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MORPHO-ANATOMICAL, HISTOLOGICAL, PHYTOCHEMICAL AND PHYSICOCHEMICAL CHARACTERIZATION *OF DICLIPTERA BUPLEUROIDES* NEES

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Abstract

Dicliptera bupleuroides Nees. a medicinal herb belongs to family Acanthaceae. Morpho-anatomical, phytochemical and physicochemical characterizations of *Dicliptera bupleuroides* Nees were determined. The anatomy of leaf, stem, and root depicted various structures including; fibres, vessels, tracheids, oil cells, starch granules, cortical cells, cork cells, phloem, collenchyma and parenchyma tissues etc. Anatomical sections of leaf, stem and root showed the arrangement of different cells, certain tissues that will serve as diagnostic characters to standardize this plant. In conjunction with these observations palisade ratio, stomatal index, vein termination, vein inlet number of leaf were also calculated. Histochemical features were studied by using Phlorogucinol, conc. HCl, Iodine solution, Ferric chloride and Sudan III solution. These reagents were used to locate the presence of Ca+2 oxalate crystals, lignin, starch, tannins and oil globules, respectively. In fluorescence analysis different colors were noted when powder of whole herb were exposed to ordinary and UV light. Phytochemical screening of methanolic extract of whole herb exhibited the occurrence of saponins, tannins, carbohydrates, flavonoids, glycosides, sterols, lipids and alkaloids. Physicochemical analysis i.e. extractive values and ash values were calculated to strengthen standardization process. These findings and estimations will help in characterization, verification and quality maintenance of *Dicliptera bupleuroides* Nees.

Key words: *Dicliptera bupleuroides* Nees, Morpho- anatomical, Histological, Physicochemical, Phytochemical.

Introduction

Standardization of herbal drugs is the major problem world widely. The significance role of medicinal plants cannot be ignored. In various developing countries, a great number of population rely on medicinal plants to fulfill basic and primary healthcare needs. Therefore historical and traditional uses of medicinal plants have frequently gained popularity (Aburjai *et al*., 2007). The effectiveness of traditional system of modern medicines depends upon the quality of these herbal medicines relies on the proper authentication of the plant material by adopting the standard procedures to standardize the herbal material. Morpho-anatomical evaluations are one of the important tools among this standardization. The initial authentication is carried out by macroscopic characterization of plant material because there are chances of adulteration during collection. This necessitates the need of plant material standardization including qualitative and quantitative estimations (Ishtiaq *et al*., 2016).

Dicliptera bupleuroides Nees. is a perennial herb. It belongs to family Acanthaceae, contain 250 genera and 2500 species. It is found in the planes of Pakistan and Afghanistan as flowering plant (Singh *et al*., 2014). Flora of India indicated it has two taxa: *D. roxburghiana* Nees and *D. roxburghiana* var. *bupleuroides*. Synonyms are *Brochosiphon* Nees and *Dactylostegium* Nees. Common name in Urdu is kaali booti (Ajaib *et al*., 2015).

This species is also arrogated to be used in traditional medicines for applying on wound of snake bite, in fever, in stomach troubles and also used in bone fracture (Panigrahi & Dubey, 1983). Traditionally it is used as general tonic, as diuretic, in skin diseases, on wound of snake bite, in fever, in stomach troubles and also in bone fracture. Flowers used for offerings and ornamental purposes. Plant poultice is good against the bites of wasps and bees (Panigrahi & Dubey, 1983; Qureshi *et al*., 2009; Ummara *et al*., 2013).

Dicliptera bupleuroides possessed antioxidant, hepatoprotective, antimicrobial and other biological activities. It contained phenols, flavonoids, ascorbic acid, lipids, starch, glycosides and many other compounds (Bahuguna *et al*., 1987; Luo *et al*., 2002; Ahmad *et al*., 2013).

