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ANTIOXIDANT PROSPECTIVE AND SECONDARY METABOLITES IN *ABUTILON INDICUM* AT DIFFERENT ENVIRONMENT

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ABSTRACT

To investigate the antioxidant property and secondary metabolites of *Abutilon indicum* Plant at various Habitats were compared with those of relevant callus cultures induced from every explant in *in-vitro*. The callus cultures were effectively initiated on Murashige and Skoog (MS) medium supplemented with 2, 4-D (1 mg/L) pooled with different concentrations (0.1–1.0 mg/L) of kinetin as plant growth regulators. The circulation of Flavonoids and phenolic compounds in the plant extracts were analyzed by using Aluminium (III) Chloride Colorimetric assay with standards. Flavonoids were found in all callus extracts in comparison with in their natural habitat plant parts at various Habitats. In this study, the antioxidant activities of the extracts were evaluated in vitro antioxidant-testing systems. The secondary metabolites of flavonoid and phenolic acid contents of *Abutilon indicum* were studied at dissimilar habitats and *in-vitro* callus culture extract. Among these studies hills and wet soil habit plants showed maximum secondary metabolites than the other habitats. The antioxidant potential was studied by dot blot assay. Between these results, we fulfilled that, the ecological stress factors such as dryness, temperature, salt and soil pH is a crucial factor for liberate secondary metabolites and antioxidant potent of *Abutilon indicum*.

INTRODUCTION: *Abutilon indicum* (Malvaceae) is spread throughout the hotter parts of India. It has been reputed in Siddha system of medicine as remedy for jaundice, piles, ulcer and leprosy [14]. The plant is reported to be having analgesic [1] and anti fertility [6]properties. The flowers of the *Abutilon indicum* are known to contain flavonoids [9]. The leaf extract of *Abutilon indicum* has been already reported for the hepatoprotective activity [11, 12]. However, the literature survey afforded no scientific investigation on hepatoprotective property of flowers of the title plant so far. Hence, the present study was undertaken. Antioxidants act as a major defense against radical mediated toxicity by protecting the damage caused by free radicals. Potential antioxidant therapy should therefore include either natural antioxidant enzymes or agents capable of augmenting the function of this oxidative free radical scavenging enzyme [2]. Medicinal herbs have been used in primary health care over many centuries before the advent of modern

medicine. There are considerable evidence for the role of plants, fresh fruits, green vegetables to prevent the occurrence of number of disease in human like diabetes and cancer.

Antioxidants are vital substances which posses the ability to protect the body from damage caused by free radical induced oxidative stress. A variety of free radical scavenging antioxidants exists within the body which many of them are derived from dietary from dietary sources like fruits vegetables, & teals. In this study the antioxidant activity and radial scavenging activity of methanolic extracts of selected plant material. Traditionally used by tranian population as folk remedies was evaluated against linoleic acid per oxidation and 2, 2-diphenyl-1-picrythydrajyl radial [4].

Natural products are important sources for biologically active drugs. There has been an increasing interest in the study of medicinal plants as natural products in different parts of the world[5]. Medicinal plants containing vigorous chemical constituents with high antioxidant property play an important role in the prevention of various degenerative diseases and have potential benefits to the civilization. The medicinal significance of these plants depends on bioactive phytochemical constituents that produce definite physiological action in the human body.

Some of the most significant bioactive phytochemical constituents include alkaloids, flavonoids, phenolics, essential oils, tannins and saponins [7]. Phenolics are usually found in medicinal plants and have been reported to have multiple biological effects, including antioxidant activity. Potential sources of antioxidants have been found in leaves, oilseeds, barks and roots. Natural antioxidants from plant sources are potent and safe due to their harmless nature; wild herbs have been investigated for their antioxidant properties.

Plants contain several compounds such as phenolics, terpenoids, flavanoids, pigments and other natural oxidants including Vitamin A, Vitamin C and Vitamin E that have been associated with protection from treatment of chronic diseases such as heart diseases, cancer, diabetes and hypertension as well as other medicinal condition [3]. These protective effects have been attributed partly to various antioxidant compounds present in fruits and plants. More than 60 flavonoids present in citrus posses a wide range of properties including anti-inflammatory, anti tumor activity, inhibition of blood clots and strong antioxidant activity [10].

Plants are the rich source of compounds which may protect organism from free radical injury and disease.

