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Pharmacognostical standardization, phytochemical analysis and *in-vitro* antioxidant activity of rhizome of *Polygonum alpinum* All.

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Abstract

Objective: The present study was designed to evaluate the pharmacognostic characters, physicochemical parameters and in-vitro antioxidant activity of rhizome of *Polygonum alpinum*.

Methods: The pharmacognostic and phytochemical screening along with physicochemical parameters were evaluated as per the recommended procedures. Antioxidant activity of the alcoholic and aqueous extract was determined using DPPH and reducing power method.

Results: The outer and inner side of the rhizome is dark brown and white brown respectively with characteristic odour and astringent taste. The rhizomes are cylindrical in shape and 5-15 cm in diameter. The TS of rhizome shows multi-layered cork cells, cortex with scattered sclerides, medullary rays, xylem vessels, phloem vessels and pith. The powder microscopy revealed the presence of epidermis, hypodermis with calcium oxalate crystals, non lignified vascular bundle, calcium oxalate crystals, cork in surface view and fragments of bordered pitted vessels. Phytochemical screening of various extracts revealed the presence of carbohydrates, cardiac glycosides, coumarin, steroids, terpenoids, flavonoids, saponins and tannins. Physiochemical analysis showed 2.325% of total ash, 0.525% of water soluble ash, 0.650% of acid insoluble ash and 3.625% of sulphated ash *w/w*. Other parameters like extractive value, foreign matter, moisture content, swelling index, foaming index, pH of different solvents, total tannin content, total fat content and fluorescence analysis were also determined. The total phenolics in alcoholic and aqueous extract was found to be 41.06 and 23.30 mg/g GAE respectively while flavonoid content in alcoholic and aqueous extract was 182.13 and 123.71 mg/g RE respectively. The percentage inhibition of DPPH by alcoholic and aqueous extracts at a concentration of 100 µg/mL was found to be 96.50% and 49.44% respectively. The reducing power of alcoholic and aqueous extracts at 100 µg/mL was found to be 0.295 and 0.175 respectively and increased to 0.765 and 0.596 respectively at 500 µg/mL. The results indicate that alcoholic extract has potent antioxidant activity as compared to the aqueous extract.

Conclusion: This is the first report on the pharmacognostic studies and antioxidant activity of *Polygonum alpinum* All. It is going to be helpful in the characterization of the crude drug.

Keywords: *Polygonum alpinum*; DPPH; reducing power; pharmacognostic characters.

1. Introduction

Polygonum alpinum All. belongs to the family Polygonaceae. It is locally known as Chuka ladur; chita-hola in Kashmiri and Alpine knotweed in English. It is a perennial herb 1.5 to 3m in height. Leaves are oblanceolate with long stipules, flowers white in much branched narrow pyramidal clusters and are hermaphrodite (have both male and female organs), pollinated by insects. Flowering occurs from June to July. Nuts are triangular in shape. The plant prefers moist soil with light (sandy), medium (loamy) and heavy (clay) for suitable growth. The plant is common on alpine slopes in temperate Himalaya. The plant is native to western Himalaya: Kullu to Kashmir, distributed westward to S. Spain, Siberia, and N. America. Water extract of the dried root powder of the herb is used to prepare rice and fed to arthritic patients. Poultices prepared from the rhizome of the herb along with the crushed seeds of *Medicago falcata* is put on aching joints for producing joint relief in these patients [1-4].

2. Materials and methods

2.1 Collection of plant material and authentication:

The rhizomes of *Polygonum alpinum* were collected in August 2013 from Aharbal area of J&K. The plant was authenticated in the center of Plant Taxonomy, Department of Botany, University of Kashmir, Hazratbal, Srinagar and specimen preserved there under voucher number 1896-KASH. The rhizomes were washed thoroughly under tape water, shade dried and then crushed to coarse powder. The powdered drug material was passed through the sieve mesh 40 and stored in air tight container for further analysis.

2.2 Macroscopic and microscopic study

Macroscopic and microscopic study of *Polygonum alpinum* rhizomes was carried out as per recommended procedures [5,6]

2.3 Phytochemical analysis

2.3.1 Qualitative phytochemical analysis

Phytochemical screening was done using standard procedures [7,8]. Extracts of Polygonum alpinum were screened for alkaloids, saponins, terpenoids, flavonoids, coumarins, lactones, anthraquinones, tannins, cardiac glycosides, phenols and phytosterols.

