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Standardization of *Clitoria annua* var. *Emarginata*. S.R. Yadav & Dhanke: Leaves

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Abstract
In recent years, the population is switching towards herbal drugs as it shows less/no side effects. The standardization of herbal drugs are necessary. *Clitoria annua* var. *emarginata*. S. R. Yadav & Dhanke is traditionally called as Surguja / Charo / Jungle-wal is indigenous to India. The leaflets are known for its hepatoprotective activities. The investigation is done for the first time to develop pharmacognostical standards of the said plant part. It involves macroscopy, microscopy, histochemistry, powder study, physicochemical and phytochemical analysis. The size and shape of the leaf is an important macroscopic characters. The microscopic features, SEM microscopy, along with powder study shows the presence of glandular and non-glandular trichomes, calcium oxalate crystals, anisocytic and paracytic stomata, etc. The physicochemical analysis and extractive values showed significant results. The phytochemical and histochemical studies, have recognized the presence of secondary metabolites. Thus, these pharmacopeial standards will be useful in authentication of said drug.

Keywords: *Clitoria annua* var. *emarginata*, leaves, pharmacognosy

Introduction
India is the home to the ancient herbal medicine system. The World Health Organization states that 80% of the world population relays on herbal medicine [1]. Concurring to the data cited on the National Health Portal, Indian forests are the largest source of collection for raw materials used in perfumeries and drug manufactory. About 8,000 herbal remedies have been codified in AYUSH systems in India. Ayurveda, Unani, Siddha and Folkmedicines are the major systems of indigenous medicines. Due to this standardization plays a key role in drug manufacture. One such indigenous plant is *Clitoria annua* var. *emarginata* S.R. Yadav & Dhanke, commonly known as Surguja / Charo / Jungle-wal is a herbaceous shrub [2, 3]. In Buldhana district of Maharashtra, the leaves are used for elephantiasis and jaundice by the local healers. The mature seeds are cooked and consumed for five days for fertility. The plant is economically important. The pods are consumed due to its medicinal properties [4, 5].
The folklore claims that the said plant part are used in hepatic disorders. Due to its therapeutic potential, the plant is investigated for the first time for its Pharmacognostic parameters. The study involves macroscopy, microscopy, histochemistry, powder study, fluorescence analysis, physicochemical evaluation and preliminary phytochemical analysis.

Material and Methods
Procurement of Materials: The leaflets of *Clitoria annua* var. *emarginata* S.R. Yadav & Dhanke were used for study. (Figures. 1 & 2). The plant was collected from Sanjay Gandhi National Park, Borivali, Mumbai. It was authenticated from the Botanical Survey of India, Pune Regional center. The fresh as well as preserved leaves/leaflets were used for investigation. For preservation F.A.A (formaldehyde: acetic acid: alcohol) solution was used. The remaining leaflets were shade dried and then grounded to moderately coarse powder for further pharmacognostic evaluation [6].

Pharmacognostic study
Macroscopic study: The fresh leaflets were used to study macroscopic character. With the help of magnifying lens and scale measurements of different parameters were noted [7]. Photographs were taken for evidence.

Microscopic study: The fresh hand cut sections were prepared for microscopic studies [8]. A few dried and fresh leaf samples were sent to Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay,

Powai, for SEM studies, and analyzed in ESEM mode. The sections were observed under the magnification of 25 X to 20,000X. The cell contents were measured using stage and ocular micrometer [9]. The leaf constsnts were also taken into considerations for the following studies such as stomatal index, vein-islet termination number, palliasde ratio and trichome density [10].

Histochemical analysis: The fresh hand cut sections of leaflets were treated with various reagents to localize secondary metabolites by standard methodology [11, 12].

Powder study: The dried powder of leaflets was treated with aqueous chloral hydrate and instilled with 50% glycerin and observed under microscope. The measurements were taken with the help of stage and ocular meter using standard procedure [13].

Fluorescence analysis: Fluorescence analysis was carried out by adding various reagents to dry powder and observed under ultraviolet (U.V.) and ordinary light by the standard procedure [14, 15].

Physicochemical analysis

For standardization of extract, various physicochemical parameters such as moisture content, ash values and extractive values were performed as per standard methodology [16].

