Original Article

Phytochemical Analysis, Histochemical localization and Antioxidant Activity of *Hoya wightii* ssp. *palniensis* and *Elaeocarpus recurvatus*

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ABSTRACT

The present investigation was carried out to determine the phytochemical constituents and their localization histochemically for two endangered plants of Western Ghats such as *Hoya wightii* ssp. *palniensis and Elaeocarpus recurvatus.* The antioxidant content of different plant parts and their free radical scavenging activity were also analyzed. This study provides referential pharmaco-botanical and phytochemical information for the conservational importance of these endangered plants.

Keywords: *Hoya wightii* ssp. *palniensis, Elaeocarpus recurvatus*, preliminary phytochemical analysis, histochemical localization, Western Ghats, free radical scavenging activity.

INTRODUCTION

Hoya wightii ssp. *palniensis* is a woody trailer belonging to the family Asclepiadaceae. It is an endangered species restricted to the Western Ghats of Tamil Nadu. Its vulnerability was due to habitat destruction[13]. It bears attractive white flowers which last for a week. *Elaeocarpus recurvatus* (Elaeocarpaceae) is a tree species, valued for its hardwood. Its endangeredness is due to poor seed germination efficiency. *In vitro* conservation measures have been taken by us in our laboratory[11,12] and *ex situ* conservation of *in vitro* regenerated plantlets has been carried out in collaboration with Vattakkanal Conservation Trust, Pambarpuram, Kodaikanal. In this materialistic world, before taking conservation measures to a plant, we are in urge to substantiate its usage or importance to the public, i.e., whether the plant species have ethnic, medicinal, industrial or ecological importance. Then, only our conservation measures will be valued and can be followed by others in order to restore the lost habitat of the particular plant species. At this juncture, we need to scrutinize the pharmacological or medicinal use of these plant species for its

botanical identity. The role of anatomical, phytochemcial and free radical scavenging activity are sought at this investigation to provide a set of diagnostic features of the drug which will help to a considerable extent to ascertain the botanical identity of the plant. Anatomical perspective of medicinal plants is an integral component of pharmocognosy, especially while proposing diagnostic protocols for establishing the botanical identity and ascertaining the quality control of raw materials[4]. The present study has been carried out to know the anatomical features of leaf for the phytochemcial localization and free radical scavenging activity to serve as a possible tool for the proper use of *Hoya wightii* ssp. *palniensis* and *Elaeocarpus recurvatus* and importance of their conservation.

MATERIALS AND METHODS

Preliminary phytochemical analysis

Preparation of leaf and stem extracts

Healthy plant leaves and stems of *H. wightii* ssp. *palniensis* and *E. recurvatus* were collected, washed thoroughly in tap water and dried in room temperature for 15 days. The dried 25 g of leaf and stem were powdered and soaked separately in 100ml petroleum ether, hexane, chloroform, ethyl acetate and water by keeping it in a shaker for 3 days (Extraction by Soxlet apparatus need large quantity of plants. *H. wightii* ssp. *palniensis* and *E. recurvatus* are endangered plants, hence extraction using soxlet was neglected). The extracts were filtered through cheese cloth and the extracts were reduced to 10% of its original volume. The organic solvent filtrates were concentrated in vacuum using a rotary evaporator, while aqueous extract was dried using water bath.

Phytochemical Screening of leaf and stem extracts

The phytochemical components of *H.wightii* ssp. *palniensis* and *E. recurvatus* from leaf and stem were screened using the methods of Brinda *et al*.,[3] and Harbone[8]. The components were alkaloids, phenolics, saponins, steroids, xanthoproteins, flavonoids, triterpenoids, catechins, anthroquinones and tannins.

Histochemical localization of metabolites

For the present study, fresh samples of leaf and stem were cut in to small pieces and fixed immediately in FAA for 24hrs. After fixation they were washed thoroughly in distilled water, dehydrated and thin hand sections were made. Histological localization of metabolites like phenolics (Reeve[16]), lignin (Calcium hypochlorite-Sodium sulphite method) (Campbell *et al*.,[5]) and Protein (Weime[21]) were made.

