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Phytochemical screening and estimation of primary metabolites in *Tridax procumbens* (L.): plant parts grown-*In vivo* & *In vitro*

Ankita Jain[1,*], DV Rao[1], Amla Batra[1] and Amita Jain[2]

[1]Department of Botany and Biotechnology, University of Rajasthan, Jaipur-302004, India.

[2]Department of Biotechnology and Bioinformatics, D.Y. Patil Vidyapeeth, Sector No-15, CBD, Belapur, Navi Mumbai - 400 614, M.S, India.

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ABSTRACT

Plants have been an important source of medicine for thousands of years. Recently, the world health organization estimated that upto 80% of people still rely mainly on traditional remedies such as herbs for their medicines. In the present study various plant parts of *Tridax procumbens* were evaluated separately for the presence of primary metabolites. *Tridax procumbens* Linn belongs to family Asteraceae, and commonly known as Dhaman grass, is a common herb. It is employed as an indigenous medicine for variety of ailments including Jaundice. The plant also has hepatoprotective activity and it is used in Ayurveda in various liver disorders. Phytochemical screening and quantification of primary metabolites in different plant parts i.e leaf and stem (*in vivo*) as well as its callus (*in vitro*) quantification revealed the presence of total soluble sugars, proteins, lipids chlorophyll and proline. It showed higher concentration of total soluble sugar, lipid and protein in leaves whereas chlorophyll content and proline was found higher in callus as compared to other parts of plant.

Introduction

In recent years, traditional system of medicine has become a burning issue of global importance. Many of the plant species that provide medicinal herbs have been scientifically evaluated for their possible medicinal applications. It has been mentioned that natural habitats for medicinal plants are disappearing fast and it is increasingly difficult to acquire plant derived compounds. This has prompted scientists to consider the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals. World health Organization has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species (Rafiq khan *et al.*, 2013). According to WHO more than 80% of world's population relies on traditional herbal medicine for their primary health care (Tijani *et al.*, 2008).

Tridax procumbens Linn is a common herb found in India. The whole plant was reported to treat various ailments such as bronchial catarrh, dysentery, diarrheoa, and to check hemorrhage from cuts (Pandey *et al.*, 2008 & Vijayan *et al.*, 2007). Pharmacological studies have shown that *Tridax procumbens* possess properties like anti-inflammatory, hepatoprotective, wound healing, immunomodulatory, antimicrobial, antiseptic, hypotensive and bradycardiac effects (Saraf *et al.*, 1991, Taddei *et a.l*, 2000 & Tiwari *et al.*,2004).

Tridax procumbens Linn (Family –Asteraceae) is a common herb found in India. It is native to tropical, subtropical and mid temperate regions worldwide. It habitats waste places, road sides and hedges throughout India. The exomorphology and histomorphology of leaf, petiole and root of the plant was studied (Suseela *et al.*, 2002).

The medicinal value of the plants lies in some active compounds called phytochemicals that produce a definite physiological action on the human body. Phytochemicals are divided into two groups, which are primary and secondary constituents according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins, lipids, chlorophyll while secondary constituents consists of alkaloids, terpenoids, flavonoids, tannins, phenolic compounds(Krishnaiah *et al.*,2007). The primary metabolites are known to play a major role in the adaptation of plant to their environment, and also represent important source of pharmaceuticals (Ramachandra & Ravishankar, 2002). In view of its medicinal value, the present study is aimed to screen and to quantify the pharmaceutically important primary metabolites from *Tridax procumbens* leaves, stem and callus and compare them which will greatly contribute to the nutraceutical industry and drug research.

To the best of our knowledge there has been no comprehensive analysis on quantification of primary metabolites in *Tridax procumbens.*

Materials and Methods:

Plant Material

Plant material of *Tridax procumbens* (RUBL211384) was collected from Chittorgarh district of Rajasthan, India. The plant was taxonomically identified and authenticated by herbarium of Rajasthan University, Jaipur. The voucher specimen of the plant was retained the herbarium.

Collected plant material was air-dried under shade at room temperature, ground with an electric grinder into fine powder using a mechanical blender (Chase *et al.*, 1949). The resultant was then subjected for successive extraction with petroleum ether, benzene, chloroform, acetone, alcohol, and water with soxhlet apparatus. The extracts were then concentrated in vacuum under reduced pressure using rotary flash evaporator and dried in desiccators. The extractive values were deduced using following formula:

Tele:
E-mail addresses: jain.aim2009@gmail.com

Yield (%) = Dry weight of extract/Dry weight of plant powder × *100*

These extracts were then subjected to preliminary phytochemical screening for the detection of various plant constituents. Each of these extracts was processed further to evaluate the presence of carbohydrates, proteins, alkaloids and flavonoids following the established protocols (Kokoshi *et al.,* 1949).