Preliminary Phytochemical analysis

The dry powder was extracted with solvents like water, alcohol, and methanol. The extracts were filtered and used for the analysis. This was carried out as per the standard procedure [17, 18].

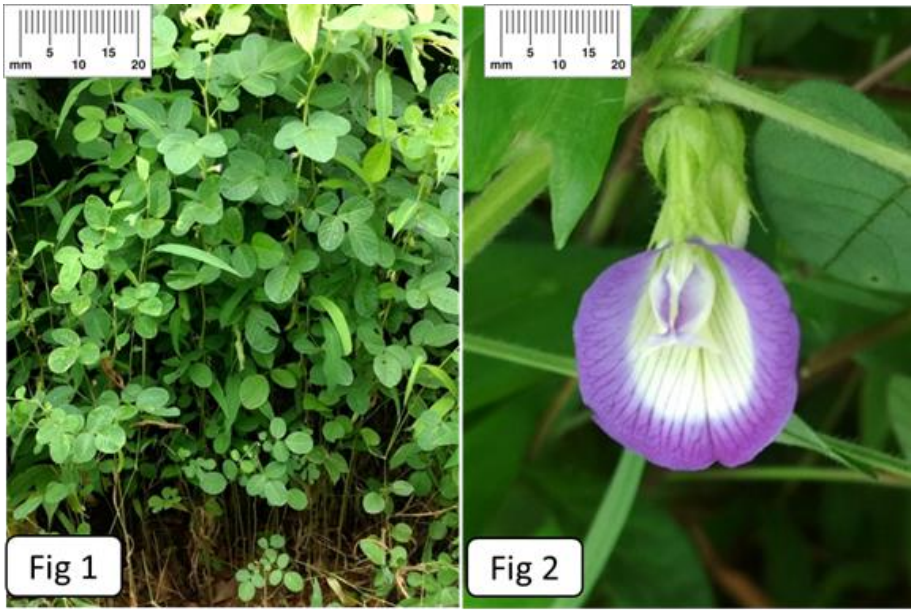

Fig 1: Habit of *Clitoria annua* var. *emarginata;* **Fig 2:** Flower of *Clitoria annua* var. *emarginata*

Results

Macros-copy of Leaves:

The leaves are alternately arranged on the main axis, each leaflet is opposite. The leaves are imparipinnately compound with 5 leaflets. The leaflets is with short petiole and stipules. The apical leaflets size decreases gradually in comparison with the basal leaflets. The length from the base of the petiole to apex of the leaves is 13.20 cms to 14.70 cms. The apical leaflet's length, from the base of the leaflet to apex is 6.50 cms to 7.60 cms, and breadth is 4.50 cms to 5.10 cms. The middle leaflets sizes from 5.4 cms to 6.00 cms in length, 3.50 cms to 4.70 cms in breadth. The length of the lower leaflet sizes from 3.30 cms to 3.90 cms, and breadth is 2.60 cms to 2.90 cms. The leaflet is ovate to elliptical in shape. The venation is unicostate reticulate. The leaflets shows entire margin with mucronate apex and symmetrical base. The surface is smooth, with hairy texture. (Figures. 3 & 4).

Fig 3: Lower surface of lamina

Fig 4: Upper surface of lamina

Organoleptic study

The colour of the upper surface of leaflets is dark green while the lower surface is light green. The leaflets are with characteristic odour and bitter taste.

Microscopic study of leaflets

The T.S of leaflet is dorsiventrally flat.

T.S of leaflet passing through lamina

Upper epidermis: The upper epidermis is single layered tangentially elongated cells measuring 9.60 to 24.20 µm in length and 7.20 to 19.20 µm in breadth. The epidermal cells shows two types of non-glandular trichomes which are bicellular uniseriate. In one type of trichome the apical cell is curved, with smooth wall & smaller in size, measuring 24.00 to 33.60 µm in length and 7.20 to 9.60 µm in breadth. The other trichome is warty, with blunt end and are seen at regular interval measuring 43.20 to 52.80 µm in length and 7.20 to 12.00 µm in breadth.

Mesophyll: The mesophyll is differentiated into palisade and spongy tissues. The mesophyll consists of single layered elongated palisade tissues measuring 26.40 to 36 µm in length and 7.20 to 12 µm in breadth. It is filled up with chloroplast, below the palisades layer, there is spongy tissue. It is 2 to 3 layered in thickness, measuring 9.60 to 19.20 µm in width. It is also chlorenchymatous, with intercellular spaces. The mesophyll is interrupted by poorly developed vascular bundles.