The antioxidants are act by preventing the formation of new radical species and reduce the rate of chain initiation. They contain enzymes like superoxide dismutase (SOD), catalase (CAT), gluthione peroxidase (GPx) and metal binding protein like ferritin and ceruloplasmin. They interrupt the free radicals generally by donating hydrogen atom to the peroxy free radicals. They include carotenoids, Vitamin E, Vitamin C, uric acid, bilirubin and albumin.

Living organisms posses non-enzymatic and enzymatic antioxidant defence system to protect against radical mediated disorders. In view of the disturbance that can be caused by free radicals, organisms have evolved not antioxidant defense to protect against them but also repair system that prevent the accumulation of oxidatively damaging molecules.

MATERIALS & METHODS: The young leaf, shoot tip, node, internodes of *Abutilon indicum* was collected from different natures of the soil conditions like hills, dry land, and wet land for this study.

Collection of plant sample:

Sampling Area (Nature of the soil)	Location	Plant
Wet Soil	Tirchirappalli, Tamil Nadu (India)	*Abutilon indicum*
Hills Region	Ooty(Nilgiris), Tamil Nadu (India)	*Abutilon indicum*
Dry Soil	Dharmapuri, Tamil Nadu (India)	*Abutilon indicum*

These samples were brought to the laboratory and kept in refrigerator for callus induction and secondary metabolite studies.

***In-vitro* Culture-Callus Formation**:

Media preparation: The most popular Murashige & Skoog (MS) media was used for this research were obtained from Hi-media laboratory Pvt.Ltd, Mumbai (India). The chief and slight nutrients were also prepared for this work. Various plant growth

regulators such as, auxins and cytokines were used in this study. The primary hormone sources 2, 4- D, and BAP plays a significant role. The auxins were added to culture medium at a concentration range varying from $0.1mgl^{-1}$ to $1mgl^{-1}$. The auxins were added for the regulation of cell elongation, tissue swelling and cell division particularly in callus formation.

Surface Sterilization: Young leaves of *Abutilon indicum* was to be found in a clean glass and were rinsed under running tap water for 30 minutes before the initiation of surface sterilization. The young leaves were wrapped up in 25% (v/v) Clorox containing three drops of Tween 20 for 10minutes. The young leaves were then rinsed with sterile distilled water several times until all traces of Clorox were eliminated. The sterilized young leaves were cut into 5 mmx5mm in size and were transferred to the medium with sterile forceps.

Basal Medium [15]: MS medium (Murashige and Skoog, 1962) was used as the basal medium for the cultivation of plants in *in-vitro* condition. Sucrose at 3% (w/v) was added into the mixture. The pH of the medium was adjusted to 5.7±0.1 with 0.1M HCl or 0.1 M NaOH followed by addition of 0.8% (w/v) agar. The medium was then autoclaved at 121^{o}C, 15 psi for 15minutes. After autoclaving, a total of 25mL of the sterile medium was poured into culture tubes in the laminar flow, and was allowed to solidify. The culture tubes were then sealed prior to the initiation of treatments.

Callus Induction in Single Auxin Treatments: The effect of various concentrations of different auxins on callus induction from the leaf explants, the MS medium was supplemented with different auxin concentrations. The auxins treated were 2, 4-Dichloro-phenoxyacetic acid (2, 4 –D) and Indole-butyric acid (IBA), at the concentrations of 0.1-0.5mg/L. MS medium devoid of plant growth regulator was used as the control.

Callus Induction in Combination Treatments: At first the auxin alone used for experimental studies, then 1 mg/L of 2, 4-D was further combined with kinetin in order to study the effects of combination of auxin and cytokinin on callus induction. The concentrations of cytokinins examined were 0, 0.1, 0.5, 1.0 and 2.0 mg/L. The control for the experiment was MS medium lacked of plant growth regulator.

Culture Conditions: All the cultures were maintained in the culture room at 25±1^{o}C, under photoperiod of 16 hours light, 8 hours dark provided by white fluorescent tubes with the intensity of 1000 lux. The cultures were incubated for 20 days and daily annotations were made to monitor the day of initial callus formation.

Data Collection: The day of initial callus formation, the morphology and color of the callus were recorded. At the end the observation period, percentage of the explants forming callus as well as the degree of callus formation was measured.

Extraction Procedure for Field-Grown Plant Parts: Leaves and stems of *Abutilon indicum* were harvested from field grown plants and shade-dried for 7 days. The dried materials were powdered using a mechanical grinder. From these ten grams of each powdered material was extracted twice with 500 ml of methanol continuously. Thereafter, the resulting methanolic extract was reduced in vacuum (40°C), freeze-dried and stored at 4°C until further use in the experiment.

