatory limits by WHO
Manganese (Mn)	0.078	No regulatory limits by WHO
Magnesium (Mg)	0.643	No regulatory limits by WHO
Iron (Fe)	27.32	20.0 ppm
Zinc (Zn)	0.928	27.4 ppm
Copper (Cu)	6.279	3.00 ppm

Table 4: Heavy Metal Residue of powdered drug of rhizomes of *Iris kashmiriana*.

Sl. No.	Test Parameters	*Iris kashmiriana* (ppm)	MDL(WHO) (ppm)
1.	Cadmium (Cd)	0.031	0.3
2.	Chromium (Cr)	0.041	2.0
3.	Nickel (Ni)	0.064	0.63
4.	Lead (Pb)	0.318	1.0
5.	Mercury (Hg)	0.035	0.1 ppm

Table 5: Percentage yield *(% w/w)* of various fraction of hydroalcoholic extract of *Iris kashmiriana* Baker.

Fractions	Percentage Yield
Hexane (IKH)	5.0
DCM (IKD)	11.5
Ethylacetate (IKE)	9.0
n-butanol (IKB)	27.0
Residual aqueous extract (IKRaq)	30.75

Table 6: Results of phytochemical screening of different extracts of the rhizomes of *Iris kashmiriana* (IK).

Tests	IKHa	IKH	IKD	IKE	IKB	IKRaq
Test for carbohydrates						
Benedict's test	++	-	+	++	+	++
Molisch's test	+	-	+	++	++	++
Fehling's test	+	-	+	++	+	+
Test for Tannins						
Lead acetate test	++	+	++	++	++	+
5% $FeCl_3$ test	+	++	++	+	+	++
Test for Phenolics						
1% $FeCl_3$ test	++	+	++	++	++	+
Test for Flavonoids						
Shinoda test	+	-	+	+	+	-
Test for saponins						
Froth test	+	+	+	+	+	+
Foam test	+	+	+	+	+	+
Test for Terpenoids						
Salkowski test	++	-	++	++	+ +	-
Test for sterols						
Libermann's test	+	-	+++	+++	+++	+
Test for aminoacids and proteins						
Ninhydrin test	-	-	-	-	-	-
Xantho-proteic test	-	-	-	-	-	-
Test for cardiac glycosides						
Legal test	+	+	++	+++	+++	-
Keller killiani test	+	+	+	++	++	-
Test for alkaloids						
Wagner's test	-	-	-	-	-	-
Mayer's test	-	-	-	-	-	-
Hager's test	-	-	-	-	-	-
Dragendroff's test	-	-	-	-	-	-

(+) Present; (-) Absent.

Table 7: Total Phenolic Content and Total Flavonoid Content of various fractions of hydroalcoholic extract of *Iris kashmiriana* Baker.

Fractions	TPC (mg GAE/g of extract)	TFC (mg RE/g of extract)
Hexane (IKH)	24.92 ± 8.33	19.2 ± 0.49
DCM (IKD)	298.08 ± 11.45	82.275 ± 2.87
Ethyl acetate (IKE)	440.76 ± 8.68	135.97 ± 4.26
n-butanol (IKB)	214.6 ± 2.49	72.7± 5.34
Residual aqueous extract (IK Raq)	124.96 ± 8.26	40.4 ± 3.73

The values are represented as mean ± SD; (n=3).

Table 8: **Reducing power of various fractions of hydroalcoholic extract of *Iris kashmiriana* Baker.**

Conc. (µg/mL)	Standard	Hexane	DCM	Ethyl acetate	*n*-butanol	Residual Aqueous fraction
100	0.73±0.13	0.14±0.31	0.29±0.15	0.43±0.16	0.13±0.26	0.28±0.16
200	1.19±0.24	0.28±0.41	0.47±0.26	0.99±0.04	0.23±0.14	0.29±0.15
300	1.87±0.43	0.44±0.36	0.80±0.53	1.57±0.57	0.65±0.32	0.37±0.83
400	2.92±0.12	0.54±0.41	1.34±0.10	2.49±0.32	0.85±0.33	0.52±0.43
500	3.89±0.13	0.88±0.33	1.97±0.29	3.65±0.16	1.54±0.17	0.68±0.53

Table 9: **DPPH quenching potency of various fractions of hydroalcoholic extract of *Iris kashmiriana* Baker.**