Estimation of Antioxidant contents

Determination of total phenolic content

The method proposed by Sadasivam and Manickam[18] was employed for quantification of total phenols. Samples (approximately 200 mg) was homogenized in a mortar by adding 80% ethanol. It is then centrifuged at 10,000 rpm for 20 min and the supernatant was filtered using filter paper Whatmann no.42. The residue was re-extracted (five times) with 80% ethanol and the supernatant collected were evaporated to dryness. Residue was dissolved in 5 ml of distilled water from which about 0.2 ml was taken and total volume was made up to 3 ml with distilled water. To this fresh Folin-ciocalteau reagent (0.5 ml) was added. After 3 min, 2 ml of Na_2Co_3 (sodium carbonate) solution (20%) was added in each tube, mixed thoroughly and placed in a hot water bath (58^0C) exactly for 1 min. It was then cooled to room temperature and then absorbance (650 nm) was measured against

blank using a spectrometer (Elico's mini Spec, India).

Determination of total flavonoids

Total flavonoid contents were determined using the method of Orden *et al*.,[15]. A volume of 0.5 ml of 2% $AlCl_2$, ethanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicates the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/l. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.0255x$, $R^2 = 0.9812$, where x was the absorbance and y was the quercetin equivalent (mg/l).

Determination of total proanthocyanidins

Determination of proanthocyanidins was based on the procedure reported by Sun *et al*.,[20]. A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-metahnol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin content were expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve: $y = 0.5825x$, $R^2 = 0.9277$, where x was the absorbance and y is the catechin equivalent (mg/g).

Free radical scavenging activity by DPPH assay

The free radical scavenging activity of the fractions was measured *in vitro* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay[14]. About 0.3mM solution of DPPH in 100% ethanol was prepared and 1 ml of this solution was added to 3 ml of the fraction dissolved in ethanol. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The scavenging activity at different concentrations was determined and was compared with that of ascorbic acid, which was used as the standard.

RESULT

Preliminary phytochemical analysis

The results of preliminary phytochemical analysis i.e., presence and absence in different plant parts of *H. wightii* ssp. *palniensis* and *E. recurvatus* was shown in Table 1, 2, 3 and 4.

Phytochemcial screening of stem of *Hoya wightii* ssp. *palniensis*

Methonolic extract showed the presence of alkaloids, phenolics, steroids, flavonoids and triterpenoids (Table 1). Ethyl acetate extract showed the presence of alkaloids only. Aqueous extract showed the presence of alkaloids, xanthoprotein, flavonoids and triterpenoids. Solvents like petroleum ether, chloroform and hexane showed no phytochemical activity.

Phytochemcial screening of leaf of *Hoya wightii* ssp. *palniensis*

Methanolic extract showed the presence of alkaloids, phenols, saponins, steroids, flavonoids and triterpenoids (Table 2). Aqueous extract showed the presence of alkaloids, saponins, steroids, xanthoproteins, flavonoids and triterpenoids.

Phytochemcial screening of stem of *E. recurvatus*

Methanolic and aqueous extract showed the presence of alkaloids, phenolics, flavonoids and tannins (Table 3).

Phytochemcial screening of leaf of *Elaeocarpus recurvatus*

Methanolic and aqueous extracts showed the presence of alkaloids, phenolics, flavonoids and tannins. Petroleum ether showed the presence of alkaloids and flavonoids (Table 4).

Histochemical localization of metabolites

Histochemical localization of metabolites was shown in Fig.1 A-F. The presence of lignin was localized at the cortical and stelar region of *E. recurvatus* (Fig.1B) and at the cortical region of *H. wightii* ssp. *palniensis* (Fig. 1A). A bright red color will develop within a few minutes but will gradually fade in 30-45 mins. The red and brown color is believed to be due to the presence of different groups of lignin in angiosperms. Not clear although it is believed that syringyl groups of lignin are the sites of reaction in angiosperms (Wardrop, 1971) Phenolics were localized at the stelar region of *E. recurvatus* (Fig.1D) and *H. wightii ssp. palniensis* (Fig.1C). Protein was localized in the stelar region of *Elaeocarpus recurvatus* (Fig.1F) and *H. wightii* ssp. *palniensis* (Fig.1 E).