2.3.2 Fluorescence analysis

Fluorescence is observed when cut surface or powder of various herbal drugs is exposed to UV light in presence of various chemical reagents [9,10]. For the study of fluorescence, a pinch of powdered drug was placed on grease free glass slide and treated with Acetone, Pet ether, $CHCl_3$, Ethyl acetate, Methanol, Glacial acetic acid, NH_3, HNO_3, HCl, H_2SO_4, Picric acid, 5% NaOH, 5% $FeCl_3$ and observed under visible day light and UV light at 254 and 365nm.

2.3.3 Physico-chemical analysis

The physicochemical analysis of *Polygonum alpinum* rhizome was carried out by determining ash values, extractive values, foreign organic matter, moisture content and pH of different solvents. Other parameter like swelling index, foaming index, total tannin content, total fat content were also determined [11, 12].

2.4 Antioxidant Activity

2.4.1 Determination of DPPH free radical scavenging

The free radical scavenging capacity of alcoholic and aqueous rhizome extracts of *Polygonum alpinum* was determined using DPPH [13,14]. Freshly prepared 1 ml DPPH (1,1-diphenyl-1-picrylhydrazyl), was taken in test tubes and extract added followed by serial dilutions (20µg/mL to 100µg/mL) to every test tube. Final volume 3 ml was made with methanol and was mixed. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. the absorbance was read spectrophotometrically at 517 nm. Ascorbic acid was used as standard. Control sample was prepared containing the same volume without extract. Methanol alone served as blank.

2.4.2 Determination of reducing power

The reductive capability of the extracts was quantified by the method of Oyaizu (1986) [15]. One ml of extract at dose levels of 100, 200, 300, 400 and 500µg/mL (both alcoholic and aqueous extracts of *Polygonum alpinum)* was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3 Fe $(CN)_6$]. Similar concentrations of standard ascorbic acid were used as standard. The mixture was incubated at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of 10% trichloroacetic acid. The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1%, w/v). Blank reagent was prepared similarly but without extract.. The absorbance was measured at 700 nm in a spectrophotometer against a blank sample. Increased absorbance of the reaction mixture indicated greater reducing power.

2.4.3 Determination of total phenol content

The total phenolic content of alcoholic and aqueous extracts of *Polygonum alpinum* was determined using Folin–Ciocalteu reagent by Yu L *et al*'s (2002) method [16]. Serial dilutions (50, 100, 150, 200, 250 µg/mL) were prepared and mixed with 2.5 mL Folin–Ciocalteu reagent and allowed to stand for 2 minutes. 2.5 mL 7.5 % sodium carbonate was added to each test tube mixed thoroughly and allowed to stand for 2 hrs at 20^{o}C. The absorbance of the mixture was read at 765 nm. These data were used to estimate the phenolic content using a standard curve obtained from various concentrations of gallic acid.

2.4.4 Determination of total flavonoid content

The total flavonoid content in extracts was determined by the method described by Chang *et al*, 2002 [17]. 1.0 mL of extract containing (200, 400, 600, 800 and 1000 µg/ml) was mixed with methanol (3 mL), aluminium chloride (0.2 mL, 10 %), potassium acetate (0.2 mL, 1 M) and final volume made up to 10 ml with distilled water. The mixture was vortexed for 30 seconds and allowed to stand for 30 minutes at room temperature. The absorbance was measured at 415 nm against blank. Methanol (1 mL) in place of extract was used as the blank and rutin was used as the standard solution. All determinations were carried out in triplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the formula:

$$X= (A \times m0)/ (A0 \times m),$$

where X is the flavonoid content, mg/mg plant extract in RE, A: the absorption of plant extract solution, A0: the absorption of standard rutin solution, m: the weight of

plant extract in mg and m0: the weight of rutin in the solution in mg.

3. Results

3.1 Pharmacognostic Study

3.1.1 Macroscopic characters of rhizomes

Table 1 and Figure 1 summarize the macroscopic characters of rhizomes of *Polygonum alpinum*. The macroscopic study showed the rhizomes dark brown (outer side) and white brown (inner side) with characteristic odour and astringent taste. The rhizomes are cylindrical shape and 5-15 cm in diameter.