Lower epidermis: lower epidermis is made up of wavy single layered, tangentially elongated cells measuring 9.60 to 28.80 µm in length and 7.20 to 16.80 µm in breadth. It is interrupted with bicellular trichomes, which are uniseriate, like that of upper epidermis. On the lower epidermis stomata and trichomes are more than that on upper epidermis.

T.S leaflet passing through midrib: The upper epidermis is single layered with thick cuticle. The cells are round or slightly elongated 7.20 to 16.80 µm in length and 9.60 to 12 µm in breadth, more trichomes are present in midrib region. Stomata are present on the epidermis of midrib.

The epidermis is followed by the collenchyma cells, it is 5 layered in thickness measuring 12 to 19.20 µm in width. The collenchyma is followed by ring of sclerenchyma which is 2 to 4 layered in thickness, measuring 7.20 to 14.40 µm in width. In the younger leaves, the sclerenchyma cells are present perpendicular to the epidermis, they are present in the form of arch and at maturity two curve are joining together to form a ring of sclerenchyma around the vascular bundles. Inner to the ring it shows the central patch of parenchymatous cells which are measuring from 14.40 to 24 µm in width. On the lower side of vascular bundle it shows phloem while on upper side xylem. Protoxylem is on the upper side and metaxylem is towards inner side. Outer to sclerenchyma above the lower epidermis, it shows the presence of 6 to 8 layered parenchyma cells measuring from 14.40 to 24 µm in width. The calcium oxalate crystals are present in parenchyma cells. The lower epidermis is single layered in thickness and slightly tangentially elongated and wavy. The lower epidermis measures from 7.20 to 14.40 µm in length and 4.80 to 12 µm in breadth. (Figure 5)

SEM: The SEM of leaflets showed cells alike of light microscope. In the magnified images of ESEM, shows the presence of wax crystalloids on both the surfaces. On the lower epidermis, crystalloids are dense and compactly arranged. Gradually at maturity, collenchyma cells show angular thickening with cellulose deposition, as soon as the thickening increases, the lumen becomes smaller. In the similar manner sclerenchyma shows the shrunken lumen because of the lignin deposition in the inner wall. The depositions and thickening can be easily observed under high magnification. The ESEM mode has also revealed the presence of xylem with annual and pitted vessels. The xylem cells also show the slight deposition of the lignin layer. (Figure. 6)

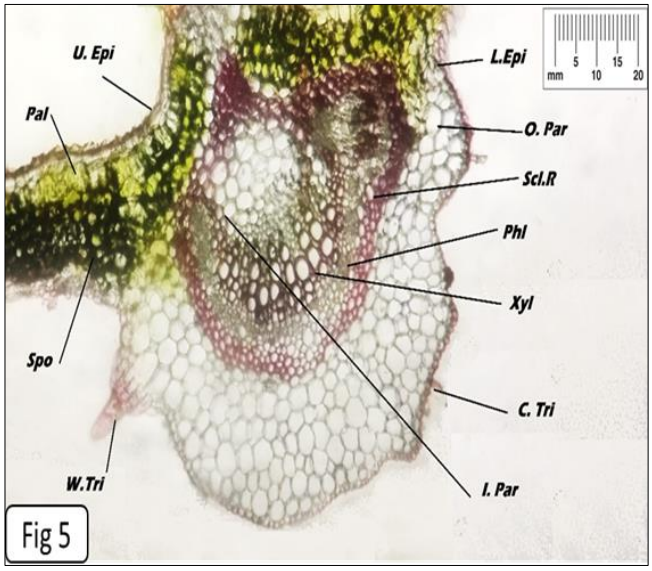

Fig 5: T.S. of leaflet passing through midrib and lamina;