Antioxidant contents of crude extract of different plant parts of *H.wightii* ssp. *palniensis* and *E. recurvatus*

The antioxidant contents like total phenolics, flavonoids and proanthocyanidins were calculated and their results were given in Table 5. Total phenolic content of plant extracts varied from 59.4µg to 248.5 µg. Stem extract of *in vitro* regenerated *H. wightii* ssp. *palniensis* showed highest phenolic content of 248.5 µg pyrocatechol equivalent/mg; followed by leaf extract of in vitro regenerated *H. wightii* ssp. *palniensis* (76.35 µg); leaf explants of *H. wightii* ssp. *palniensis* (71.47 µg); *H.wightii* ssp. *palniensis* leaf callus (71.39 µg) and stem extract of *E.recurvauts* showed low phenolic content (59.4 µg).

Flavonoid content of plant extracts varied from 33.3µg to 139.3 µg. Stem extract of *in vitro* regenerated *H. wightii* ssp. *palniensis* showed highest phenolic content of 139.3 µg quercetin equivalent/mg; followed by leaf extract of *in vitro* regenerated *H. wightii* ssp. *palniensis* (42.8 µg); leaf explants of *H. wightii* ssp. *palniensis* (40.05 µg); *H. wightii* ssp. *palniensis* leaf callus (37.15 µg) and stem extract of *E.recurvatus* showed low flavonoid content (33.3 µg).

Proanthocyanidin content was high in leaf of *E.recurvatus* (207.9 µg) followed by leaf callus of *E.recurvatus* (125 µg); *in vitro H.wightii* ssp. *palniensis* leaf (97.2 µg); *H.wightii* ssp. *palniensis* leaf callus (89.4 µg). Lowest proanthocyanidin content was observed on stem of *E.recurvatus* (41.9 µg).

DPPH activity

DPPH radical scavenging activities of plant extracts varied from 0.289 mg/ml of Ascorbic acid equivalent to 0.481 mg/ml. The stem extract of *in vitro* regenerated *H.wightii* ssp. *palniensis* showed the highest antioxidant activity (0.481 mg/ml DPPH inhibition); followed by stem of *E. recurvatus* (0.425 mg/ml); leaf extract of *in vitro* regenerated *H.wightii* ssp. *palniensis* (0.415 mg/ml); *H.wightii* ssp. *palniensis* leaf extract (0.393 mg/ml) and *H.wightii* ssp. *palniensis* stem extract showed the lowest DPPH activity (0.289 mg/ml).

DISCUSSION

The results revealed that among the organic solvents used, methanol was more suitable to fractionate the metabolites from the plant parts of *H. wightii* ssp. *palniensis* and *E.recurvatus.* Both the plants showed the presence of phenolics, flavonoids and proanthocyanidins. The plant metabolites

were histochemically localized and were quantitatively measured.

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decreases cardiovascular complications (Yen *et al*., 1993). The crude phenolic extracts from plant materials are composed of a complex mixture of phenolics differing in the number and arrangement of both hydroxyl and methoxy groups on the aromatic rings of phenolic acids, flavonoids and other related compounds, as well as their degree of polymerization. The total content of phenolics was determined using the Folin-Ciocalteau reagent, which is sensitive to many classes of phenolic compounds[10]. In this study, quantity of phenolics was more in *H. wightii* ssp. *palniensis* than *E.recurvatus* but was invisible. Additionally, the total content of flavonoids and proanthocyanidins were estimated.

Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as anti-inflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral and anticancer activities. They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants[6]. In view of their wide pharmacological and biological actions, they have a greater therapeutic potential. The presence of high phenolic and flavonoid content in the fractions has contributed directly to the antioxidant activity by neutralizing the free radicals. Flavonoids of *in vitro* regenerated *Hoya wightii* ssp. *palniensis* showed high flavonoid content than other plant parts. This might be due to the morphological adaptations carried during their acclimatization process.

