International Journal of Applied Research 2016; 2(9): 450-454

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(9): 450-454
www.allresearchjournal.com
Received: 03-07-2016
Accepted: 04-08-2016

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Preliminary pharmacognostic and phytochemical evaluation of *Hybanthus stellarioides* (Domin) P. I. Forst

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Abstract
Hybanthus stellarioides (Domin) P. I. Forst. (Violaceae) is a seasonal herb recently reported from Karnataka state. So far phytochemical and pharmacognostic characters of this plant have not been studied. Therefore present study was focused on macroscopic characters, microscopic characters and physico-chemical properties.

Keywords: *Hybanthus stellarioides,* Violaceae, pharmacognosy, phytochemical analysis

1. Introduction
The genus *Hybanthus* Jacq. with 100 species is distributed throughout tropics (Mabberley, 2008) [11]. The genus is represented by six species in India (Kamble *et al.* 2014) [6]. During an extensive survey of medicinal plants on the hill tops in Belgaum district, Karnataka State *H. stellarioides* was found on the Saudatti hills (Kulkarni *et al*. 2016) [10]. The species grows in the crevices of sand stones on the rocky flat hill tops (15°47' N and 75 ° 07' E. 610m MSL). It is a seasonal herb but is a very good forage plant for sheep and goat. To further evaluate the plant, pharmacognostic and phytochemical studies were made.

2. Materials and methods
2.1. Macroscopic and microscopic analysis
Morphological key characters of the plant were observed using dissecting as well as compound microscopes. Transverse sections of the plant parts (leaf, petiole, stem and root) of 10 µm thickness were obtained and are double stained with Safranin (4%) and fast green (2%) reagents. Stomatal index of the leaf and powder microscopy of the whole plant was also calculated as per the standard methods (Kokate, 1985) [8].
Microphotographs were taken using Olympus trinocular microscope (model CX21) at different magnifications (×100 and ×200) with inbuilt analogue camera (DIGI-EYE-330) to study the anatomical features.

2.2. Physico-chemical analysis
The physico-chemical constants of the plant powder were carried out to evaluate the quality and purity of the drug. The various parameters such as moisture content, ash values and extractive values were calculated as per standard methods (Chase and Prett, 1949; Anonymous, 1996 and Mukherjee, 2002) [3, 2, 13].

2.3. Extraction of the plant material and preliminary phytochemical analysis
The collected plant material was cleaned, shade dried and coarsely powdered. Hot percolation method was used for extraction using Soxhlet apparatus. A known amount of powdered material (50g) was refluxed sequentially in petroleum ether, benzene, chloroform, acetone, methanol, ethanol and water (250ml each), in the order of increasing dielectric constant of the solvents, for 6-8 hours. All the extracts were dried and stored at 4 ^{0}C for

further use. The extracts were subjected to preliminary phytochemical screening as per standard methods (Kokate, 1994; Horborne, 2007 and Khandelwal, 2007) [8, 4, 7].

2.4. Calculation of % Extractive value

Five gram coarse powder of the plant was refluxed in soxhlet apparatus with petroleum ether, benzene, chloroform, acetone, methanol, ethanol and water (100ml each) sequentially. Each extract was evaporated to dryness in a china dish and weight was noted. The percentage extractive value with reference to the air-dried drug was calculated using the formula:

$$\% \text{ Extractive value} = \frac{\text{Weight of the extract obtained}}{\text{Weight of powdered sample}} \times 100$$

2.5 Fluorescence analysis

The plant extracts and the plant powder treated with different chemical reagents were observed under visible light as well as UV light (254 nm and 366 nm), as per the methods of Chase and Prett (1949) [3], for the Fluorescence analysis.

3. Results

3.1. Morphological characters

H. stellarioides (Domin) P. I. Forst. is a small erect, unbranched or rarely branched seasonal herb, growing to a height up to 30cm and is covered with spreading hairs. Leaves are simple, alternate, linear-lanceolate in shape and subsessile; 1.2–8 cm long and 0.2–0.8 cm wide; with attenuate base, acute apex, entire but distantly denticulate margin; mid vein is prominent but lateral veins are obscure; stipules are small, to 1 mm long with hairy margins. Flowers are solitary in the axil; stalk is up to 9 mm long, filiform and jointed; peduncle is divaricate but pedicel is curved and drooping. Sepals 5, linear-lanceolate, unequal, uninerved, slightly keeled and hairy mostly on the keels. Petals 5, highly unequal, the upper two are small, recurved, pale; the laterals are falcate, pale, the lower biggest and clawed; limb is 5 mm long and 12 mm broad, orange colored with 3 central long and 2 lateral small deep colored veins which produce dichotomously branched lateral nerves running almost to the margin; limb is with a white blotch at base near the claw. Stamens 5, didynamous; filaments of two stamens are with hairy appendages below; connective is distinctly prolonged beyond anthers. Pistil 3-4 mm long; ovary is superior, ovoid, 6–12 ovuled; stigma is spathulate. Capsule is ovoid, three angled when mature, 5-8 x 3-5 mm in size and 4-10 seeded.