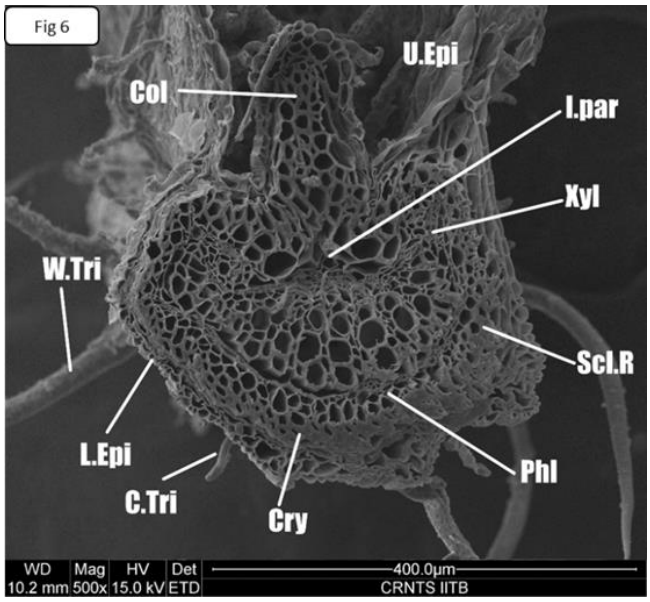

Fig 6: T.S leaflet passing through midrib under Electron Microscope

Abbreviations: U. Epi– Upper epidermis, L.Epi – Lower Epidermis, Pal – palisade tissue, Spo – spongy tissues, Col – collenchyma, Scl.R – Sclerenchyma Ring, O.Par – Outer parenchyma, I.Par – Inner parenchyma, Xyl- Xylem, Phl- Phloem, C.Tri – Curved trichome, W. Tri – Warty trichome. Cry- Crystal.

Leaf constants:

The fresh leaflet were studied for the leaf constant. The results obtained are given in Table 1.

Table 1: Leaf constants of *Clitoria annua* var. *emarginata.* S.R.Yadav & Dhanke leaflets

S. N	Leaf Constants		Observations
1	Type of stomata		Anisocytic and Paracytic type
2	Stomatal index. (Figures. 7& 8)	Upper	7.33%
		Lower	18.76%
3	Measurement	Length	14.40 to 16.80 µm
		Breath	14.40 to 16.80 µm
4	Palisade Ratio		05
5	Trichome Density	Upper	13 to 17
		Lower	08 to 14
6	Vein-islet termination number (Figure. 9)		11 to 15