Conc. (µg/mL)	Standard	Hexane	DCM	Ethyl acetate	*n*-butanol	Residual Aqueous fraction
50	43.9±0.31	20.2±0.01	31.43±0.14	35.04±0.01	12.63±0.10	8.87 ±0.03
100	56.05±0.01	28.83±0.01	57.87±0.26	55.44±0.07	28.85±0.02	12.1 ±0.04
150	73.32±0.11	33.65±0.06	73.98±0.33	71.46±0.07	53.47±0.12	14.2 ±0.01
200	87.55±0.18	38.43±0.20	86.22±0.01	83.44±0.03	71.76±0.02	17.4 ±0.23
250	98.25±0.03	45.84±0.02	91.44±0.15	95.12±0.02	89.01±0.06	20.78 ±0.26

Phytochemical analysis

The first phytochemical research conducted on several fractions of *Iris kashmiriana* demonstrated the existence of many secondary metabolites like glycosides, polysaccharides, tannins, saponins, sterols, phytosterols, amino acids, terpenoids, and flavonoids in all the fractions and extract (Table 6).

Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content results of different *Iris kashmiriana* showed variation from 24.92 to 440.76 mg GAE/g dry weight. Ethyl acetate fraction showed the highest concentration 440.76 followed by DCM (298.08) and n-butanol (214.6) respectively. Similarly, the total flavonoid content results of different *Iris kashmiriana* fractions showed variation from 19.2 to 135.97 mg RE/g dry weight. Ethyl acetate fraction showed the highest concentration 135.97 followed by DCM (82.27) and n-butanol (72.7) respectively (Table 7).

In vitro antioxidant screening

The antioxidant properties of different fractions of *Iris kashmiriana* Baker rhizomes were evaluated using reducing power assay and DPPH free radical scavenging activity.

Reducing power assay

In comparison of ascorbic acid, all the fractions of *Iris kashmiriana* revealed concentration dependent reducing powder. However, At the concentration of 500 µg/mL, the highest activity was demonstrated by ethyl acetate fraction (3.65±0.16) followed by DCM (1.97±0.29) and *n*-butanol (1.54±0.17). Standard Ascorbic

Figure 2: Dried and fresh rhizomes of *Iris kashmiriana* Baker; Transverse section of *Iris kashmiriana* Baker.