Figure 1: Showing Photographic image of Rhizome (cut into pieces) of *Polygonum alpinum*

Table 1: Showing macroscopic characters of rhizome of *Polygonum alpinum*

Parameters	Observations
Color	dark brown (outside) white brown (inside)
Odour	Characteristic
Taste	Astringent
Shape	Cylindrical
Size	5-15 cm in diameter
Fracture	Splintery

3.1.2 Microscopic characters of rhizomes of *Polygonum alpinum*

The microscopical study (TS) of the rhizome showed multi layered outer cork, inner cortex, sclerides scattered in ring shape in the cortex, phloem right below the cortex and above the xylem vessels, xylem vessels, pith at the center and multiserrate (3-5) medullary rays emerging from xylem vessels up to the middle of cortex (Figure 2). The powder microscopy also revealed the presence of epidermis, hypodermis with calcium oxalate crystals, non lignified vascular bundle, calcium oxalate crystal, cork in surface view and Fragments of Bordered pitted vessels (Figure 3).

Figure 2: Showing T.S of rhizome of *Polygonum alpinum*

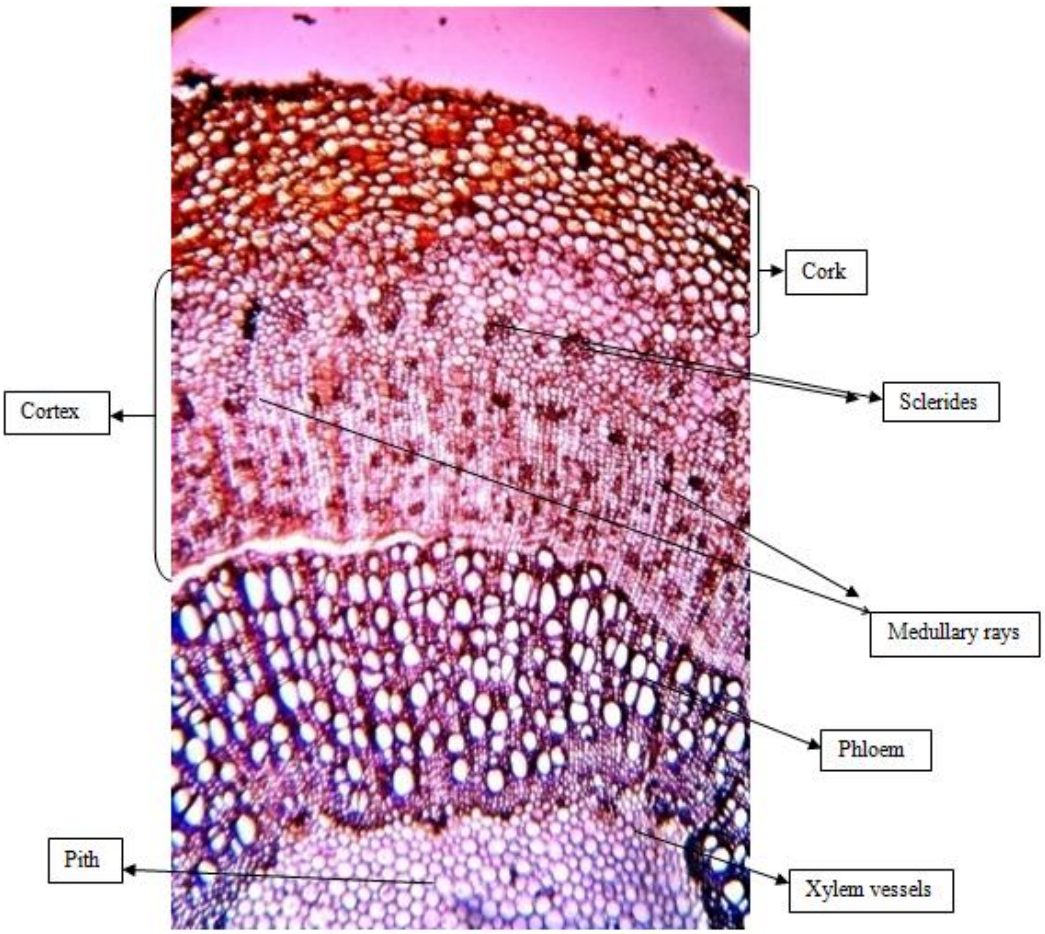
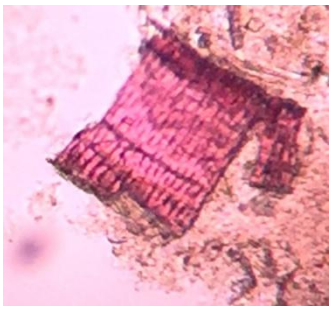
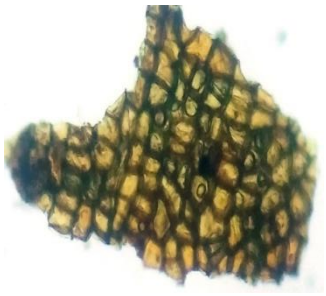