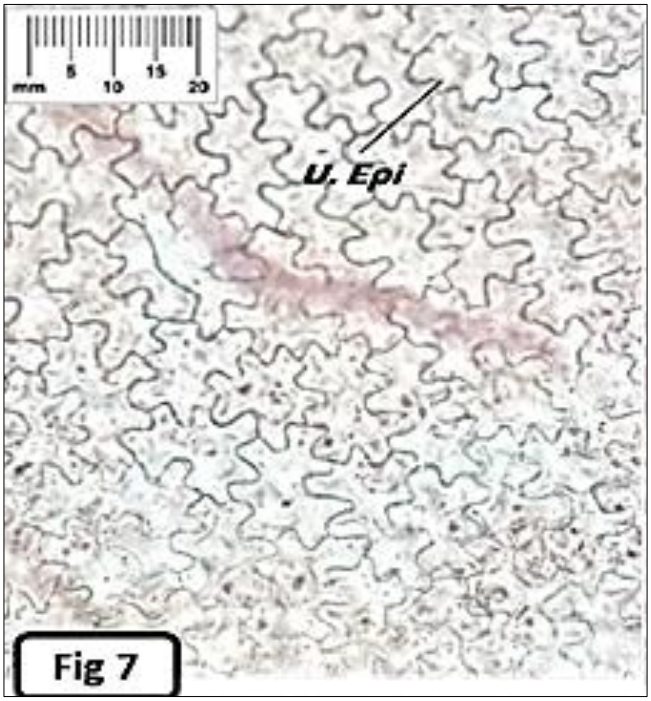

Fig 7: Upper Epidermis of lamina

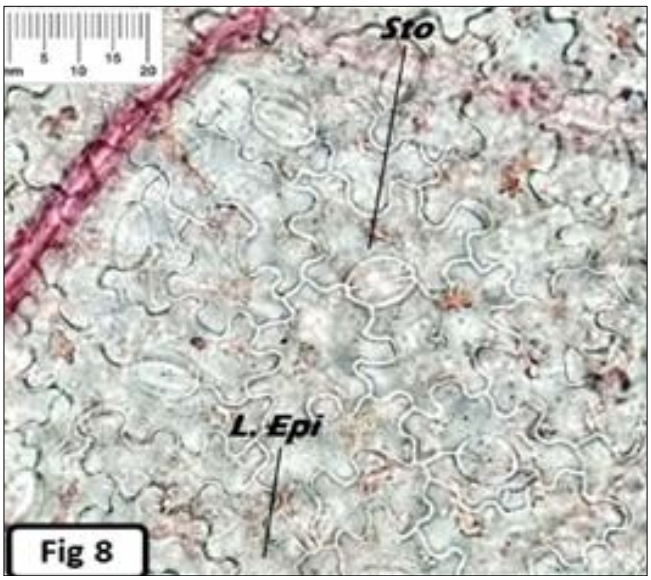

Fig 8: Lower Epidermis of lamina

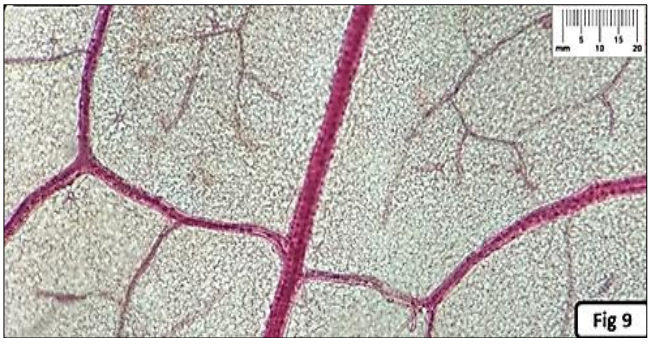

Fig 9: Vein-islet termination

Histochemical analysis

The section of the fresh leaflet was treated with different reagents to study the location of different metabolites. The results are given in Table 2.

Table 2: Histochemical analysis of *Clitoria annua* var. *emarginata.* S.R.Yadav & Dhanke leaflets

Sr. No.	Plant constituent Tests	Observations
1	Test for Starch	-
2	Test for Lipids	-
3	Test for Proteins	+
4	Test for Tannins	+
5	Test for Alkaloids	+++
6	Test for Saponins	+++
7	Test for Glucosides	+
8	Test for Mucilage	+++
9	Test for Calcium oxalate crystals	++

"+++" High concentration, "++" Moderate concentration,"+" Less concentration, and "-" Absent.

Powder study: The dried coarse powder of leaflets is green in colour. After treating powder with basic procedure, the observation leads to some keystone results. The powder shows the presence of simple tissues such as palisade cells measuring 31.20 to 36.00 µm length and 9.60 to 12.00 µm breadth and spongy tissues 7.20 to 9.60 µm width, collenchyma cells measuring 9.60 to 16.80 µm length, sclerenchyma cells measuring 7.20 to 12.00 µm length. The two types of stomata i.e. anisocytic and paracytic, measuring 14.40 to 19.20 µm length and 9.60 to 12 µm breadth. Epidermal cells measuring 16.80 to 24.00 µm length and 7.20 to 16.80 µm breadth and two types of trichomes. The two types of trichomes observed are; A trichome which is bicellular, pointed and with curved apex measuring 07.20 to 12.00 µm length and 4.80 to 09.60 µm breadth with smooth surface and second bicellular with the blunt and straight apex, warty surface, measuring 43.20 to 52.80 µm length and 4.80 to 12.00 µm breadth. The Complex tissues such as Xylem with spiral thickening were also noted. The calcium oxalate crystals are observed measuring 7.20 to 9.60 µm length and 12.00 to 14.40 µm. (Figures. 10 – 18).