Materials and Methods

Plant collection: The plant was collected from Bhimber (shmani), Kotli, Azad Kashmir and got authenticated by Dr. Uzma Hanif, Department of Botany, Government College University (GCU), Lahore, Pakistan. A specimen of plant was deposited in herbarium of GCU under voucher No: GC. Herb.Bot.3402. The plant was dried under shade for about 15 days and then pulverized. Powder was stored in amber colored bottles.

Morpho- anatomical evaluations

Morphological evaluation: Macroscopic evaluations were carried out on 3 samples of each part. The taxonomical description was made according to the related articles and the data given in books (Parveen *et al*., 2020).

Anatomical evaluation: Fresh leaves, stem and root were immediately fixed in formalin: acetic acid: 70% alcohol (5:5:90) for 24 h. TS of leaf, stem and root were made by using microtomes. Sections were stained with safranin and fast green dye (Jaiswal *et al*., 2014).

Histochemical evaluation: The sections of leaf, stem and roots were taken and they were treated with various chemicals to note the chemical reaction and color change. Phlorogucinol, concentrated HCl, Iodine solution, Ferric chloride and Sudan III solution were used to locate the presence of Ca+2 oxalate crystals, lignin, starch, tannins and oil globules, respectively. Inferences were compiled according to the observations made during the histochemical analysis. During analysis sections were

observed under light microscope and their photographs were taken using digital camera (Christodoulakis *et al*., 2015, Zulfiqar *et al*., 2020).

Micrometery of anatomical sections: Chloral hydrate (75%) solution was used as clearing reagent prior to powder microscopy. Slides of powdered leaves stem and roots were prepared according to the prescribed procedures. Micrometer was to measure the size of different cellular structures observed in each sample. Photographs were captured through the microscope with digital camera (Parveen *et al*., 2020).

Quantitative evaluation: Microscopic examination is employed for the quantitative evaluation and for this purpose some cell size measured by using micrometer (Sonibare & Olatubosun, 2015; Ishtiaq, 2016) and specific histological features including, stomatal index, vein-islet and vein termination number and palisade ratio were noted (Najafi & Deokule, 2010; Kumar *et al*., 2008).

Fluorescence analysis: Fluorescence analysis of crude powder of *Dicliptera bupleuroides* Nees was performed by using reagents. The analysis was carried out in ordinary light and UV light *i.e*. short wavelength of 254 nm and long wavelength of 365nm. The results of this analysis are given in (Table 4). These results showed variation in colour which may be helpful in discrimination of original herbal products with adulterated or substandard herbal product (Schoor *et al*., 2015).

Preliminary phytochemical analysis: The preliminary phytochemical analysis of whole herb was carried out according to the standard procedures (Ulukuş & Tugay, 2020).

Physicochemical analysis: This analysis included the extractive values *i.e*.in methanol, aqueous, chloroform, *n*-hexane, butanol, dilute alcohol along with the total ash, water soluble ash, acid insoluble ash and sulphated ash values. All the test were carried out according to standard procedure (Evans, 2003; Ishtiaq *et al*., 2016).

Results

Marphological evaluation: *Dicliptera bupleuroides* Nees is commonly called by local people as krech or kali booti, is perennial herbaceous plant. 1-2 feet in its height, it may vary upto 6 feet. Leaves are ovate (2-6cm) long. Flowers are purple or pink color. Individual flowers reach from 1.5-2 inches (3.8-5.1 cm) long (Table 1).

Anatomical evaluation: TS of leaf showing upper most epidermal cell 3-5 layers of spongy mesophyll, vascular bundles with xylem and phloem and lower mesophyll palisade. TS of stem showing outer boundary of epidermal cells, hexagonal cortex cell, vascular bundles and central pith region. The TS of root also disclosed the presence of epidermis, medullary rays, pericycle fiber, cortex and pith in the center (Fig. 1).