To grow the plant parts *in vitro*, explants taken were sterilized and inoculated on MS media. The callus cultures obtained were maintained for 6 months by periodic subculturings maintained on the same media at 26±2ºC with photoperiod of 16 hr with 55% humidity and 2000-3000 lux light intensity. The callus grown on media [MS7] supplemented with IAA: NAA (1:3 mg/l), BAP: IBA: NAA (3:3:0.5 mg/l) and 2,4D: kin (5:0.2 mg/l) gave best results. Plant regeneration via shoot and root morphogenesis was observed, when callus was transferred on media with various combinations and concentrations of hormones. The callus grown was dried at room temperature, powdered and stored.

Preliminary screening

Phytochemical analysis was carried out in petroleum ether, benzene, chloroform, alcohol and aqueous extract of leaves, stem and callus of *Tridax procumbens* using standard procedures. (Khandelwal ,2009,Kokate, 2000 & Kumar *et al.,* 2009)

Proteins

Xanthoproteic test: Extract was treated with few drops of concentrated HNO_3 formation of yellow indicates the presence of proteins.

Biuret test: To 3 ml of the extract few drops of 10% NaCl and 1% copper sulphate was added for the formation of violet purple colour.

Millon's test: To 3ml of the extract few drops of Millon's reagent was added for the formation of red colour.

Amino acids

Ninhydrin test: To the 2 ml extract 2 ml on ninhydrin reagent was added & boil for few minutes, formation of blue colour indicates the presence of amino acid.

Carbohydrates

Molisch test: To a small amount of the extract few drops of Molisch reagent was added followed by the addition of conc. Sulphuric acid. The mixture was then allowed to stand for 2 min and then diluted with 5ml of distilled water. Formation of red or violet colour at the inter phase of two layers indicates the presence of carbohydrates.

Fehling's test: The extract was treated with 5ml of Fehling's solution (A and B) and kept in boiling water bath. The formation of yellow or red colour indicates the presence of reducing sugar.

Flavonoids

Shinoda' S Test- To 2 ml of the test solution a piece of Mg++ ribbon and concentrated HCl was added drop by drop. The resulting pink/ scarlet crimson or occasionally green/blue colour indicated the presence of flavonoids.

Alkaloids

Each of the test samples was acidified with 2% HCl heated (600) for 2 h which was later, cooled and filtered. A formation of white precipitate on addition of 2-3 drops of following reagents to 2 mL of the above solution indicated the presence of alkaloid.

1. Modi Mayer's reagent- Prepared by mixing 1.35g $HgCl_2$ and 3.95 g KI in 100ml of distilled water.
2. Wagner's reagent- Prepare by mixing 1.27g I2 and 2.00 g KI in 100 ml distilled water.
3. Bourchardt's reagent- Prepare by mixing 2.0g I2 and 4g KI in 100 ml distilled water.

Analysis of Primary metabolites in *Tridax procumbens* (L.):

Total soluble sugars: ***Extraction***: The dried test materials (50 mg each) were homogenized in a mortar and pestle with 20 ml of 80% ethanol separately and left overnight. Each sample was centrifuged at 1200 rpm for 15 min; the supernatants were collected separately and concentrated on a water bath method of Loumis & Shull (1937). Distilled water was added to make up the volume up to 50 ml and processed further for quantitative analysis.

Total proteins: ***Extraction:*** The test samples (50 mg each) were separately homogenized in 10 ml of cold 10% trichloroacetic acid (TCA) for 30 min and kept at 4ºC for 24 h. These mixtures were centrifuged separately and supernatants were discarded. Each of the residues was again suspended in 10 ml of 5% TCA and heated at 80ºC on a water bath for 30 min. The samples were cooled, centrifuged and the supernatants of each were discarded. The residue was then washed with distilled water, dissolved in 10 ml of 1N NaOH, and left overnight at room temperature (Osborne, 1962).

