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Quality control standardization of *Blumea lacera (Burm.f.) DC.* Leaves

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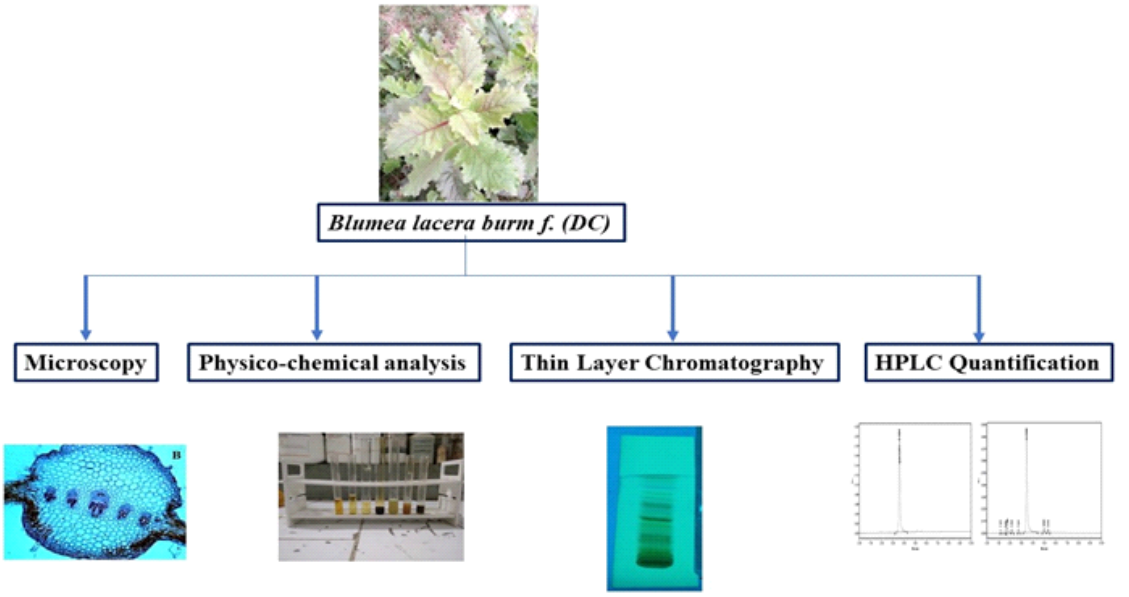
Graphical Abstract



Abstract

Background: Blumea lacera (Burm.f.) DC. (Asteraceae) is one of the annual herb indigenous to plains of North-west India, Tropical Africa and South East Asia**.**

*Hypothesis:*Despite the popular exploitation of this herb as medicine, still no proper study has been reported regarding the pharmacognostical standardization of its leaves. Thus, the aim of the present study was to scientifically establish a standard monograph based on pharmacognostical and phytochemical aspects as per World Health Organization guidelines.

Method: The diagnostic characters of the *Blumea lacera (Burm.f.) DC.* Leaves were evaluated based on the macroscopical and microscopical characters. Heavy metal content, microbial load assesment, fluorescence drug analysis and preliminary phytochemical screening of different fractions were also carried out.

Results: Determination of various physicochemical parameters such as total ash (23.412% w/w), water soluble ash (1.615% w/w), acid insoluble ash (14.625% w/w)**,** alcohol soluble extractive (8.28% w/w), water soluble extractive (19.56% w/w), hexane soluble extractive (2.3% w/w), loss on drying (17.533 % w/w) and crude fibre content (2.75% w/w) were ascertained.Total phenols (367.4779±7.83 mg/g gallic acid equivalent), tannins (29.039±1.109 mg/g tannic acid equivalent), flavonoids (244.751±3.003 mg/g rutin equivalent, RE), flavonols (48.284±1.465 mg/g RE) and, total alkaloidal content (25.56±1.926 mg/g atropine equivalent) were quantified from the ethanolic extract of the leaves of *Blumea lacera (Burm.f.) DC.* (EBL).Quantification of quercetin in the EBL was assessed by HPLC analysis and was found to contain 31.603±1.141 mg/g.

Conclusion: The obtained qualitative and quantitative standards will provides referential information for correct identification and standardization of this medicinal plant.