Histochemical analysis of TS: Histochemical analysis of the leaf, stem and root was carried out. The transverse sections were exposed to conc. HCl, phlorogucinol, ferric chloride, iodine solution and Sudan III. The histochemistry of leaf showed the presence of Ca^{+2} oxalate crystals, tannins and starch granules as it gave effervescence with conc. HCl, megnata coloration with phlorogucinol and blue coloration with iodine solution. However, it didn't show any change when exposed to Sudan III and ferric chloride which confirms the absence of oil globules and lignins. Histochemistry of stem demonstrated the presence of Ca^{+2} oxalate crystals, tannins, lignins and starch granules as an effervescent response was observed conc. HCl, megnata coloration when treated with phlorogucinol, greenish black coloration in case of ferric chloride solution and purple coloration in case of iodine solution. It showed red colouration when exposed to Sudan III, which confirms the presence of oil globule in the stem. On the other hand, transverse sections of root showed the presence of Ca^{+2} oxalate crystals, tannins, oil globule and starch granules. Production of tiny bubbles which were seen on the slide under the microscope, megnata coloration of the section and dark coloration of the cells were clear signs of the presence of Ca^{+2} oxalate crystals and tannins respectively. In addition to that, on exposure to iodine solution, blue shade appeared within the slide in certain cells. This proved the presence of starch granules in the root. It was further verified in the powder microscopy of root. Unlike stem, the TS of root is devoid of lignins.

Table 1. Morphological study of leaf, stem and root of *D. bupleuroides*.

Parameters	Leaf	Stem	Root
Taste	Characteristic	Acrid	Bitter
Odor	Pungent	Pungent	Not defined
color	Dark green	Light brown	Light brown
Texture of powder	Fine	Coarse	Coarse
Shape	Ovate	Cylindrical	Fibrous tap
Margin	Linear	-	-
Apex	Obtuse	-	-
Venation	Parallel	-	-
Nature	-	woody	-
Direction of growth	-	Decumbent.	-
Length	5-17 ± 2.68 mm	14-45 ± 5.40 cm	3-7 ± 0.91cm
Width	1.2-2.2 ± 0.216 cm	0.6-0.8 ± 0.04 cm	-
Thickness	0.1-0.4 ± 0.06 mm	0.3-0.7 ± 0.09cm	-
Surface	Smooth	Rough	Rough

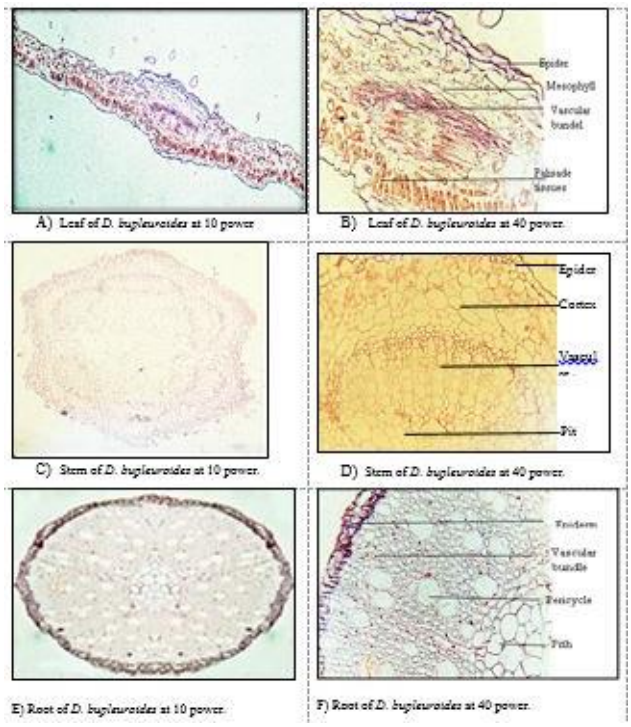

Fig. 1. A and B showing TS of leaf, C and D showing TS of stem, E and F showing TS of root of *D. bupleuroides* nees.