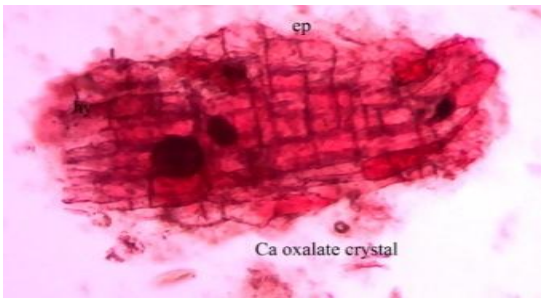
Figure 3: Powder microscopy of *Polygonum alpinum* showing variousmicroscopic structures



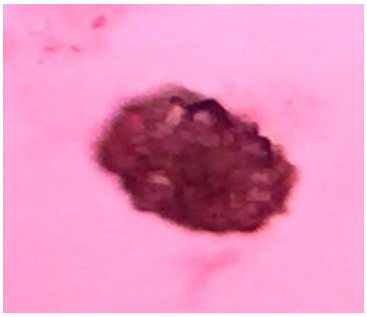
Fragments of Bordered pitted vessels



Cork in surface view



Epidermis (ep.) and hypodermis (hy.) in surface view, with calcium oxalate crystal inthe hypodermis



Calcium oxalate crystal

Epidermal layer with non lignified Vascular bundle

3.2 Phytochemical analysis

The result of qualitative phytochemical analysis of crude powder of *Polygonum alpinum* are shown in table 1. The rhizome showed the presence of carbohydrates, cardiac glycosides, coumarin, steroids, terpenoids, flavonoids, saponins and tannins.

Table 1: Showing Phytochemical screening of *Polygonum alpinum*

Plant constituent Test reagent used	Pet. ether extract	Chloroform extract	Ethyl acetate	Methanol extract	Aqueous extract
Alkaloids	-	-	-	-	-
Carbohydrates	-	+	+	+	+
Anthraquinone glycosides	-	-	-	-	-
Cardiac glycosides	-	+	+	+	+
Coumarin	-	+	+	+	+
Proteins and amino acids	-	-	-	+	+
Flavonoids	-	+	+	+	+
Steroids and terpenoids	-	-	+	+	+
Saponins	+	+	+	+	+
Resin	-	-	-	-	-
Tannins	-	+	+	+	+

+ sign indicates Present and - sign indicates Absent

3.3 Physicochemical Study

The physicochemical characterisation of *Polygonum alpinum* rhizome included ash values shown in figure 4. The powdered rhizome pieces were extracted with different solvents by three different methods viz. cold extraction, hot extraction and sucessive extraction. The values are shown in figure 5. Other physicochemical parameters included loss on drying, foreign organic matter, foaming index, swelling index, pH of different solvents, total tannin content and total fat content. These were determined as per WHO guidelines. The results are presented in table 2.

Figure 4: Shows Ash values of *Polygonum alpinum* root

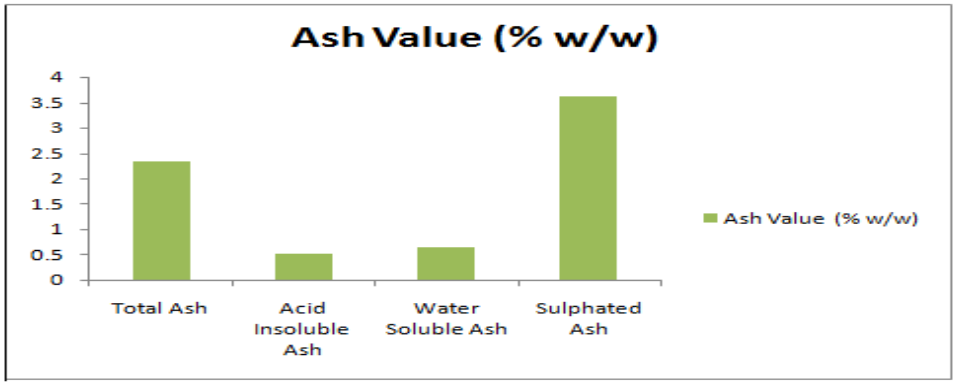
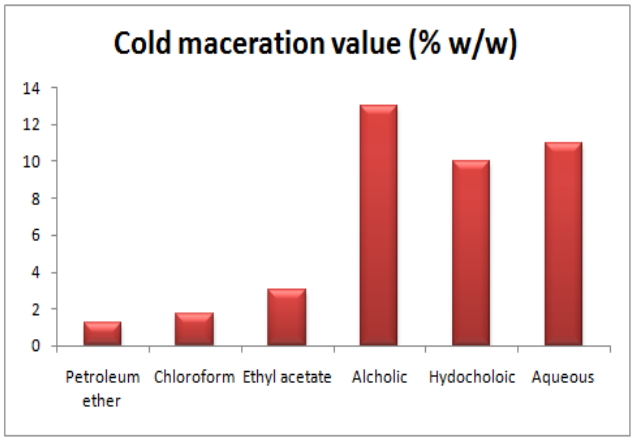
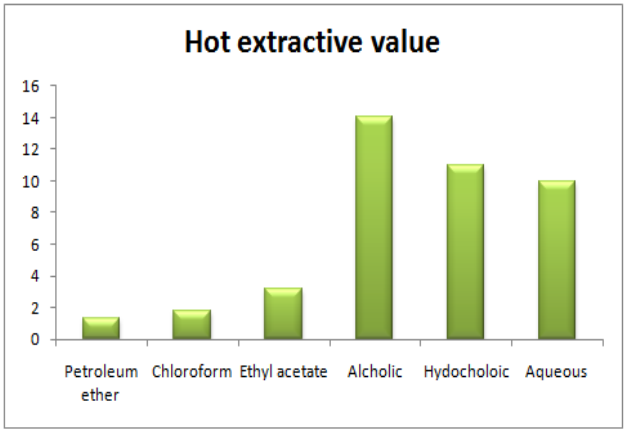