Keywords: *Blumea lacera (Burm.f.) DC.*, pharmacognosy, quercetin, HPLC

Introduction

From the time, immemorial herbs have been used in all cultures throughout history to cure diseases (Doughari, 2012). The evidence of the use of herbal remedies some sixty thousand years ago by Neanderthal man was revealed in 1960 in a cave in Northern Iraq (Solecki and Shanidar, 1975). As per WHO, 25% of modern medicines are derived from the herbalorigin, and almost 80% of world's population is dependent on herbal medicine for their primary care treatment more abundantly in developing countries like India. Though, herbal drugs are of great interest still the complete systematic information of all the herbs used as medicine is not yet documented. Thus, herbal drugs lack wide acceptance.

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Safety and effectiveness are the basic need for all medicines, whether they are synthetic or of plant origin (Anonymous,1998,1998a,2000). The term "herbal drugs" denotes the plants or plant parts that have been converted into phytopharmaceuticals by processes such as harvesting, drying, and storage(Anonymous,1998). Hence, the occurrence of variation is common. This variability may also be due to differences in growth, geographical location, and time of harvesting. Standardization of herbal medicines is the assurance of quality, efficacy, safety, and reproducibility by prescribing a set of standards, constant parameters and different qualitative and quantitative tests. Hence, standardization is a tool in the quality control process of the herbal drugs.

*Blumea lacera (Burm.f.) DC.*is an annual herb with a strong odor, distributed throughout the plains of North-west India (up to 2,900 m altitude), and is also found in Tropical Africa, South East Asia, Bangladesh, Nepal, Indonesia, Malaysia, Philippines, Thailand, Vietnam (Khare, 2004). In traditional literature, several names had been given to this plant viz. Ayurvedic: Kukundara, Kukkuradru, Tamrachuda, Mriducchada; Kukrondaa. Unani: Kakrondhaa. Siddha: Narakkarandai. *Blumea lacera (Burm.f.) DC.* is a herbaceous plant, tomentose, capitula in axillary and terminal dense to lax panicles, achene, ribbed, Pappus whitish, camphor-like odor, highly variable, grown in grassland, field, roadside and forest edge (Pornpongrungrueng et al,2016). The stems are erect, simple or branched, very leafy of this hairy or glandular herb and are 1-2 ft in height. The leaves are 5-12 cm long, 2-6 cm wide, obovate or oblanceolate, stalked, and toothed or (rarely) lobulated at the margins smaller toward the top, leaves are eaten as a vegetable. The bright yellow flowering heads are about 8 mm across, borne on short axillary cymes, and collected in the terminal, spike-like panicles. According to Bhaavaprakaasha, the herb can cure fever, bronchial infections, vitiated blood. Traditionally, it is used internally and externally as a styptic and anti-inflammatory agent. The juice of leaves is mixed with black pepper (*Piper nigrum*) & given to cure bleeding piles. This herb is also given as anthelmintic, particularly for threadworm. Leaves and roots are used as astringent, diuretic and febrifuge. Bruises and ulcers can also be treated by applying fresh juice or extract (Khare, 2004 and Quattrocchi, 2012).

Materials and Methods

Pharmacognostical evaluation:

Morphological, histological and powder evaluation:

The leaves of *Blumea lacera (Burm.f.) DC.*were collected after monsoon from botanical garden of Banaras Hindu University, Varanasi & authenticated by the Department of Botany, Banaras Hindu University. Macroscopic examination such as Size, Color, Texture, Odour was done as per WHO; the fresh leaves were cut into suitable sizes for performing microscopy as per WHO. Numerous free hand sections of fresh leaves were taken, these sections were stained with safranin and phloroglucinol then observed under a compound microscope at projection 10X and 40X then photographs of different magnifications were taken with Nikon Eclipse E200 microscopic units.For studying the crystals, fibers, and lignified cells, the polarized light was employed. Since these structures have birefringent property, under polarized light, they appeared bright against a dark background. For studying the isolated cells, leaves were macerated with concentrated nitric acid and potassium chlorate, washed with distilled water and mounted in glycerin. For micromorphological investigation viz. for leaf constants, fresh material was used, and standard peel study was followed. Stomatal index, palisade ratio, vein-islet and vein termination numbers were calculated (Wallis, 1984).