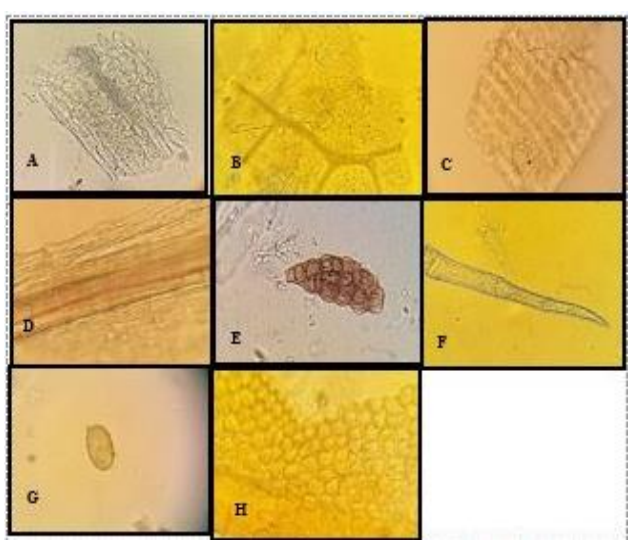

Fig. 2. *D. bupleuroides* leaf A: epidermal tissue, B: epidermis with trichome base, C: epidermis with stomata, D: vessels in leaf lamina, E: head of glandular trichome, F: glandular trichome, G: pollen grain, H: palisade tissue.

Anatomical analysis of leaf, stem and root: The anatomy of leaf shows; epidermal cells with numerous stomata, epidermal tissue contain various layers of palisade tissues, oil cavity, group of vessels, numerous glandular trichomes with base attached to the surface of epidermal cells (Fig. 2). Stem anatomy shows; thin walled cork cells, sclerides, lignified xylem parenchyma cells, widely distribution of reticulate vessels through the region, glandular trichomes with covered head and calcium oxalate crystals (Fig. 3). Root shows epidermal cells with few stomata, pith cells, part of cork and phelloderm, thin wall parenchyma cells and phloem cells with (Fig. 4).

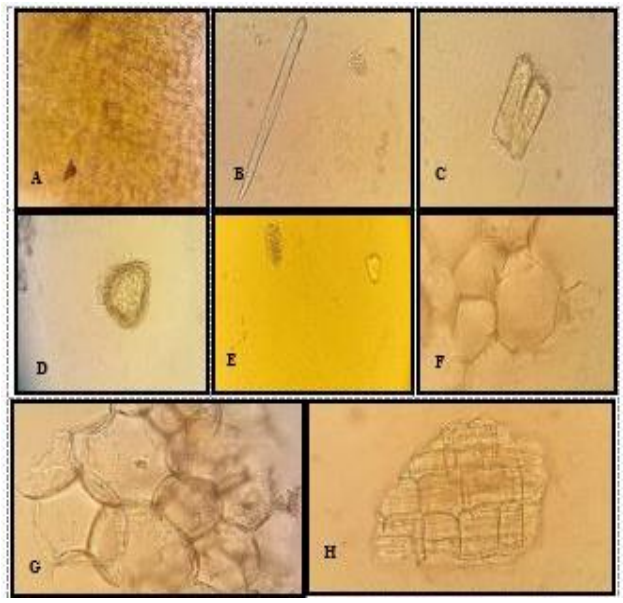

Fig. 3. *D. bupleuroides* stem A: epidermal tissue, B: spindal shape fiber, C: sclerides, D: oil duct, E: prism shape crystals, F and G: medullary rays, H: cork and cortex.