3.2. Anatomical features

The transverse section of leaf, petiole, stem and root of *H. stellarioides* plant shows following anatomical features:

3.2.1 Leaf

The leaf lamina is dorsiventral and amphistomatic. The adaxial epidermis is thick walled, tangentially elongated or barrel shaped, compactly arranged and cells contain mucilage, with distinct cuticular striations. The abaxial epidermis is thin walled, tangentially elongated, compactly arranged, slightly wavy, lack cuticular striations but some cells possess mucilage. The mesophyll tissue is differentiated into two to four layers of palisade parenchyma cells of which the two lower layers are running transversely like transfusion tissue and four to five layers of spongy parenchyma tissues. Some of the mesophyll cells are filled with brown pigment in addition to chlorophyll. Along the margins of the lamina sharp pointed, spiny glands are present. Conjoint collateral vascular bundles are in the centre of midrib and it is surrounded by parenchymatous tissues (Fig. 1B).

3.2.2. Stomatal Index

The stomata are anisocytic, scattered on both epidermis but, more abundant on abaxial surface than on the adaxial surface. Stomata are 28-31 mm long & 19-26 mm broad. Stomata frequency is 3-5/mm^2 on the adaxial side and 21-22/mm^2 on the abaxial side. The stomatal index is 9.5-15.5 on the adaxial and 21.5-24.5 on the abaxial side (Fig. 1F).

3.2.3. Petiole

Transverse section of petiole is convex in outline. Distal part of the petiole is spindle shaped with two lateral wings. Epidermis is single layered, cells are tubular shaped, compact, with thin cuticle and glandular trichomes. Various forms of calcium oxalate crystals are found below the epidermis. Vascular bundles are scattered in the parenchyma tissue. In the proximal part, three vascular bundles are present of which median is large and other two laterals are smaller. The middle region of the petiole is slightly winged. At the basal part of petiole the xylem and phloem are fused to form a single shallow arc (Fig. 1C).

3.2.4. Stem

The T.S. of stem shows uneven circular outline with thin single layered epidermis having barrel shaped, compactly arranged cells which are with thin cuticle and uniseriate striations. Hypodermis is collenchymatous, 1-2 layered; cortex is composed of 4-6 layers of parenchymatous tissues and calcium oxalate crystals are scattered; the vascular cylinder consists of 1-2 layers of discontinuous patches and varies from perivascular sclereids followed by a narrow zone of phloem and dense cylinder of xylem. Xylem consists of narrow circular, thick walled radially arranged vessels of 15-24 µm diameter and thick walled fibres usually vary from 3-10 µm in diameter, endarch. Pith is wide and parenchymatous (Fig. 1D).

3.2.5 Root

The T.S. of root shows narrow uniform outer zone of phellem (cork) composed of tangential cells, followed by cortex composed of less compact parenchyma cells and secondary phloem is in small radial masses. Secondary xylem is dense and forms compact circular cylinders. It consists of scattered circular vessels of 25-50 µm in diameter and the fibres are fairly thick walled with wide lumen. Calcium oxalate crystals are also present in the cortex (Fig. 1E).

3.3. Powder microscopy

Powder of the plant treated with a mixture of phloroglucinol and concentrated HCl (1:1) showed the presence of tracheary elements, sieve tube, trichome, tylosis, secretory gland, vessels and calcium oxalate crystals (Fig. 1G-P).