Extraction of Callus: Callus induced on MS medium was harvested and dried, and the dry weight was determined [13]. One gram (dry weight) of callus was soaked in 10 ml of 80% methanol for 3 hours and sonicated in an Ultrasonic Sonicator at 20 pulses for 20 minutes. The extract was centrifuged at 10000 rpm for 10 minutes. Then, the supernatant was concentrated under vacuum (40°C), freeze-dried, and stored at 4°C until further use in the experiment.

Analysis of secondary metabolites in Callus Culture and Fresh Leafs:

Estimation of Total Flavonoids Content: In order to compare the total flavonoid content between leaf-derived callus and *in vivo* leaves of *Abutilon indicum* by aluminum (III) chloride, colorimetric assay was carried out. The samples or the biochemical tests were in *vivo* young leaves, and Month-old calli induced and maintained in MS medium supplemented with 1mg/L of 2, 4-Dichlorophenoxyacetic acid (2, 4 –D).Total of 0.5 g of samples were weighed and extracted with 50 mL of 80% (v/v) methanol. The mixtures were then ultrasonicated for 20 minutes followed by centrifugation at 12,000 revolutions per minute (rpm). Using a pipette, 1mL of the supernatant as collected into a test tube, and 4mL of deionized water as added.

After that, 0.3 mL of 10% (w/v) $NaNO_2$ was added to the test tubes, and was left to react for 5 minutes. Then, 0.3 mL of 10% (w/v) $AlCl_3$ was added and was left for 1 minute to exact. Lastly, 2 mL of 1M NaOH was added and the mixtures were shaken.

A total of 2 mL of the mixtures were transferred to a cuvette, and the absorbance values of both types of samples were measured using spectrophotometer at 510 nm. A mixture of 1mL of 80% v/v) methanol, 4mL of deionized water, 0.3 mL of 10% w/v) NaNO2, 0.3 mL of 10% (w/v) AlCl3 and 2 mL of M NaOH were prepared as the blank.

Catechin assured as a standard in determining the total flavanoids content. From a Catechin tock concentration of 100 mg/L, several dilutions were added to prepare a series of concentrations at 0, 10, 20, 40, 60, 80 and 100 mg/L. A standard curve was constructed with the optical density at 510 nm against the concentrations of Catechin.

The total flavonoids content of the samples were then estimated from the standard curve and further expressed in milligram of catechin equivalent per gram of sample fresh mass (mg/g).

Total Phenol Analysis; Estimation of Total Phenols: Total phenol was estimated by the method of Malick and Singh [8].

Extraction: 500 mg of fresh plant tissue was ground in a pestle and mortar with 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was evaporated to dryness. The residue was dissolved with 5 ml of distilled water and used as extract.

Estimation: To 2 ml of the extract, 0.5 ml of Folin-Ciocalteau reagent was added. After 3 minutes, 2 ml of 20% Na CO solution was mixed thoroughly. The mixture was 2 3 kept in boiling water for exactly one minute and after cooling the absorbance was read at 650 nm. The total phenol was determined using a standard curve prepared with different concentration of gallic acid. The results were expressed in milligrams per gram fresh weight.

Total Steroid Analysis:

Extraction: 500 mg of fresh plant tissue was ground in a pestle and mortar with 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was evaporated to dryness. The residue was dissolved with 5 ml of distilled water and used as extract.

Estimation: 5-10g of the fresh leaf sample. Saponified for about 30 minutes in a shaking water bath at 37^0C with extracting the sample in 12% alcoholic potassium hydroxide. Transferred the saponified extract into a separating funnel containing 10-15ml of petroleum ether and mixed gently, taken up the steroids pigment into the petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and petroleum either extract containing the steroid to an amber colored bottle. Then, repeated the extraction of the aqueous phase similarly with petroleum ether, until it is colorless, discarded the aqueous phase. To the petroleum ether extract added a small amount of anhydrous sodium sulphate to remove turbidity. Noted the final volume of the petroleum either extracts and diluted if need by a known dilution factor. The absorbance of the extract at 450nm and 503 nm was noted in spectrophotometer using petroleum either as a blank.