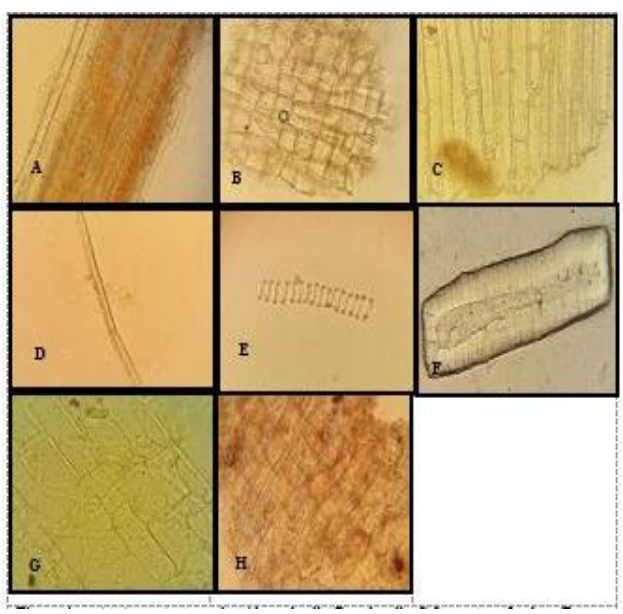

Fig. 4. *D. bupleuroides* root A: epidermal cell, B: cork cell, C: fragments of xylem, D: part of fiber, E: reticulate vessel, F: sclerides, G and H: parenchymal cells.

Quantification of anatomical analysis: In powder microscopy of leaf shows epidermal cell length 70µm (73.533 ± 4.347) and width is 18µm (17.733±2.171). Trichome length is 122µm (122.66±1.440) and width is 20µm (19±1.699), reticulate vessels show length 60-92µm (78.33±8.277) and width 13-18µm (14.33±0.720). Size of stomata is 22µm (22.66±1.44) length wise and width is 11 µm (11.66 ± 0.720). Parenchyma cell has length of 45µm (43.8±0.748) and width of 44.5µm (44±0.471). Head of trichome has length 31µm (26.33±2.76) and width 22µm (19.33±1.08). Also contain pollen grain various sizes with length 40sµm (30± 4.714) and width of 18µm (16.33±0.720).

In stem powder different structures are found and their measurements taken by using micrometer. Cork cell length is 39.9µm (13.33±1.186) and width is 11µm (6±0.942), reticulate vessels length is 133µm (78.33±8.277) and width is 11µm (14.33±0.720). Length of parenchyma cell is 44.4µm (43.8±0.748) and width is 45µm (44± 0.471). Epidermal cell length is 84 µm (73.533±4.347) and width is 13µm (17.733±2.171). Length of oil duct is 25-30µm (27.33±1.186) and width is 18-26µm (22±1.885).

In powder microscopy of root show different cells, epidermal cell length is 66.6µm (73.533±4.347) and width is 22.2µm (17.733±2.171). Schlerides legth is 66.6µm (24.66±11.55) and width is 16µm (16.33±0.720). Cork and phelloderm cell length is 27µm (13.33±1.186) and width is 15.5µm (6.0±0.942). Parenchyma cell length and width is 44.4µm (43.8±0.748), 11µm (44±0.471) respectively. Also contain fibers of various sizes with length 133µm (124.93± 5.894) and width of 10µm (10±0.942) (Table 2).

The quantitative evaluation helped in establishing leaf constants including; palisade ratio (5.7±0.346), stomatal index (11±1.212), vein-islet number (13.25±1.125) and vein termination number (28.7±0.923) (Table 3).

Fluorescence analysis: The fluorescence analysis of whole herb revealed various colors of the extracts under ordinary light, Short wavelength (254 nm) UV light, and Long wavelength (365 nm) UV light indicating the presence of fluorescent compounds in the methanolic extract (Table 4).

Preliminary phytochemical analysis: The phytochemical screening of whole herb mainly revealed the presence of terpenoids, tannins, glycosides, flavonoids, alkaloids, proteins, carbohydrates, saponins, fats and fixed oils (Table 5).

Physicochemical constants: The extractive value of whole herb in methanol was high, followed by dilute alcohol. The n-hexane extractive value was the lowest as compare to other solvents (Table 6). The ash values of whole herb showed high content of total ash and water-soluble ash followed by acid insoluble ash (Table 7).