Proanthocyanidins, refer to a large class of polyphenols called flavonols. Proanthocyanidins have antioxidant properties *in vitro*. Foods rich in proanthocyanidins have high oxygen radical scavenging capacity, an *in vitro* measures with unproven relationship to antioxidant effects *in vivo*. Proanthocyanidins possess cardio protective effects including of platelet activity and activation of endothelial nitric oxide synthase[7] and anticarcinogenic activity[20].

Rice-Evans *et al*.,[17] and Brand-Williams *et al*.,[2] demonstrated that the antioxidant activities of polyphenolic compounds are largely dictated by their molecular structure. This complicates both the determination of the antioxidant activity for complex mixtures of phenolics such as plant extracts and the interpretation of experimental data. Consequently, evaluation of the antioxidant activity of plant phenolic extracts is limited to an estimation of the total antioxidant activity of the system. Numerous methods have been proposed to evaluate/estimate the antioxidant potential of natural sources of antioxidants[19]. Of these, DPPH radical scavenging activity was employed in this study. The DPPH assay is commonly employed to evaluate the ability of antioxidants to scavenge free radicals. The change in absorbance at 517nm is used as measure of the scavenging effect of a particular extract for DPPH radicals. The highest free radical scavenging activity by DPPH assay was observed on *Hoya wightii* ssp. *palniensis* stem *in vitro* and *E.recurvatus* stem. The scavenging effect of antioxidants is influenced by their concentration and type of radical in question as well as the molecular structure and kinetic behavior of the phenolics involved. Amarowicz *et al*.,[1] pointed out; however, that caution must be exercised when interpreting such results. The reactions that DPPH can undergo are not simple and straight forward, and much is still to be

learned about its chemistry. Moreover, one cannot arbitrarily assume that the decrease in absorbance at the 517nm absorption maximum is solely attributed to the antioxidant donating a hydrogen atom or an electron to DPPH. The strong free radical scavenging capacities displayed by crude phenolic extracts of different plant extracts may also possess a strong antimutagenic activity. Hochstein and Atallah[9] associated the ability of antioxidants to scavenge free radicals with their antimutagenic activity.

CONCLUSION

Based on the results obtained, the antioxidant action of the different extracts of *H.wightii* ssp. *palniensis* and *E.recurvatus* may be ascribed to their free-radical scavenging capacity as well as their chelation of metal ions in the presence of ortho-, dihydroxy- and ketol subsistents. The methonalic extract of antioxidant content exhibited strong antioxidant properties. Further research is needed to evaluate the antioxidant activity of various phenolic and flavonoid fractions of these extracts and to isolate and identify the active compounds.

ACKNOWLEDGEMENTS

We acknowledge financial support from the University Grants Commission, New Delhi and Mr.R.W.Stewart and Mrs. Tanya Balcar and their Vattakanal Conservation Trust, Kodaikanal for the help rendered during the field survey.

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Table 1. Phytochemical screening of stem extracts of *Hoya wightii* ssp. *palniensis*

S.No	Name of the compound	M	PE	C	H	EA	W
1	Alkaloids	+	-	-	-	+	+
2	Phenolics	+	-	-	-	-	-
3	Saponins	-	-	-	-	-	-
4	Steroids	+	-	-	-	-	-
5	Xanthoproteins	-	-	-	-	-	+
6	Flavonoids	+	-	-	-	-	+
7	Triterpenoids	+	-	-	-	-	+
8	Catechins	-	-	-	-	-	-
9	Antroquinones	-	-	-	-	-	-
10	Tannins	-	-	-	-	-	-

'+' indicates Presence; '-' indicates absence; PE – Petroleum Ether: M – methanol:
C – Chloroform: H – Hexane: EA – Ethyl Acetate:W – Water

Table 2. Phytochemical screening of leaf extracts of *Hoya wightii* ssp. *palniensis*