Figure 5: shows Extractive values of *Polygonum alpinum*





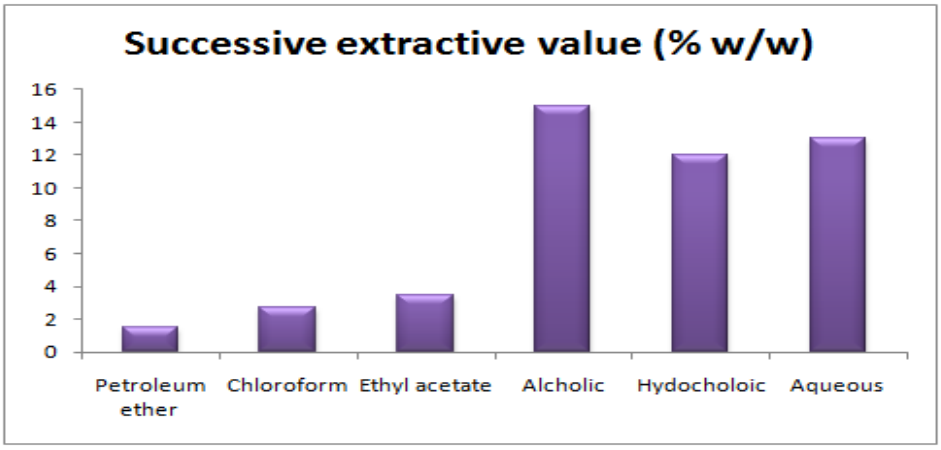


Table 2: shows Other pharmacognostic parameters of *Polygonum alpinum*

Loss on drying	7%
Foreign organic matter	0.8%
Foaming index	Less than 100
Swelling index	2.5 mL
pH	1% (5.70), 10% (5.60)
Tannin determination	26.125%
Fat content	0.68%

3.4 Fluoresence analysis

The fluoresence analysis of powdered drug which plays a vital role in the determination of quality and purity of drug material is summerised in table 3.

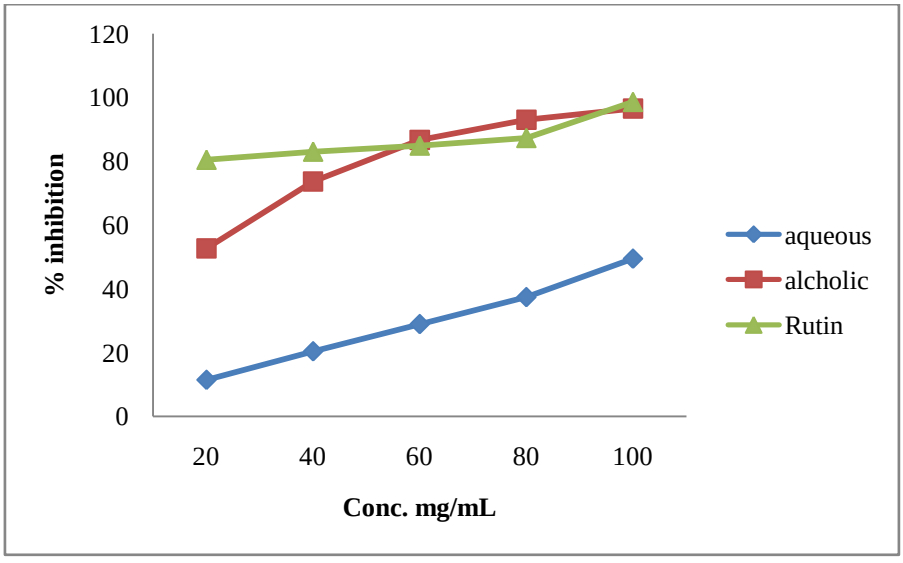
Table 3: Shows Fluorescence analysis of *Polygonum alpinum*