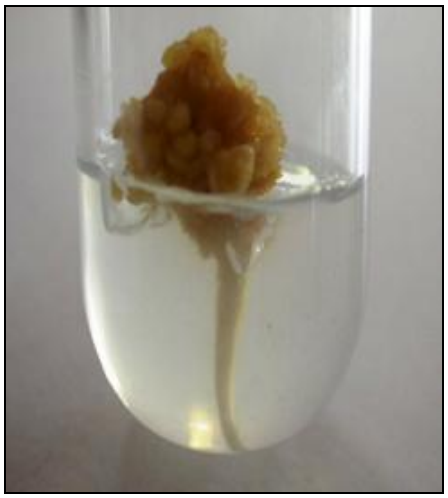
Dot-plot Rapid Screening Method: According to Soler-Rivas *et al.,* (2000), the Dot-blot test is easy, fast and reliable way to compare radial scavenging capacity of various plant extracts. 3µl of aliquots of fresh and sunshade dried powder sample extract of leafs and *in-vitro* callus of *Abutilon indicum* were spotted on the TLC plate and allowed to air dry. The TLC plate bearing the dry spots was placed upside down for 10 s in a solution of DPPH (0.1mM/l) in methanol. The spot exhibiting radial scavenging antioxidant activity showed up as yellow spot against a purple background.

RESULTS:

Callus Induction: Most naturally occurring antioxidants are secondary metabolites, which include flavonoids, Steroids and phenolic acids. The flavanoids, phenolic compounds and their antioxidant activity were studied from *Abutilon indicum* plant in their leaf and callus culture. The plants were collected from different locations like Hills, Dry and wet soil (**Figure a, b, c**).

A. HILLS REGION

B. DRY SOIL



C. WET SOIL

In the present investigation, the percentage of callus induction from leaves and stems was depend on the concentration of 2, 4-D, IBA used (**Table 1**). Leaf explants of *Abutilon indicum* on MS medium supplemented with 2, 4-D (1mg/L), IBA 0.1 mg/L) showed the maximum callus induction, but the same hormone was insufficient for the induction of callus tissues from internodes explants of this plant.

TABLE 1: EFFECT OF VARIOUS CONCENTRATIONS OF AUXINS ON CALLUS INDUCTION - USING LEAF, STEM EXPLANTS OF *ABUTILON INDICUM*

Hormone concentration (mg/l)		Culture response (%)		Callus formation (Day)	
2, 4-D	IBA	Leaf	Stem	Leaf	Stem
0.1	0.1	99	85	9	12
0.5	0.5	99	100	8	10
1.0	1.0	100	99	7	9

Similarly the plant leaf explants showed maximum (99%) culture response in the cultures supplemented with the combination of 2, 4-D (1 mg/L) and kinetin 0.1 (mg/L), but the same combination was insufficient for maximum culture response from the stem explants. In contrast to leaf explants, the stem explants of holy basil showed the maximum (100 and 99%) culture response on MS medium supplemented with the combination of 2, 4-D (1mg/L) and kinetin (0.5 mg/L) (**Table 2**).

TABLE 2: CALLUS INDUCTION FROM THE LEAF EXPLANTS *ABUTILON INDIUM* AFTER 4 WEEKS OF CULTURE IN MS MEDIUM SUPPLEMENTED WITH 1mg/l OF 2, 4-D AND KINETIN AT DIFFERENT CONCENTRATION

Hormone concentration (mg/L)	Culture response (%)		Callus formation (Day)	
2, 4-D+kinitin	Leaf	Stem	Leaf	Stem
1+0.1	99	85	8	10
1+0.5	100	100	7	9
1+1.0	100	100	7	9

In this study, the total flavonoids content of *Abutilon indium* were successfully estimated using aluminium (III) chloride colorimetric assay where by a total of 65mg/g of total flavonoids in the leaf from hills and the total flavonoids content then the other samples. The leaf in hills showed higher amount of flavonoids than others and they were expressed in **figure 1 and Table 3**.