Nutritional content analysis and physicochemical evaluation:

Nutritional content analysis was done for powdered leaves in order to collect information about vitamins (vit.K and vit.C), calories, total protein and fats. All the above contents were determined by using Association of Official Analytical Chemist Method. Physicochemical constants such as Foreign organic matter, Loss on drying, moisture content, Water soluble extractive, Alcohol soluble extractive, swelling index, Foaming index, microbial count, heavy metal analysis, ash values such as total ash, water-soluble ash, acid-insoluble ash were carried out on shade dried powdered drug as per the official methods described in WHO guidelines and Indian Herbal Pharmacopoeia (Anonymous, 2002,2002a). Volatile oil content determination was done as per the method described by Clevenger and WHO (Anonymous, 2002, Clevenger,1928). Fluorescence analysis study of powdered drug was carried out in visible/daylight and UV light (254nm & 365nm) as per the standard procedures(Kokoski, et al,1958). Determination of crude fiber was done according to Dutch method (Khandelwal, 2007).

Phytochemical evaluation:

Preliminary phytochemical screening:

Shade dried leaves of *Blumea lacera (Burm f.) DC.* was coarsely powdered (150g) and extracted with 95% ethanol (400ml) by soxhlation until complete exhaustion of the plant material. The resulting extract was then filtered and concentrated under reduced pressure using Rota evaporator (IKA) to obtain the crude extract of *Blumea lacera (Burm f.) DC.* (EBL). Further, the extract was subjected to preliminary phytochemical tests to examine the presence of various class of phytoconstituents (Khandelwal, 2007).

Quantitative estimation of various classes of phytoconstituents:

Total phenolic content and total tannin content were estimated as per Folin-coicalteau calorimetric method with a few modifications using gallic acid and tannic acid respectively as a

standard compound (Hagerman, et al, 2000). Total flavonoid and flavonol content were estimated using rutin as a standard compound (Kumaran and Karunakaran,2007). Total alkaloid content was estimated using the simple spectrophotometric method based on the detection of the yellow colored complex (470 nm) formed by the reaction of Bromo cresol green with alkaloids (Shamsa, et al 2008). All the tests were performed in triplicates.

Thin layer chromatography (TLC) analysis:

TLC of EBL was done on precoated aluminum silica gel plates F-254 as the stationary phase. In fingerprinting analysis, optimized mobile phase used for developing the chromatogram was Hexane: Chloroform: Ethyl acetate in the ratio of 7: 2: 1. For detection of quercetin, co-TLC was performed using standard quercetin and mobile phase used was Toluene: Ethyl acetate: acetic acid in the ratio of 2.5: 7: 0.25. Detection of the spots was done by spraying various detecting reagents followed by observation under UV at 254 and 365nm.

Standardization of EBL using HPLC:

HPLC analysis was carried out by using Waters 1500-series pump (Milford, MA, USA) attached to Waters 2998 photodiode array detector and data were analyzed by waters Breeze software (Waters, USA). The mobile phase was filtered through a 0.45 μm membrane filter using solvent filtration apparatus (Millipore, USA) and de-aerated prior to use. Samples were also filtered by using a 0.45 μm membrane filter (Waters, USA) for HPLC analysis. Analysis of the sample was carried out in Lichro CART® 250-4 C18 column, and methanol-acetonitrile-water (40:15:45 v/v/v) were used for better peak shape and resolution at UV 368 nm. The chromatographic conditions were as follows: isocratic flow with a flow rate of 1.0 mL/min. Detector's wavelength was 368 nm, and the injection volume was 10 μl, and all chromatographic operation was carried out at room temperature. The presence of quercetin in analyte was confirmed by comparing retention time and UV spectra with that of standard quercetin. Quantification of quercetin in EBL was done in triplicate by using following equation- $y = 146949x + 230111$.

RESULTS

Pharmacognostical evaluation:

Morphological, histological and powder evaluation:

The color of leaves is dark green (upper surface like copper color) with strong camphor-like odor and bitter taste. Leaves are 7-12 cm long, 3-5 cm wide, simple, obovate-oblanceolate with acute apex with a serrate margin. Long pilose and glandular hairs are found on both the surface of the leaf (Fig. 1).