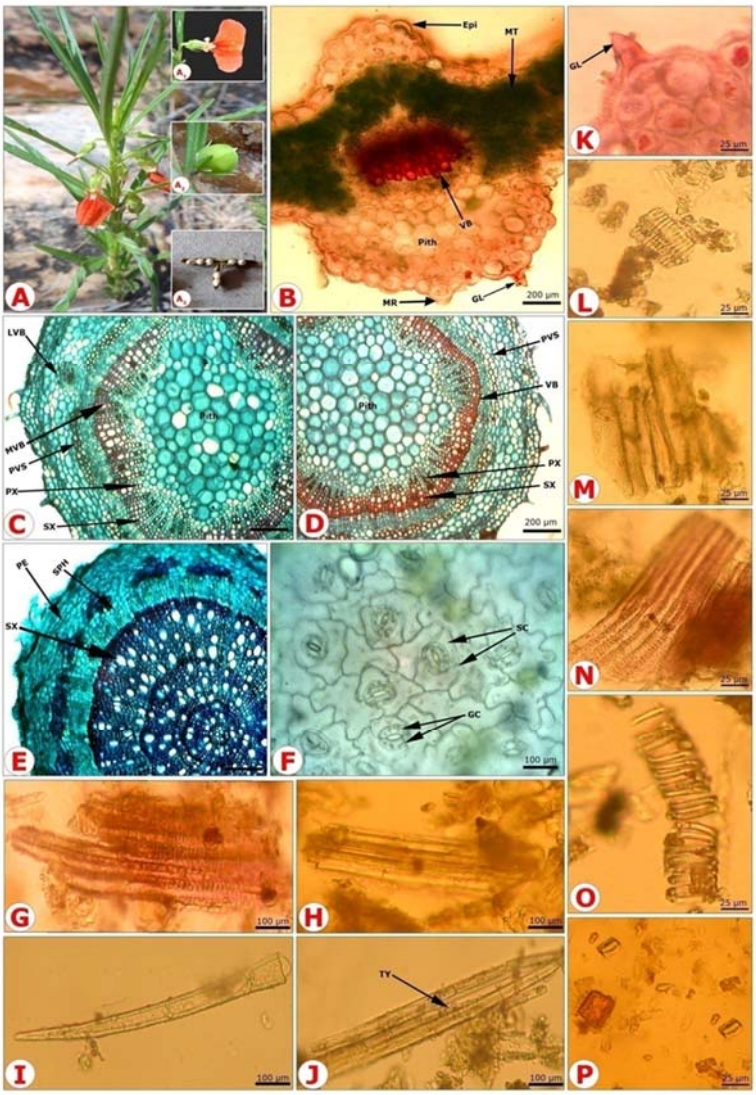

Fig 1: (A) Habit; (A1): Flower; (A2): Fruit; (A3): Seeds; B: Transverse section of leaf; C: Transverse section of petiole; D: Transverse section of stem; E: Transverse section of root; F: Stomata; G: Tracheary elements; H: Sieve tube; I:Trichome; J: Tylosis; K: Leaf marginal gland; L: Annular vessel; M: Pitted vessel; N: Scalariform vessel; O: Spiral vessel; P: Calcium oxalate crystal. Epi- Epidermis; MT-Mesophyll tissue; VB-Vascular bundle; LVB- Lateral vascular bundle; MVB- Median vascular bundle; PVS- Perivascular sclereid; Px- Primary xylem; Sx- Secondary xylem; PE- Periderm; SPH- Secondary phloem; SC- Subsidiary cells; GC- Guard cells; TY-Tylosis; GL-Gland.

3.4. Physicochemical parameters

The results of physico-chemical analysis such as total ash, acid insoluble ash, water soluble ash, sulphated ash, moisture content by loss on drying, ethanol soluble extractive and petroleum-ether extractive values are presented in Table 1.

Table 1: Physico-chemical characters of *H. stellarioides* plant

Sl. No.	Particulars	Percentage of Value (% w/w)
1	Total ash	13.0
2	Acid insoluble ash	2.0
3	Water soluble ash	3.0
4	Sulphated Ash	18.0
5	Moisture content by Loss on drying	3.0
6	Ethanol Soluble extractive	20.0
7	Petroleum-ether extractive	8.0
8	**Extractive values (Successive extraction)**	
	a)Petroleum ether (40-60 ºC)	0.9
	b)Benzene (70-80 ºC)	0.46
	c)Chloroform (50-60 ºC)	0.54
	d)Acetone (50-55 ºC)	1.43
	e)Methanol (60-65 ºC)	5.90
	f)Ethanol (60-65 ºC)	0.32
	g)Aqueous (40-60 ºC)	2.65

3.5. Fluorescence analysis

The plant extracts and the plant powder treated with different chemical reagents were observed under visible light as well as UV light (254 nm and 366 nm). The colour change of both plant powder and the extract was observed and compared with the colour under visible light. The observations, showing the variation in colour, are presented in Table 2.

Table 2: Fluorescence characters of *H. stellarioides* plant powder

Sl. No.	Particulars of the treatment	Visible light	Under UV light	
			(254nm)	(366nm)
1	Powder as such	Greyish green	Green	Brick red
2	Powder + 1N NaOH (aqueous)	Green	Brown red	Brick red
3	Powder+1NNaOH (Ethanolic)	Dark Green	Green	Reddish green
4	Powder+ 1N HCl	Blackish Green	Brown red	Red
5	Powder+ H_2SO_4 (1:1)	Green	Brown	Dark Brown
6	Powder+ HNO_3 (1:1)	Yellow	reddish	Red
7	**Extracts**			
	a) Petroleum ether (40-60 ºC)	Light green	Algal green	Salmon red
	b) Benzene (70-80 ºC)	Door counter green	Algal green	Salmon red
	c) Chloroform (50-60 ºC)	Light green	Green	Dark salmon red
	d) Acetone (50-55 ºC)	Green	Algal green	Salmon red
	e) Methanol (60-65 ºC)	Green	Green	Dark reddish brown
	f) Ethanol (60-65 ºC)	Green	Green	Dark Salmon red
	g) Aqueous (40-60 ºC)	Algal green	Dark green	Black