Discussion

Literature revealed that both qualitative and quantitative studies are important for identification of raw material during drug manufacturing (Kumar *et al*., 2011, Singh *et al*., 2010). Herbal drug's purity, safety, identity and quality can be maintained by means of standardization which involve in both the qualitative and quantitative parameters (Ishtiaq *et al*., 2018). Among the qualitative parameters powder microscopy is the simplest and more economical measure to identify and standardize the plant material. Quantitative parameter included determination of the size of different cells by using micrometer, stomatal index, vein islet, vein termination number and palisade ratio are specific for certain species and measure. In the findings about *D. bupleuroides* Nees the transverse section of leaf showed arrangement of epidermis, spongy mesophyll, vascular bundles and lower palisade tissues. The abundant stomata were also observed in the upper epidermis. The TS of stem vascular bundles arranged in circular form along with pith cells, cortex and epidermis. The TS of root also disclosed the presence of epidermis, medullary rays, pericycle fiber, cortex and pith in the center (Fig. 1).

Histochemical assessment of transverse sections were carried out at cellular level which demonstrated the existence of tannins, lignin, Ca+2 oxalate crystals and starch granules in the leaf, stem and root. Effervescent response of all parts indicated the presence of Ca+2 oxalate crystals. Blackish coloration in all parts indicated the existence of tannins. Megnata coloration in all parts except stem indicated the presence of lignin. Similarly blue color indicated the presence of starch granules in all the parts. Pinkish red coloration only in the stem section indicated the presence of oil cells.

The powder microscopy of leaf of the herb showed the epidermal cells with numerous stomata, epidermal tissue contain various layers of palisade tissues, group of vessels, numerous glandular trichomes with base attached to the surface of epidermal cells (Fig. 2). The powder microscopy of the stem showed the thin walled cork cells, lignified xylem parenchyma cells, widely distribution of reticulate vessels through the region, glandular trichomes with covered head, and calcium oxalate crystals, oil cavity and oil cells with associated tissues (Fig. 3). The powder study of root revealed the epidermal cells, thick walled schlerides cells, pith cells, part of cork and phelloderm region, thin wall parenchyma cells and fibers (Fig. 4).

Table 2. Micrometry of some cells of *D. bupleuroides* ness.

Type of cells	Length in micrometer (µm)	Width in micrometer (µm)
Epidermal cell	73.533 ± 4.347	17.733 ± 2.171
Glandular trichome	122.66 ± 1.440	19 ± 1.699
Reticulate vessels	78.33 ± 8.277	14.33 ± 0.720
Sclerides	24.66 ± 11.55	16.33 ± 0.720
Stomatal cell	22.66 ± 1.44	11.66 ± 0.720
Parenchyma cell	43.8 ± 0.748	44 ± 0.471
Cork cells	13.33 ± 1.186	6 ± 0.942
Pollen grain	30 ± 4.714	16.33 ± 0.720
Fibers	124.33 ± 5.894	10 ± 0.942
Oil duct	27.33 ± 1.186	22 s ± 1.885

Table 3. Leaf constants of *D. bupleuroides* nees.

Leaf constants	Range	Mean ± SEM
Palisde ratio	5.1-6.3	5.7 ± 0.346
Stomatal index of lower epidermis	8.9-13.1	11 ± 1.212
Vein-islet number	11.3-15.2	13.25 ± 1.125
Vein termination number	27.1-30.3	28.7 ± 0.923

Table 4. Fluoorescence analysis of *D. bupleuroides* nees.