Fig. 1 Plant of *blumea lacera (Burm.f.) DC*

Midrib region, in cross-section shows distinct biconvex outline and is projected out towards the lower epidermis (Fig. 3B). The size of wavy walled epidermal cells is same on both upper and lower surface in midrib as well as lamina. Single layered, thin-walled, and mostly tangentially elongated cells of epidermis on either side is covered with cuticle. Palisade layer is discontinuous near the midrib region. The vascular bundles are surrounded by parenchymatous cells forming a bundle sheath. The vascular bundles are closed, collateral type, 3 to 5 in midrib region. The xylem is arranged in rows where metaxylem is projecting towards upper epidermis. Phloem is present just below the xylem. Lamina in cross-section presents the structure of dorsiventral type of leaf (Fig. 2). Below epidermis single layer of palisade cells are observed in the mesophyll followed by spongy parenchyma cells are situated up to lower epidermis (Fig. 3A) which is traversed by large number of vessels (Fig. 3D). The palisade cells at extreme margin are replaced by spongy parenchymatous cells. The vascular bundle is closed, collateral type. Interestingly, stomata observed are predominantly of "anomocytic," but a few are also anisocytic (Fig. 3C). On the epidermis, both types of trichomes are seen, uniseriate, multicellular covering trichomes and biseriate, multicellular (3-4) glandular trichomes but the latter is lesser in number. Results obtained for powder microscopy and quantitative microscopy are shown in Fig. 4 and Table no. 1 respectively which revealed the presence of prismatic calcium oxalate crystals and simple starch grains.

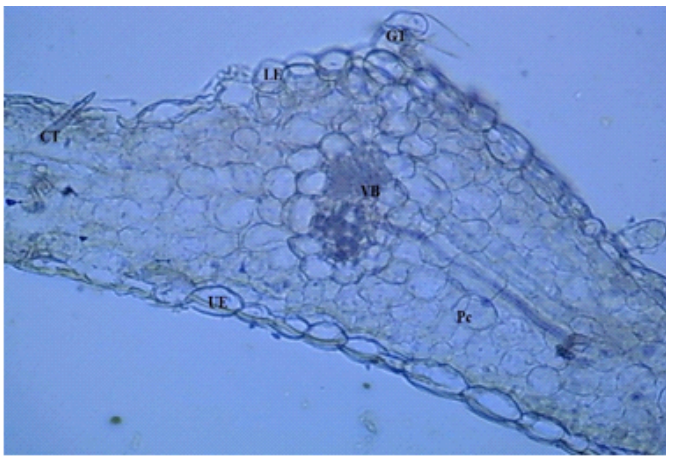

Fig 2- Transverse Section of the leaf through lamina. LE & UE: Lower & Upper Epidermis, VB: Vascular bundles, Pc: Parenchyma, CT: Covering trichomes, GT: Glandular trichomes.

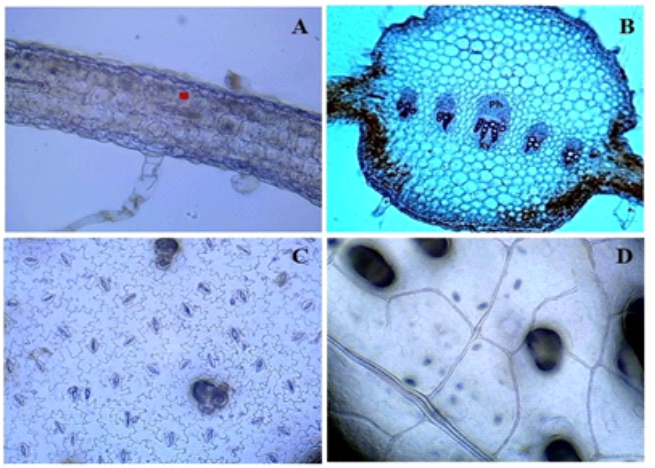

Fig 3- A: T.S. of Lamina (Palisade cells); B: T.S. of Midrib (Ph-Phloem, Xy- Xylem); C: Stomata(Lower Epidermis); D: Veinlet in young leaf.