3.6. Phytochemical analysis

Preliminary phytochemical analyses of successive extracts revealed the presence of different phytoconstituents and are presented in Table 3. Petroleum ether extract showed positive result only for steroids whereas, benzene extract showed presence of steroids and alkaloids. Chloroform extract was devoid of all the phytochemicals tested and acetone extract showed positive results for carbohydrates, tannins and phenolic compounds. Both methanol and ethanol extracts showed the presence of all the tested phytochemical constituents except proteins, amino acids and triterpenoids. However, water extract tested positive for carbohydrates, glycosides, saponin glycosides, tannins and phenolic compounds.

Table 3: Phytochemical investigation of extracts of *H. stellarioides* Plant (+ = Present, - = Absent)

Sl. No.	Phytochemicals	Extracts						
		Petroleum ether	Benzene	Chloroform	Acetone	Methanol	Ethanol	Aqueous
1	Carbohydrates	-	-	-	+	+	+	+
2	Proteins	-	-	-	-	-	-	-
3	Amino acids	-	-	-	-	-	-	-
4	Steroids	+	+	-	-	+	+	-
5	Triterpenoids	-	-	-	-	-	-	-
6	Glycosides	-	-	-	-	+	+	+
7	Saponin glycosides	-	-	-	-	+	+	+
8	Flavonoids	-	-	-	-	+	+	-
9	Tannins & Phenolic compounds	-	-	-	+	+	+	+
10	Alkaloids	-	+	-	-	+	+	-

4. Discussion

Of the six species occurring in India, *H. enneaspermus* is the only plant having traditional medicinal value (Jain, 1991; Yoganarasimhan, 1996) [5, 16]. This plant is used for curing vomiting, burning sensation, urinary calculi, painful dysentery and as an external application for wounds (Mozhi *et al*. 2013) [12]. It is also reported that the plant possesses anti-inflammatory, antiarthritic, antiplasmodial and antimicrobial properties (Amutha priya *et al*., 2011) [1]. Comparatively *H. stellarioides* and *H. enneaspermus* are very closely related except that the former is having distantly serrate margin of the leaf and orange coloured petals of the flower. Further a comparison of the anatomical studies of leaf, petiole, stem and root of *H. enneaspermus* carried out by Retnam and Britto (2007) [15] indicates that the characters are similar to that of *H. stellarioides*. Secretary glands in the leaf margins, parenchymatous tissues scattered with calcium oxalate crystals in stem and thick walled secondary xylem fibres forming a compact circular cylinder with wide lumen in the root are the major characters in both the plants. Apart from these, both the plants show similar characters in the petiole in having three vascular bundles in the proximal part, of which median is large and other two laterals are smaller. At the basal part of the petiole the xylem and phloem are fused to form a single shallow arc. Similarly, the physicochemical and phytochemical parameters are also similar in both the species. The fluorescence study revealed that powder of both the plant have same colour pattern under visible light as well as UV light possibly due to the presence of similar chemical entities.

The phytochemical study revealed the presence of carbohydrates, steroids, glycosides, saponin glycosides, flavonoids, tannins and phenolic compounds and alkaloids in *H. stellarioides*. In general saponin acts as antibacterial and anti-neoplastic. Flavonoids are reported to have anti-allergic, anti-inflammatory and anti-cancer properties. The alkaloids possess a good analgesic, anti-inflammatory and anti-oxidant activity (Pradhan *et al.* 2013) [14]. It is possible that *H. stellarioides* is also having the similar medicinal properties as that of H. *enneaspermus* which has to be further confirmed through various comparative biological activities.

5. Conclusion

Though *H. stellarioides* is closely allied to H. *enneaspermus*, a well known medicinal plant, so far the plant is not in traditional medicinal use. The preliminary pharmacognostic and phytochemical studies carried out confirmed that these two species are almost similar with respect to the parameters studied, proving the possible medicinal use of *H. stellarioides* also.

6. Acknowledgment

The authors are thankful to the authorities of Karnatak University, Dharwad for the facilities provided. First author acknowledge the co-operation and encouragement of the Principal, KLE's P.C. Jabin Science College, Hubballi and also the UGC, New Delhi for the financial assistance in the form of FDP grant. Second author gratefully acknowledge the UGC, New Delhi for the research grant in the form of UPE: Herbarium/Botanical Garden Project.

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