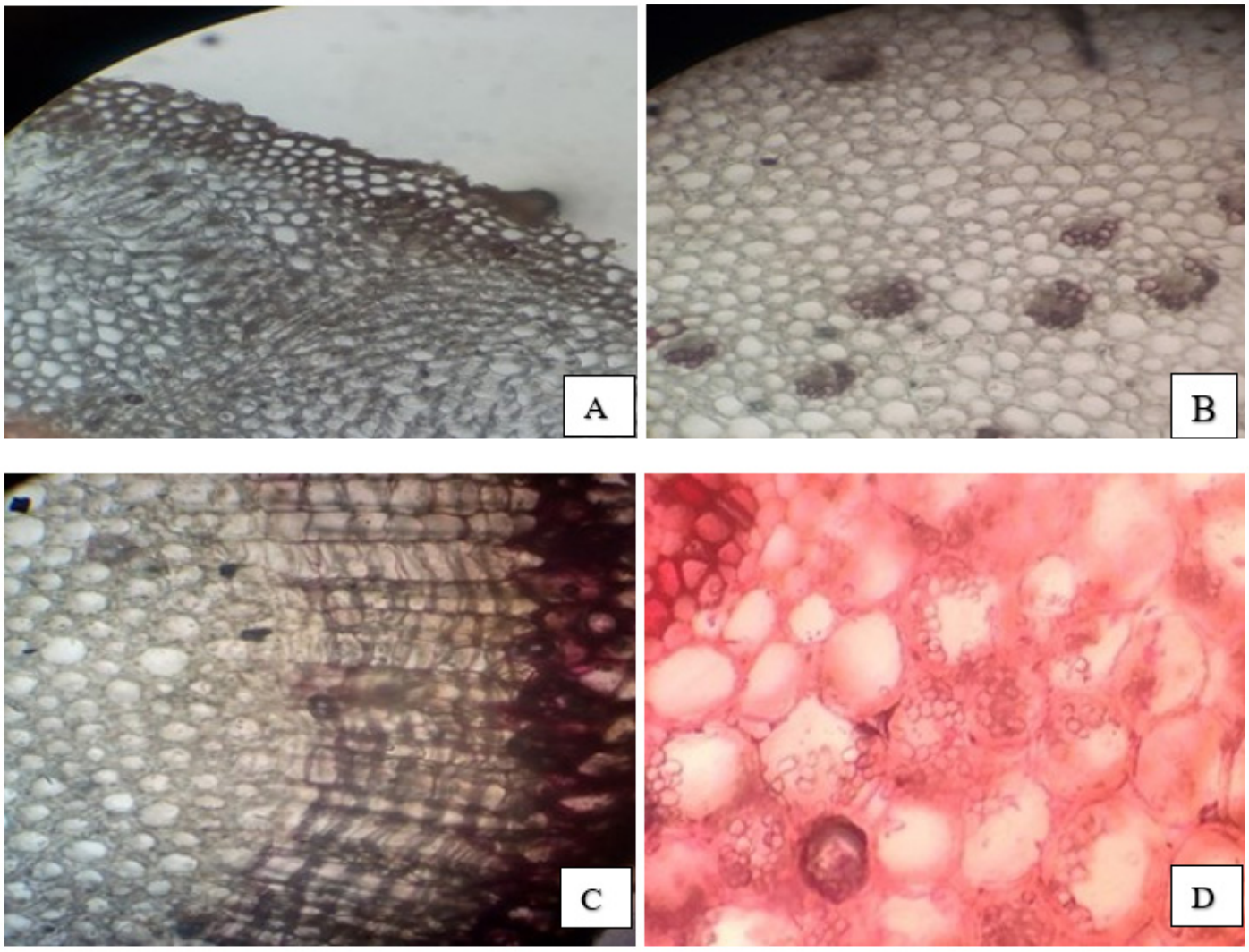

Figure 3: Transverse section of *Iris kashmiriana* Baker showed the presence of (A) Cork cells, (B) Ground tissue with scattered vascular bundles, (c) Cork cells with phelloderm (d) Starch granules; Powder Microscopy of the rhizomes of *Iris kashmiriana* Baker.

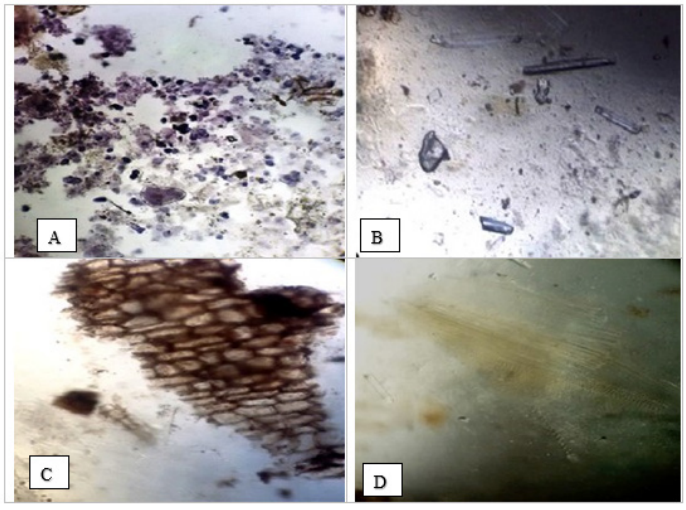

Figure 4: Powder microscopy of *Iris kashmiriana* Baker rhizomes revealed the presence of (A). Starch Granules; (B). Prismatic Calcium oxalate Crystals; (C). Cork cells; (D). Spiral xylem vessels.

acid at the same concentration demonstrated 3.89±0.13 reducing power (Table 8).

DPPH assay

Various fraction of hydroalcoholic extract of *Iris kashmiriana* rhizomes demonstrated dose dependent DPPH quenching potency as compared to the standard drug. Among the various fractions, ethyl acetate fraction at 250 µg/mL showed maximum potency (95.12±0.02) followed by DCM (91.44±0.15) and n-butanol (89.01±0.06) at the same concentration. Standard drug showed (98.25±0.03) activity at the same conditions and concentration (Table 9).

CONCLUSION

This study represents the inaugural investigation into a comprehensive analysis of the macroscopic and microscopic attributes, phytochemical screening, and quantification of total phenolics and antioxidant activities of the *Iris kashmiriana*

species. This study also revealed that all the fractions of the plant rhizomes possess good antioxidant activity which However, additional research is required in order to have a comprehensive understanding of the underlying mechanism through which fractions function as an antioxidant drug. Furthermore, the data derived from the pharmacognostic and phytochemical examination might serve as a benchmark for the purpose of ensuring the quality control of this particular plant.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Cite this article: Farooq S, Mohi-ud-din R, Mir PA, Mir RH, Kumar N, Bhat ZA. Pharmacognostic Standardization, Heavy Metal Analysis and Antioxidant Potential of *Iris kashmiriana* Baker Rhizome. Pharmacog Res. 2024;16(1):123-31.