TABLE 3: TOTAL FLAVONOIDS ANALYSIS

Secondary metabolites	Habitats			
	Hills	Dry land	Wet land	Callus
Flavonoids (mg/g)	65	48	45	40

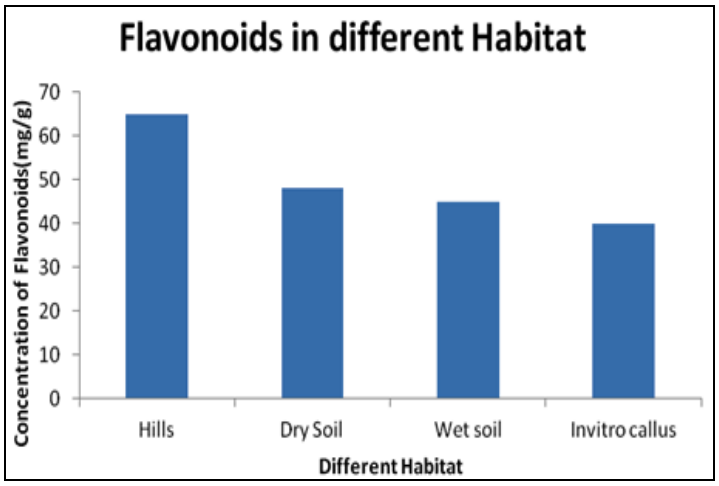


FIGURE 1: FLAVONOIDS IN DIFFERENT HABITAT

In this study, we evaluated the TPC of methanolic extract of *Abutilon indicum* leaves and the callus cultures in various habitations. The phenolic content of leaves in hills habitats was 60 mg/g and callus culture possess 55mg/g of extract. Among these results the callus in hills habitat showed maximum yield of phenolic compound than others. The comparative representation was expressed in **Table 4**.

TABLE 4: QUANTITATIVE ANALYSIS OF TPC

Secondary metabolites	Plant material	Habitats			
		Hills region	Dry soil	Wet soil	Callus
Phenolic content (mg/g)	Fresh leaf	62	50	45	48

In this study, the total steroids content of *Abutilon indicum* were successfully estimated using aluminium (III) chloride colorimetric assay where by a total of 45mg/g of total steroids in the leaf from hills and the total steroids content then the other samples. The leaf in hills showed higher amount of steroids than others and they were expressed in **figure 2 and Table 5.**

TABLE 5: TOTAL STEROIDS ANALYSIS

Secondary metabolites	Plant materials	Habitats			
		Hills	Dry soil	Wet soil	*In-vitro* Callus
Steroids (mg/g)	Fresh leaf	40	31	19	14

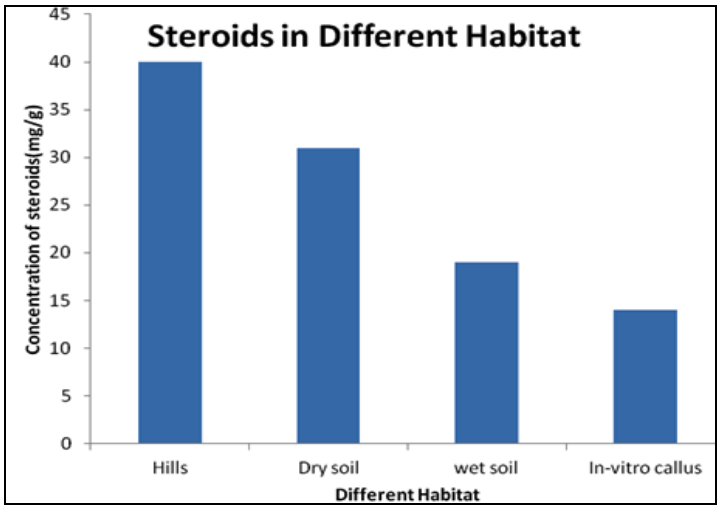


FIGURE 2: STEROIDS IN DIFFERENT HABITAT

DISCUSSION: The plant samples which were collected from hill region posses more secondary metabolites then the dry and wet soils. Because, increase in high concentration mostly is entailed with elevated temperature or low water accessibility, and brought environment often are associated with high concentrations in the soil. The effects of drought and salt stress on the accumulation of secondary metabolites plant products can be made.

In a whale array of experiments it could be shown the plants which are exposed to drought stress indeed produce higher amount of secondary metabolites. The plant sample contain a wide variety of compounds with secondary metabolites content phenolic compounds (Flavonoids and phenols) nitrogen compounds (Alkaloids, Chlorophyll derivatives and amines), carotenoids, steroids and terpenes were reported to posses secondary metabolites in suppressing the initiation or propagation of the chain reactions.

Flavonoids and phenolic compounds are the main secondary metabolite compounds of fresh leafs and vegetables. In this study *Abutilon indicum plant* extracts showed secondary metabolites, however, the magnitude of secondary metabolites potency varies with the types of extracts. This could be due to the different in concentrations and type of secondary metabolite compounds present in these extracts [6].

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