Experiment	Visible/ Daylight	UV Light (254nm)	UV Light (365nm)
Powder as such	Light brown	Greyish brown	Black
Drug + water	Light brown	Greyish brown	Black
Drug+ Acetone	Light brown	Greenish black	Black
Drug+ Pet ether	Brown	Dark brown	Black
Drug+ $CHCl_3$	Brown	Greyish black	Black
Drug + Ethyl acetate	Brown	Greyish black	Black
Drug+ Methanol	Brown	Greyish black	Black
Drug+ Glacial acetic acid	Brown	Greyish black	Black
Drug+ NH_3	Greyish brown	Dark brown	Black
Drug+ HNO_3 (Conc.)	Reddish brown	Greenish brown	Black
Drug+ HNO_3 (dil.)	Reddish brown	Greenish brown	Black
Drug+ Hcl (Conc.)	Dark brown	Black	Black
Drug+ Hcl (dil.)	Dark brown	Blackish brown	Black
Drug+ H_2SO_4	Greyish brown	Greenish brown	Black
Drug+ H_2SO_4 (dil.)	Brown	Greyish brown	Black
Drug + Picric acid	Yellowish brown	Greenish brown	Black
Drug + 5% NaoH	Black Brown	Greenish brown	Black
Drug + 5% $FeCl_3$	Greenish Black	Greenish Black	Black

3.5 DPPH free radical scavenging

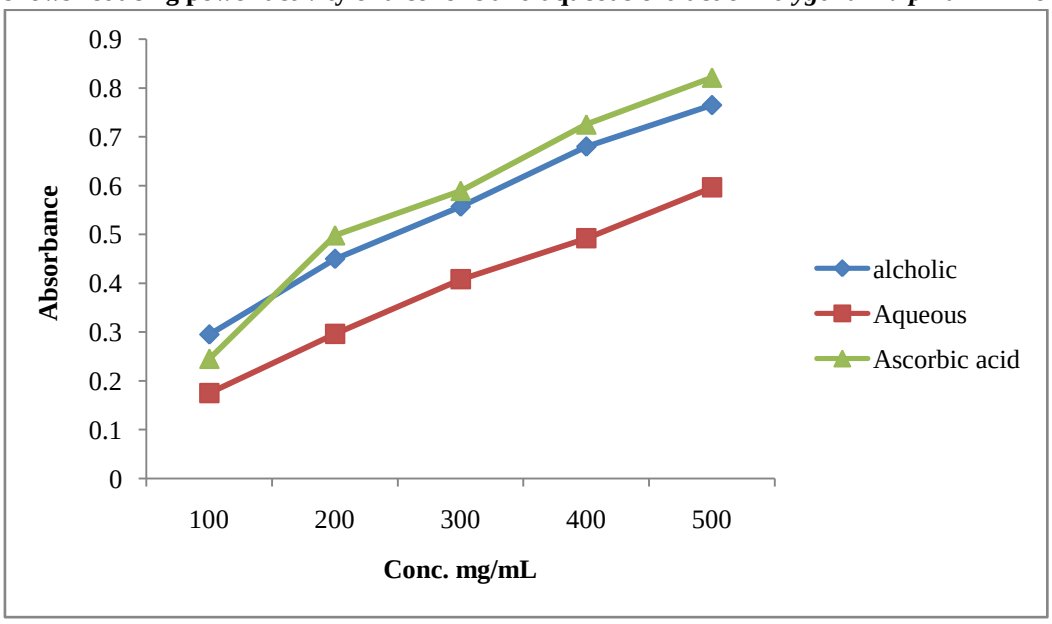
The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical with a maximum absorption at 517 mm is quintessentially considered as a stable radical that can readily undergo reduction by an antioxidant. Due to the universally accepted practice of being this reaction as easy and convenient to manage, it now has widespread use in the free radical-scavenging activity assessment. The DPPH free radical scavenging test is carried out to measure the hydrogen atom or electron donor capacity of the extracts to the stable DPPH radical formed in solution [18]. This method is widely used to evaluate the free radical scavenging ability of various samples [19]. DPPH free radical scavenging of alcoholic and aqueous rhizome extract of *Polygonum alpinum* was found to be 96.50% and 49.44% respectively at concentration 100 µg/mL, where as rutin at the same concentration produced an inhibition of 98.59%.