Protocol	Observations		
	Ordinary light	Short wavelength (254nm)	Long wavelength (365nm)
Powder	Light brown	Light brown	Green
$FeCl_3$	Orange	Yellow	Dark brown
Conc. HCl	Light brown	Light yellow	Dark yellow
Conc. HNO_3	Light orange	Light yellow	Pale green
Conc. H_2SO_4	Choclate brown	Dark green	Purple brown
1M NaOH	Lemon yellow	Dark green	Light yellow
$AgNO_3$	Light yellow	Cream	Off white
Water	Light yellow	Green	Light green
CCl_4	Light green	Light orange	Light pink
Methanol	Light green	Light brown	Pink
CH_3COOH	Light green	Light green	White
Xylene	Brown	Light yellow	Creamy white
NH_3	Brown	Yellow	green
I_2	Redish brown	Dark yellow	Orange
Br-Water	Dark orange	Light yellow	Dark purple
Aniline	Red	Dark orange	Purple
Pet-ether	Light brown	Light yellow	Yellow
Ethyl Alcohol	Light green	Light brown	Pink
Picric acid	Yellow	Dark yellow	Dark orange
KOH	Colorless	Light brown	White
n-hexane	Light brown	Light yellow	Light green
Acetone	Colorless	Yellow	Pink

Table 5. Preliminary phytochemical analysis of *D. bupleuroides* nees.

Phytochemical group	Name of test	Crude extract
Terpenoids	Salkowaski test	+ + + +
	Liebermann's test	+ + + +
Tannins	Ferric Chloride test	+ + + +
	Bromine water test	+ + + +
Glycosides	Bromine water test	+
	Keller killani test	+
	Legal 's test	+ +
Flavonoids	Alkaline reagent test	+ + +
	Lead acetate test	+ + +
Alkaloids	Mayer 'test	+ +
	Wagner 'test	+ +
	Hager 's test	+ +
Proteins	Millon 's test	+ +
	Ninhydrin test	+ +
Carbohydrates	Molisch 's test	+ + +
	Benedicts 's test	+ + +
Saponins	Foam test	+
Fats and Fixed oil	Spot test	+ +

Table 6. Extractive values of *D.bupleuroides* Nees.

Parameters	Percentage yield ± SEM
Methanolic	5.72 ± 0.066
Aqueous	10.156 ± 0.073
Chloroform	2.23 ± 0.0094
n-hexane	0.07 ± 0.0047
Butanol	1.166 ± 0.027
Dilute alcohol	3.23 ± 0.168
Pet.ether	2.803 ± 0.167

Table 7. Ash values of *D. bupleuroides* nees.

Parameter	Value % (w/w)
Total ash	35.55 ± 0.336
Acid insoluble ash	16.28 ± 0.171
Water soluble ash	1.936 ± 0.028
Sulphated ash	39.806 ± 0.057

The quantitative evaluation with help of micrometer and with TS of leaf helped in the establishment of leaf constants that proved to be a helpful tool to aid in the authentication and confirmation process of this therapeutically important drug (Tables 1-3). The fluorescence analysis is an appreciated, easy and direct method for the identification of fluorescent compounds.

Different compounds give fluorescence when they are exposed to short and long wavelength UV light (Joshi, 2012). The powder of whole herb gave various colors when observed under ordinary daylight, in short and long wavelength of UV light (Table 4). The preliminary phytochemical screening of methanolic extract of *D. bupleuroides* has been done (Table 5). Extractive values of whole herb powder in methanol, aqueous, chloroform, *n*-hexane, butanol, dilute alcohol and petroleum ether the results are tabulated in (Table 6). The ash values for the powdered plant are calculated to figure out the amount of siliceous material left over in the residue and to determine the extraneous material adhered to the plant while its collection (Mukherjee, 2002). The ash values of whole herb were carried out and the results are presented in (Table 7).

Conclusion

The study of morpho-anatomical features of *D. bupleuroides* had shown the standards, which will be effective parameters in the identification and recognition of its purity and genuineness. Physicochemical parameters such as ash values, extractive values, phytochemical, histochemical and fluorescence analysis are indicators of the quality of material. These evaluations and physicochemical characterization all are anatomical features helpful for a researcher in their research work for authenticity of this plant material.

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