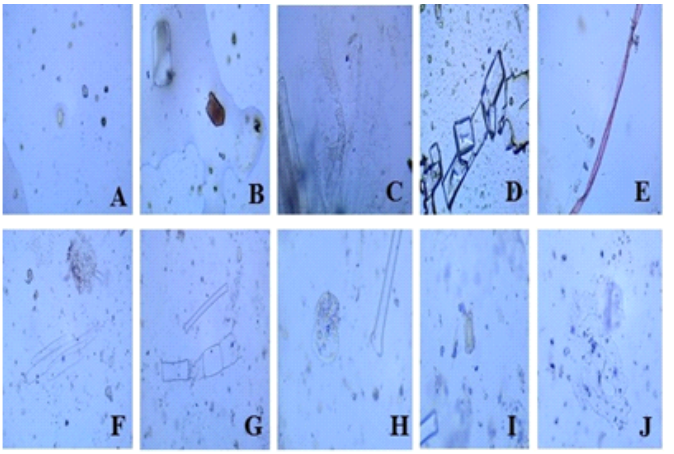

Fig 4- Powder microscopy- A: Oil glands, **B:** Starch grains, **C:** Vascular bundles, **D:** Ca oxalate crystals, **E:** Fibers, **F:** Phloem Fibers, **G-H:** Covering & Glandular trichomes, **I:** Stone cells, **J:** Epidermis

Table No. 1- Leaf constants

LEAF CONSTANT*	UPPER EPIDERMIS	LOWER EPIDERMIS
STOMATAL NUMBER (PER SQ. MM)	9.933±1.75	20.867±3.270
STOMATAL INDEX	18.023±3.206	26.623±2.757
VEIN ISLET NUMBER (PER SQ. MM)	4.72±1.95	
VEIN TERMINATION NUMBER (PER SQ. MM)	1.36±0.49	
PALISADE RATIO	5.5±0.00	

*values expressed as mean±SD

Nutritional content analysis and physicochemical evaluation:

The results obtained after nutritional content analysis shows that the leaves are rich in vitamin K (502.29 µg/100g) showing that *Blumea lacera (Burm f.) DC.* Can be a good source of vitamin K; however, vitamin C was present in appriciable amount (<0.1mg/100g). The results like total carbohydrates, sugar, protein, fat and energy shown that *Blumea lacera (Burm f.) DC.* can be used as food (Table no. 2). The result of various physicochemical evaluation tests is shown in Table no. 3. Results of microbial limits test and heavy metal analysis are shown in Table no. 4 and 5 respectively, and fluorescence analysis result is shown in Table no. 6.

Preliminary phytochemical analysis:

Preliminary phytochemical screening results of EBL shown the presence of phenol, phytosterols, flavonoids, tannins and alkaloids as major constituents and also carbohydrates, reducing sugars, proteins and amino acids. Whereas, hexose sugars, saponins, cardiac glycosides, and anthraquinone glycosides were found to be absent.

Table No. 2- Nutritional content analysis

NUTRITIONALCONTENT	METHOD	RESULTS
PROTEIN	ISO 5983:2005	18.82 g/100 g
TOTAL CARBOHYDRATES	ISO 1656:2007	43.26 g/100 g
TOTAL FAT AS ETHEREXTRACT	AOAC 920.97	1.93 g/100 g
SUGARS	AOAC 945.66	<0.5 g/100 g
ENERGY (CALCULATED)	EASI-GEN-SOP-14: 2015	265.69 kcal/100 g
VITAMIN K	AOAC 999.15	502.29 µg/100 g
VITAMIN C (TOTAL)	EASI-CHE-SOP-112	<0.1 mg/100 g

Table No. 3- Physicochemical parameters

S. NO.	PARAMETERS	VALUES*
1	Swelling index	1.333ml
2	Foaming index	
3	Loss on drying	17.533 % w/w
4	Moisture content (%)	11.75% w/w
5	Water soluble extractive value (%)	19.56% w/w
6	Ethanol soluble extractive value (%)	8.28% w/w
7	Hexane soluble extractive value (%)	2.3% w/w
8	Total ash value (%)	23.412% w/w
9	Acid insoluble ash value (%)	1.615% w/w
10	Water soluble ash value (%)	14.625% w/w
11	Crude fiber content (%)	2.75% w/w

*mean value of three independent readings

Quantitative estimation of various classes of phytoconstituents:

Quantitative estimation confirmed the total phenolic content as 367.4779±7.83 mg/g gallic acid equivalent while total tannin content was found to be 29.039±1.109 mg/g tannic acid equivalent in EBL. Total flavonoids and flavonol content were found to be 244.751±3.003 mg/g and 48.284±1.465 mg/g equivalent to rutin respectively. Total alkaloidal content in leaf was found to be 25.56±1.926 mg/g equivalent to atropine.