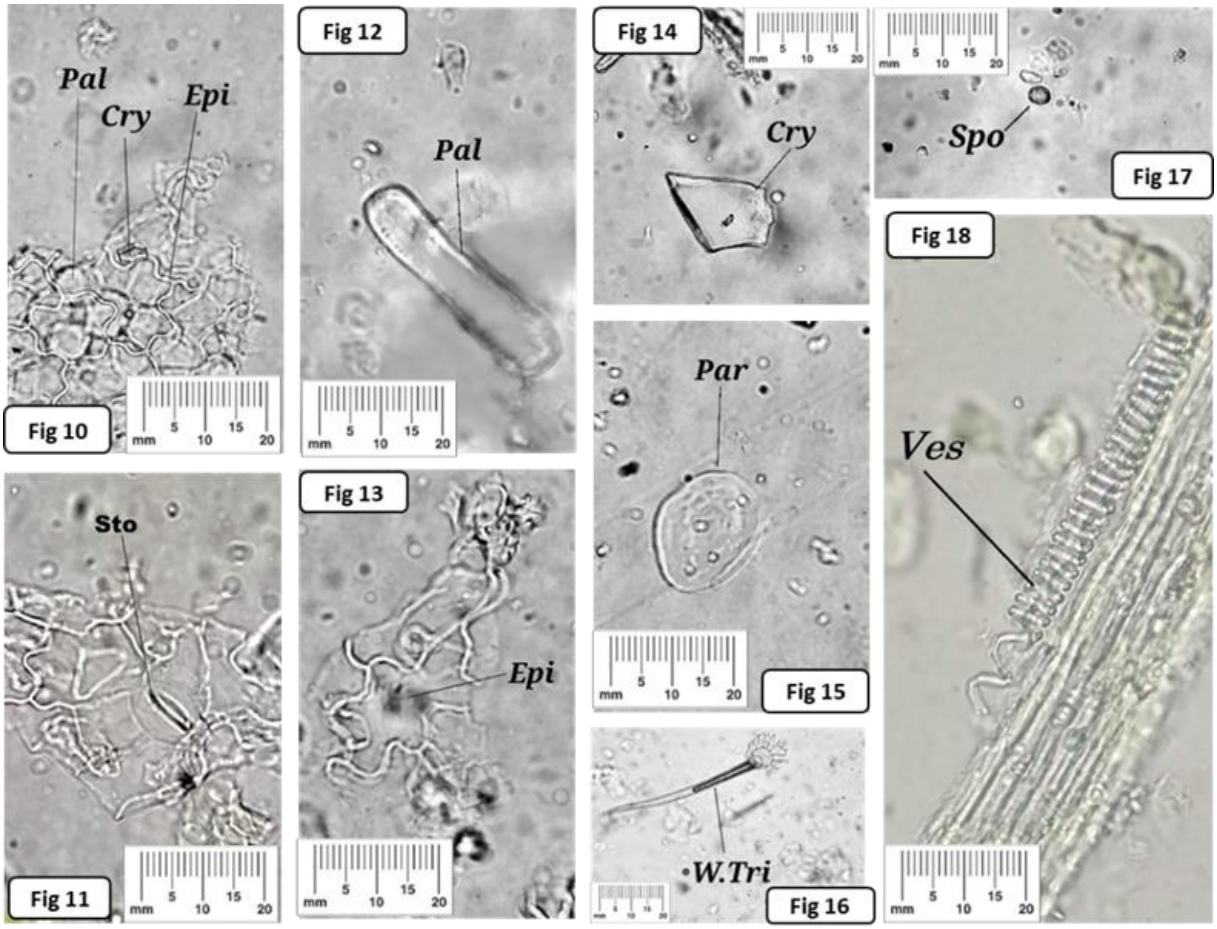

Fig. 10-16: Powder study - Fig 10: palisade, calcium oxalate crystal, epidermal cells x 450; Fig 11: stomata x 450; Fig 12: palisade cell x 450; Fig 13: epidermal cells x 450; Fig 14: calcium oxalate crystal x 450; Fig 15: parenchyma x 450; Fig 16: warty trichome x 100; Fig 17: spongy cell x 450; Fig 18: annular vessels x 450

Abbreviations

Epi- Epidermis Pal – palisade tissue, Spo – spongy tissues, Par – parenchyma, Sto- Stomata, W.Tri – Warty trichome. Cry- Crystal, Ves – Vessels.

Physicochemical evaluation

The physicochemical values such as moisture content, ash values (total ash, water soluble, acid insoluble ash and sulphated ash) and extractive values using various solvents were established for the powder drug. It is given in Table 3.