S.No	Name of the compound	M	PE	C	H	EA	W
1	Alkaloids	+	-	-	-	+	+
2	Phenolics	+	-	-	-	-	-
3	Saponins	+	-	-	-	-	+
4	Steroids	+	-	-	-	-	+
5	Xanthoproteins	-	-	-	-	-	+
6	Flavonoids	+	-	-	-	-	+
7	Triterpenoids	+	-	-	-	-	+
8	Catechins	-	-	-	-	-	-
9	Antroquinones	-	-	-	-	-	-
10	Tannins	-	-	-	-	-	-

'+' indicates Presence; '-' indicates absence; PE – Petroleum Ether: M – methanol: C – Chloroform: H – Hexane: EA – Ethyl Acetate:W – Water

Table 3. Phytochemical Screening of stem extract of *Elaeocarpus recurvatus*

S.No	Name of the compound	M	PE	C	H	EA	W
1	Alkaloids	+	+	-	-	-	+
2	Phenolics	+	-	-	-	-	-
3	Saponins	-	-	-	-	-	-
4	Steroids	-	-	-	-	-	-
5	Xanthoproteins	-	-	-	-	-	-
6	Flavonoids	+	+	-	-	-	+
7	Triterpenoids	-	-	-	-	-	-
8	Catechins	-	-	-	-	-	-
9	Antroquinones	-	-	-	-	-	-
10	Tannins	+	-	-	-	-	+

'+' indicates Presence; '-' indicates absence; PE – Petroleum Ether: M – methanol: C – Chloroform: H – Hexane: EA – Ethyl Acetate:W – Water

Table 4. Phytochemcial screening of leaf extract of *Elaeocarpus recurvatus*

S.No	Name of the compound	M	PE	C	H	EA	W
1	Alkaloids	+	-	-	-	-	+
2	Phenolics	+	-	-	-	-	-
3	Saponins	-	-	-	-	-	-
4	Steroids	-	-	-	-	-	-
5	Xanthoproteins	-	-	-	-	-	-
6	Flavonoids	+	-	-	-	-	+
7	Triterpenoids	-	-	-	-	-	-
8	Catechins	-	-	-	-	-	-
9	Antroquinones	-	-	-	-	-	-
10	Tannins	+	-	-	-	-	+

'+' indicates Presence; '-' indicates absence; PE – Petroleum Ether: M – methanol: C – Chloroform: H – Hexane: EA – Ethyl Acetate:W – Water

Table 5. Antioxidant content and DPPH radical scavenging activity of crude extract of *Hoya wightii* ssp. *palniensis* and *Elaeocarpus recurvatus*

S. No	Name of the sample	DPPH (mg/ml DPPH inhibition)	Proanthocyanidins (µg ascorbic acid/mg)	Flavonoids (µg quercetin equivalent/mg)	Phenolics (µg pyrocatechol equivalent /mg)
1	HWP leaf	0.393±0.6bc	86.38±0.8bc	40.05±0.2bc	71.47±0.7c
2	HWPstem	0.289±0.5g	53.3±0.5e	37.15±0.4d	66.27±0.6d
3	HWP callus (L)	0.415±0.3b	89.4±0.2bc	40.02±0.3bc	71.39±0.4c
4	HWP callus (S)	0.481±0.7a	72.8±0.7d	37.05±0.8d	66.09±0.5d
5	*In vitro*HWP (L)	0.368±0.4d	97.2±0.9b	42.8±0.5b	76.35±0.3b
6	*In vitro*HWP (S)	0.425±0.2b	81.5±0.4c	139.3±0.6a	248.5±0.2a
7	*E.recurvatus* (L)	0.352±0.5e	207.9±0.3a	37.02±0.3d	66.04±0.8d
8	*E.recurvatus* (S)	0.320±0.6f	41.9±0.6f	33.3±0.7e	59.4±0.4e

HWP – *Hoya wightii* ssp. *palniensis*; S – Stem; L – Leaf; ± indicates standard error.