Table No. 4- microbial contamination test

PARAMETER	SPECIFIED LIMIT	VALUE
TOTAL PLATE COUNT	<1000 cfu/g	119 cfu/g
YEAST AND MOULD	<100 cfu/g	31 cfu/g
E. COLI	Negative	Negative
SALMONELLA	Negative	Negative
STAPHYLOCOCCIN	Negative	Negative

CFU- colony forming per unit

Table No. 5- Heavy metal content

HEAVY METAL	SPECIFIED LIMIT (PPM)	OBSERVED LIMIT (PPM)
ARSENIC(AS)	<2	0.073
LEAD(PB)	<2	0.029
CADMIUM (CD)	<2	0.0007
MERCURY (HG)	<2	0.091
ZINC (ZN)	<2	0.012

PPM- Parts per million

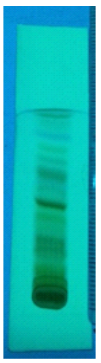

Fig 5- TLC fingerprint of EBL

Thin layer chromatography (TLC) analysis:

TLC fingerprinting analysis (Fig. 5) has shown 13 spots with different Rf values given in Table no. 7. The presence of quercetin was confirmed in EBL by comparing Rf value with that of standard quercetin (Rf. 0.798).

Standardization of EBL using HPLC:

Chromatogram of separated quercetin in EBL and standard is reported in Fig. 6.Under optimum operating conditions, the HPLC method was applied for the identification of quercetin in EBL, and the presence of quercetin was confirmed by direct HPLC–PDA using standard. A peak with retention time at 3.468 minutes was confirmed as quercetin in EBL. The quantity of quercetin in EBL was found to be 31.603±1.141 mg/g.

Table No. 6- Fluorescence powder drug analysis of Blumea lacera (Burm.f.) DC.

S. NO.	POWDER + CHEMICAL	FLUORESCENCE IN DAYLIGHT	LUORESCENCE AT AMAX254 NM	FLUORESCENCE AT AMAX 365 NM
1.	Powder as such	Juniper	NF	Black
2.	Powder + 1N NaOH in methanol	Pear	Shamrock	NF
3.	Powder + 1N NaOH in water	Olive green	Basil	Juniper
4.	Powder + 1N HCl in methanol	Dark fern	Basil	Dark green
5.	Powder + 1N HCl in water	Oldlace	NF	Purple
6.	Powder + 1N HNO3 in methanol	Sage green	Shamrock	Pine
7.	Powder + 1N HNO3 in water	Olive	Old chiffon	NF
8.	Powder + Iodine (5%)	Reddish brown	Seaweed	Dark brown
9.	Powder + FeCl3 (5%)	Lime	Emerald	Pine
10.	Powder + KOH (50%)	Olive	Shamrock	Sage
11.	Powder + NH3 (25%)	Sage	Basil	NF
12.	Powder + Picric Acid	Lime	Green	Fern
13.	Powder + Acetic Acid	Dark fern	Purple	Reddish green