Figure 6: DPPH scavenging activity of alcoholic and aqueous rhizome extract of *Polygonum alpinum*.



The ability of alcoholic and aqueous rhizome extract to reduce the Fe^{3+}/ferricyanide complex by forming ferrous products, monitored by measuring the formation of Perl's Prussian blue coloration at 700 nm. Increased absorbance is a natural phenomenon indicating strong reducing power at this wavelength. Fig. 3 shows the reductive capability of alcoholic and aqueous rhizome extract of *Polygonum alpinum* All. Confirming an increase in reducing property with concentration compared to ascorbic acid as standard. The reducing action is considered as an important mechanism of phenolics and can be strongly correlated with the presence of reductones, having the ability to donating hydrogen atom and exert antioxidant action by breaking the free radical chain [20,21].

Figure 7: shows reducing power activity of alcoholic and aqueous extract of *Polygonum alpinum* rhizome.



3.6 Total flavonoid content

Flavonoids are polyphenolic compounds with important medicinal value. These exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, anti-ulcer, anti-allergic, anti-viral and anti-cancer activities and are also capable of effectively scavenging the reactive O2 species because of their phenolic hydroxyl groups [22]. The total flavonoid content of alcoholic and aqueous extracts of *Polygonum alpinum* rhizome extract was 182.71 ± 5.20 and 123.13 ± 3.72 mg/gm respectively in terms of rutin equivalent (RE) (Table 5).

3.7 Total phenolic content

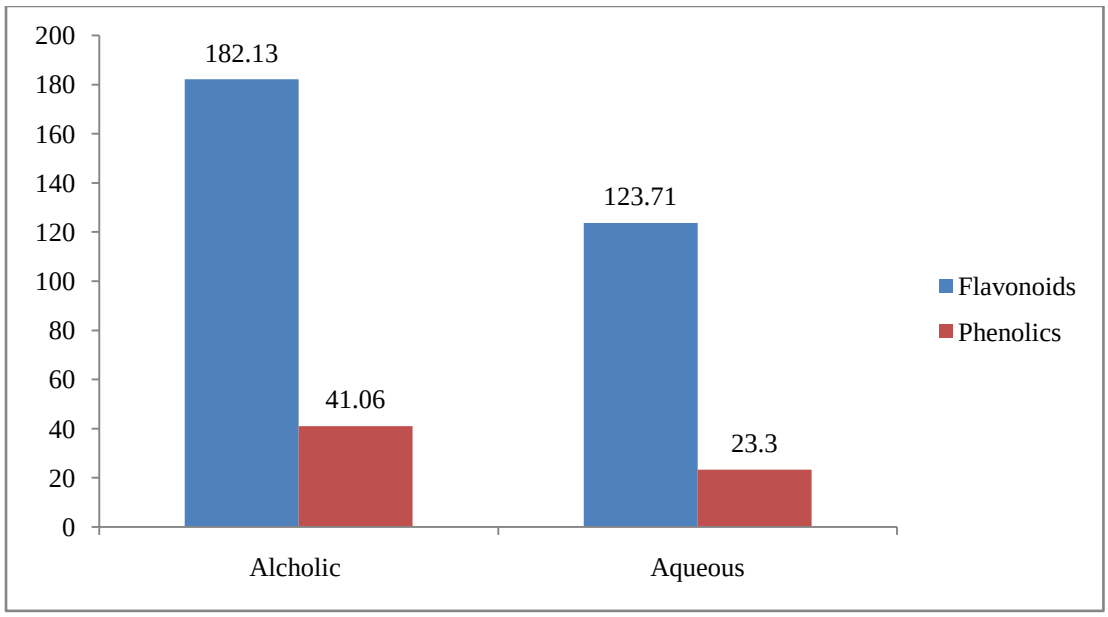
Phenolic compounds are important plant constituents for their powerful chain breaking antioxidant action because of their scavenging ability which is due to their hydroxyl groups [23]. The total phenolic content of alcoholic and aqueous extracts of *Polygonum alpinum* rhizome was 41.06 ± 4.61 and 23.30 ± 6.39 mg/gm respectively in terms of gallic acid equivalent (Table 5).