Table 3: Physicochemical evaluation of *Clitoria annua* var. *emarginata.* S.R.Yadav & Dhanke leaflets

Physico-chemical Parameters		Observations	
Moisture content %		7.14	± 0.48
Ash Values			
i.	Total ash % w/w	9.40	± 0.29
ii.	Water soluble ash % w/w	6.98	± 0.36
iii.	Acid insoluble ash % w/w	2.15	± 0.31
iv	Sulphated ash % w/w	7.40	± 0.14
Extractive Values			
i	Water soluble extractive	6.09	± 0.63
ii	Alcohol soluble extractive	7.25	± 0.53
iii	Acetic acid soluble extractive	7.91	± 0.79
iv	Butanol soluble extractive	1.38	± 0.45
v	Chloroform soluble extractive	1.58	± 0.07
vi	Methanol soluble extractive	17.78	± 0.81
vii	Benzene soluble extractive	1.68	± 0.17
viii	Ethyl acetate soluble extractive	9.94	± 0.43
ix	Acetone soluble extractive	1.58	± 0.29

Fluorescence analysis

The dried powder was treated with different regents and exposed to U.V light (short and long). These observations are tabulated in Table 4.

Table 4: Fluorescence analysis of *Clitoria annua* var. *emarginata.* S.R.Yadav & Dhanke leaflets

S.N	Test	Wavelength		
		Visible	254 nm	365 nm
1	The dry powder was treated with Nitrocellulose in amyl acetate.	Greenish Yellow	Yellowish Green	Moderate Orange
2	The dry powder was treated with Methanolic Sodium hydroxide	Yellowish Green	Light Green	Brown
3	The dry powder was treated with Nitrocellulose in amyl acetate and Methanolic Sodium hydroxide.	Greenish Yellow	Yellowish Green	Light Brown
4	The dried powder was treated with Dil. Hydrochloric acid.	Dark Green	Dark Green	Dark Brown
5	The dry powder was treated with Nitrocellulose in amyl acetate and Dil. Hydrochloric acid.	Greenish Yellow	Yellowish Green	Yellowish Orange
6	The dry powder was treaed with Aqueous Sodium hydroxide.	Yellowish Green	Light Green	Brown
7	The dry powder was treated with Dil.Nitric acid.	Pinkish Orange	Light Yellow	Brownish Green
8	The dry powder was treated with Dil.Sulphuric	Brownish Green	Dark Green	Green

Preliminary phytochemical analysis

The qualitative phytochemical analysis of powder drugs revealed the presence of various primary and secondary metabolites. The results are displayed in Table 5.

Table 5: Preliminary Phytochemical Screening of *Clitoria annua* var. *emarginata.* S.R.Yadav & Dhanke. Leaflets

Tests	Extracts		
	Water	Alcohol	Methanol
Test for starch	++	+	+
Test for carbohydrates	++	+	+
Test for mucilage	+	+	+
Test for proteins	+	+	+
Test for aleurone grains	-	-	-
Test for amino acids	+	+	+
Test for fats and oil	-	-	-
Test for tannins and phenolic compounds	+	+	+
Test for steroids	-	+	+
Test for flavonoids	+	+	+
Test for cardiac glycoside	-	-	-
Tests for anthraquinone glycosides	-	-	-
Tests for cyanogenic glycoside	-	-	-
Test for coumarin glycosides	+	+	+
Test for saponin glycosides	++	-	-
Tests for alkaloids	+	+	+
Test for terpenoids:	+	+	-

Key:"++" High concentration,"+" Less concentration, and "-" Absent.

Discussion

The leaflets of *Clitoria annua* var. *emarginata* S.R. Yadav & Dhanke is used as medicament by the aboriginals. The leaflets are known for its curative assets in hepatic disorders. For this reason, plants need to be standardized. The present work will be an aid to pharmacognostical analysis. The macroscopic studies will assist to identify the plant in its observable morphology. Microscopic evaluation shows the distinct sclerenchymatous ring around vascular bundles, which is a notable key feature. The microscopy and powder analysis, both witness the presence of two types of trichomes, warty, blunt trichomes and curved, pointed trichomes. It also shows the anisocytic and paracytic stomata, Calcium oxalate crystals. To detect the presence of adulterants, the above-mentioned physico-chemical analysis and fluorescence analysis will be useful. The findings of histochemical and phytochemical analysis have confirmed the presence of secondary metabolites. The data mentioned in the phytochemical studies could be projectile for further extraction and analysis. The detailed phytochemistry and Pharmacology are in progress.

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