NF- No Fluorescenc

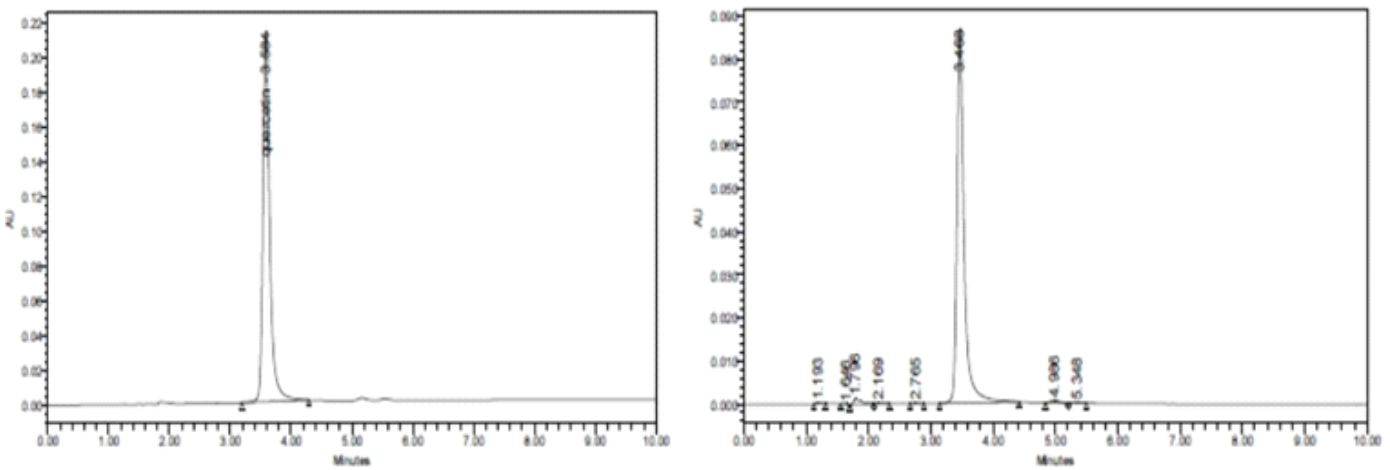

Fig 6- HPLC chromatogram of standard quercetin and EBL

Table No. 7- TLC studies of EBL

S. No.	Rf value	S. No.	Rf value
1.	0.092	8.	0.636
2.	0.138	9.	0.705
3.	0.227	10.	0.735
4.	0.295	11.	0.8
5.	0.409	12.	0.877
6.	0.477	13.	0.923
7.	0.545		

Discussion

Recently, standardization of the traditional medicinal plant has become crucial to establish them as a valuable remedy and to impose them in modern therapy. As per WHO, for diagnosing any herbal drug, pharmacognostic standards may be the primary step, which includes, macroscopic as well as microscopic evaluation of that specific plant/plant part. Further, histological evaluation enhances the understanding of the specific characters and their occurrence in the plant tissue, which is also helpful in identifying the plant/plant part also helps in avoiding misidentification. Therefore, the present study was focused on laying down the standards which could be helpful in proper authentication and identification of *Blumea lacera burm f. (DC).* The pharmacognostic study revealed several diagnostic features in *Blumea lacera burm f. (DC)* which can be an important tool in maintaining the genuine nature of this plant. The specific diagnostic character present of leaf showed the presence of a single layer of undifferentiated palisade cells on the upper epidermis (dorsiventral leaf). Furthermore, the presence of numerous uniseriate, multicellular covering as well as biseriate, multicellular glandular trichomes on both the surface, prismatic calcium oxalate crystals.

In order to detect adulteration and improper handling of the drugs, physicochemical evaluation can serve as a major tool. The presence of various impurities viz. carbonate, oxalate, and silicate which may be naturally occurring or deliberately added to the crude drug as a form of adulterant can be determined by ash values which are quantitative standards. Total ash includes both physiological as well as non–physiological ash, whereas acid insoluble ash consists of mainly silica and shows contamination with the earthy material. The amount of inorganic elements present in drugs can be estimated by water soluble ash (Evans,2002, Kokate, et al,2006). Extractive values are useful to find out the amount of active chemical constituents present in the plant/plant parts using different solvents. Loss on drying indicates the safety of the drug regarding any growth of bacteria, fungi, and yeast. Quantitative estimation of pharmacognostic parameters is an efficient tool to set up standards for crude drugs. Heavy metal (As, Pb, Ca, Hg and Zn) and microbial load for powder drug were found within the limits of WHO guidelines, ensuring the safety of *Blumea lacera burm f. (DC)* to be used free from any unwanted contaminations. Preliminary phytochemical screening revealed the presence of phenols, alkaloids, flavonoids, flavonols and tannins in *Blumea lacera burm f. (DC)*. Such phytochemical screening is helpful in predicting nature of phytoconstituents present in the tested drugs since phytochemicals are proven to be responsible for the pharmacological activity of the drugs. Moreover, the chemical standardization of EBL was also performed with the help of HPLC, and the amount of quercetin was quantified as a chemical marker which is for the first time for this plant. Recently, there has been an increase in demand for plant-derived products in developed countries. These products are increasingly being utilized as medicinal products, nutraceuticals, and cosmetics (Patel, et al,2006). To avoid the use of harmful plant material, pharmacognostic studies and phytochemical screening serve as a basis for proper

identification, authentication, collection, and investigation of the plant. These parameters can be useful in the preparation of the herbal monograph for its proper identification.

Conclusion

Hence, the present study sets all possible standards for *Blumea lacera burm f. (DC)* which will serve as useful information with respect to the identification, authentication, and standardization of *Blumea lacera burm f. (DC)* herb. Also, identification and quantification of quercetin for the first time in this plant may be useful for better exploitation of *Blumea lacera burm f. (DC).*

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