Table 4: Shows Total amount of phenolic and flavonoid content of alcoholic and aqueous rhizome extracts of *Polygonum alpinum* All.

	Total phenolic mg/g plant extract (in GAE)	**Total flavonoid mg/g plant extract (in RE)**
Alcoholic	41.06 ± 4.61	182.71 ± 5.20
Aqueous	23.30 ± 6.39	123.13 ± 3.72

Data represented as [Mean ± S.E.M. a] of *three determinations performed simultaneously.*

Figure 8: Shows Total Phenolic and Total Flavonoids in alcoholic and aqueous extracts of *Polygonum alpinum* rhizomes



4. Discussion

Proper identification, quality assurance and establishing pharmacognostic standards of crude drugs are very important in their evaluation. Microscopic characters, physicochemical studies and fluorescence analysis of crude drugs are prime steps for their evaluation [24]. According to WHO, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken [25]. In this regard, the microscopic study of the rhizomes was carried out. It showed the presence of epidermis, hypodermis with calcium oxalate crystals, non lignified vascular bundle, calcium oxalate crystals, cork in surface view and fragments of bordered pitted vessels. Other physicochemical parameters studied in this study also serve as a valuable source for judging the purity and quality of the drug. The extractive value obtained by different solvents gives an idea about the chemical constitution of the extracts. The ash value study gives an idea about the presence of earthy matter, inorganic composition and other impurities like adulteration in the drug.

In this study we for the first time are laying down the pharmacognostic standards for the rhizome of *Polygonum alpinum.* These standards can be used as diagnostic tool for the standardization of this medicinal plant and will be helpful in characterization of the crude drug.

Antioxidants play an important role in delaying or preventing degenerative diseases caused by oxidative damage of living cell components as a result of free radicals [26]. The ethanol extract showed a higher scavenging percentage than the aqueous extract. In the studies by Othman *et al* [27], the solvent significantly influenced the measurement of antioxidant properties of an extract. Furthermore, Scalzo *et al* [28] and Giorgi *et al* [29] have shown that there is a different correlation between antioxidant activity and total phenolic content (TPC). Our findings showed a positive correlation between TPC and DPPH for ethanolic extract. The same was previously reported by Gorinstein *et al* [30].

The total phenolic content of alcoholic and aqueous extracts of Polygonum alpinum rhizome was 41.06 ± 4.61 and 23.30 ± 6.39 mg/gm respectively in terms of gallic acid equivalent. TPC for ethanolic extract was higher than corresponding aqueous extract. This is in accordance with the findings of Ling *et al*[31], that predicted higher TPCs in ethanol extracts compared to aqueous extract. In addition, Moure [32] demonstrated that high polarity of solvent yields high amounts of polyphenolics. Therefore, the significant TPC differences of this study may be attributable to the solvents of the extracts shown.

The total flavonoid content of alcoholic and aqueous extracts of *P.alpinum* rhizome was 182.71 ± 5.20 and 123.13 ± 3.72 mg/gm respectively in terms of rutin equivalent (RE). Flavonoids are polyphenolic compounds with important medicinal value. These exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, anti-ulcer, anti-allergic, anti-viral and anti-cancer activities and are also capable of effectively scavenging the reactive O2 species because of their phenolic hydroxyl groups [22]. The reducing action is

considered as an important mechanism of phenolics and can be strongly correlated with the presence of reductones, having the ability to donating hydrogen atom and exert antioxidant action by breaking the free radical chain [33,34].

5. Conclusion

The macroscopic and microscopic description of this medicinal plant is the first step towards establishing its identity and purity and had to be carried out before any tests are undertaken. In this regard, the microscopic study showed the presence of epidermis, hypodermis with calcium oxalate crystals, non lignified vascular bundle, calcium oxalate crystals, cork in surface view and fragments of bordered pitted vessels. Other physicochemical parameters studied in this study also serve as a valuable source for judging the purity and quality of the drug. The extractive values obtained by different solvents give an idea about the chemical constitution of the drug. The ash value study gives an idea about the presence of earthy matter, inorganic composition and other impurities like adulteration in the drug. In this study we are laying down the pharmacognostic standards for the rhizome of *Polygonum alpinum.* These standards can be used as diagnostic tool for the standardization of this medicinal plant and will be helpful in characterization of the crude drug. The antioxidant activity of the extracts is due to presence of Phenolics and flavonoids. Alcoholic extract shows more activity than aqueous extract**.**

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