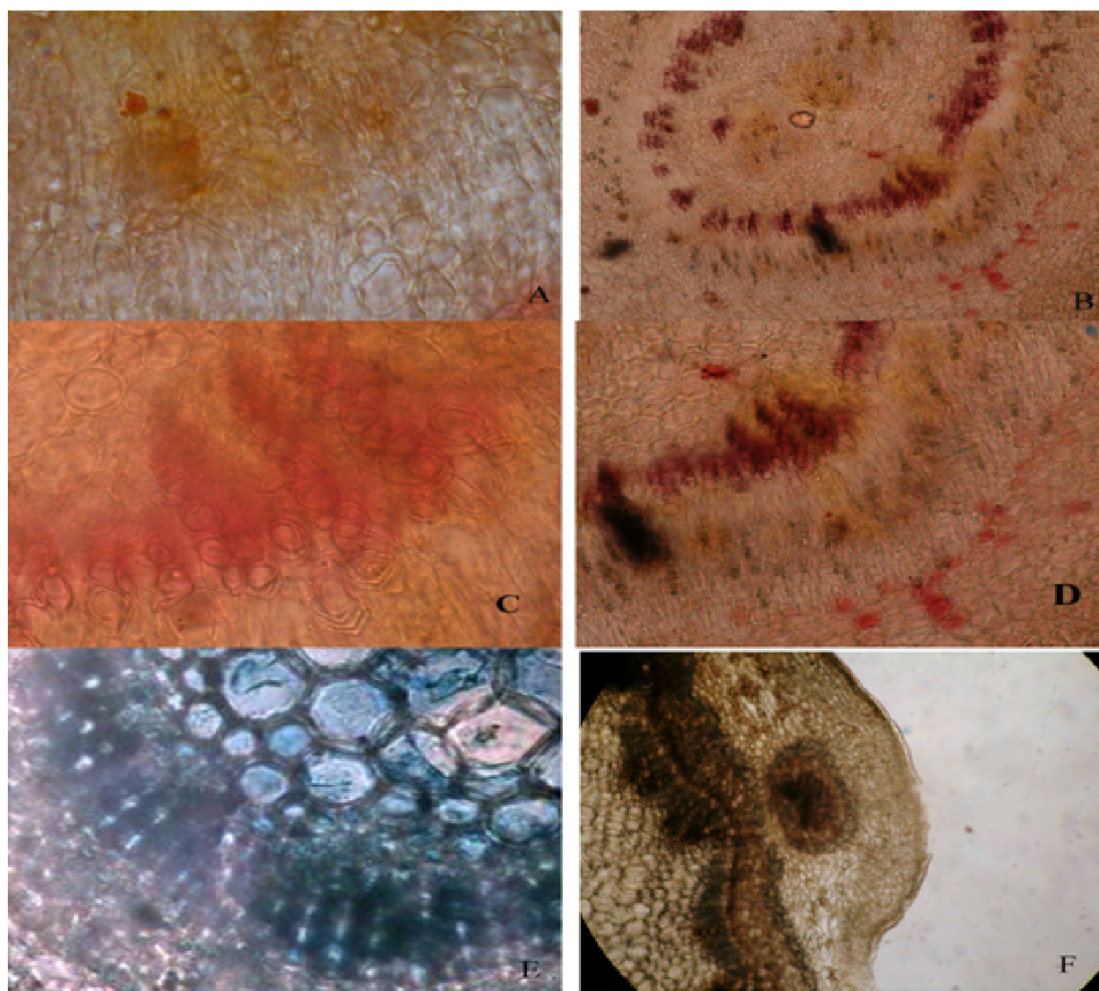

Figure 1. Histochemical localization of phytochemicals

A-Lignin of *Hoya wightii* ssp. *palniensis*
B-Lignin of *Elaeocarpus recurvatus*
C-Phenolics of *Hoya wightii* ssp. *palniensis*
D- Phenolics of *Elaeocarpus recurvatus*
E-Protein of *Hoya wightii* ssp. *palniensis*
F-Protein of *Elaeocarpus recurvatus*