Quantitative Estimation of sugars and proteins: Each of the above samples (1ml) was taken and the total protein content was estimated using the spectrophotometer by Lowry method. A regression curve of the standard protein (bovine serum albumin, BSA) was prepared. A stock solution of BSA (Sigma Chem. Co., St. Louis, USA) was prepared in 1N NaOH (1mg/ml). Eight concentrations (ranging from 0.1 to 0.8 mg/ml) were separately measured in test tubes and the volume of each was made up to 1ml by adding distilled water. To each, 5ml of freshly prepared alkaline solution (Prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4$. 5 H_2O in 1% Sodium potassium tartarate) was added and kept at room temperature for 10 min. In each sample 0.5 ml of Folin-Ciocalteau reagent (commercially available reagent was diluted with equal volume of distilled water just before use) was added rapidly with immediate mixing and optical density of each sample was measured after 30 min at 750nm using spectrophotometer against the blank (Lowry *et al.,* 1951). Five replicates of each concentration were taken and the average value was plotted against their respective concentrations to compute a regression curve.

All samples were processed in the same manner and the concentration of the total protein content in each sample was calculated by referring the optical density of each sample with standard curve. Five replicate samples were taken in each case and mean value was calculated.

Lipids ***: Extraction and Quantification:*** The test samples were dried, powdered and 100 mg was macerated with 10 ml distilled water, transferred to a conical flask containing 30 ml of chloroform and methanol (2/1: v/v Jayaraman, 1981) The mixture was thoroughly mixed and left overnight at room temperature in dark for complete extraction. Later, 20 ml of chloroform mixed with 2 ml of water were added and centrifuged. Two layers separated, the lower layer of chloroform, which contained all the lipids, was carefully collected in the pre weighed glass vials and the coloured aqueous layer of methanol which contained all the water soluble substances and thick pasty inter face layer were discarded in each test sample. The chloroform layers were evaporated to dryness and weighed. Each treatment was replicated thrice and their mean values calculated.

Chlorophyll: ***Extraction and Quantification***

Chlorophyll content was observed by Arnon's (1949) method using 80% acetone and the absorbance was read at 663 nm and 645 nm using spectrophotometer. The value of chlorophyll content was calculated by the following formula.

Table 1: Physico-chemical Evaluation of *T. procumbens* (L.) from different Plant Parts and Callus Cultures

Parameter		Organic Solvents Used				
		Pet. Ether	Benzene	Chloroform	Alcohol	Water
Physical Appearance	Plant Parts	Yellow Green Sticky	Bright Red Oily	Yellowish Orange Oily	Red Brown Sticky	Brown Dusty Viscous
Carbohydrates	Leaves	-	+	++	++	++
	Stem	-	-	+	++	+
	Callus	+	+ +	+ +	+ + +	++
Proteins	Leaves	++	-	+	++	++
	Stem	+	+	+	+++	+
	Callus	+	-	+ +	+ +	+ + +
Flavonoids	Leaves	-	++	+++	++	+++
	Stem	-	-	+	++	+
	Callus	-	+	-	+ + +	+ +
Alkaloids	Leaves	+	+ +	+	+ +	+
	Stem	-	-	+	++	+
	Callus	-	++	+	++	+

- absent; + trace amount; ++ moderate amount; +++significant amount

Table 2: Table of primary metabolites Total levels of various Primary metabolites (mg/gdw) in *Tridax procumbens* (L.)

Plant Parts	Total Soluble Sugar	Chlorophyll	Proteins	Lipids	Proline
Leaves	40.45 ±0.43	14.11 ±1.14	134 ±0.05	89 ±0.74	10.08 ±1.01
Stem	38.45 ±0.42	12.23 ±1.02	78 ±1.05	68 ±0.68	5.64 ±1.06
Callus	32.23 ±0.39	18.17 ±1.17	90 ±0.67	74 ±0.81	14.87 ±1.72

Each value is the mean of three replicates ± Standard Error

$$\text{Chlorophyll a} = \frac{(12.3 \times \text{OD at } 663 - 0.86 \times \text{OD at } 645) \times V}{1000 \times W}$$

$$\text{Chlorophyll b} = \frac{(19.3 \times \text{OD at } 645 - 3.6 \times \text{OD at } 663) \times V}{1000 \times W}$$

V= Final volume of chlorophyll extract in 80% acetone
=Fresh weight of tissue extract

1 gm plant material was macerated in 80% acetone and centrifuged, thrice, at 5000rpm for 5 min. The superanatnt was pooled and final volume was made to 4ml. OD was taken at 645 and 663 nm against 80% acetone as blank. OD was taken at 645 and 663 nm against 80% acetone as blank.

Estimation

Results are presented as the average of three replicates and the chlorophyll content was expressed as mg/gdw fresh weight

Proline: *Extraction:*

50mg plant sample (stem, leaf and callus) was powdered, macerated and homogenized in 3% sulphosalicylic acid (10ml) and then it is centrifuged at 1000 rpm for10-15 mins. The supernatant was cooled and final volume was made to 10ml sulphosalicylic acid.

Estimation

2ml of filtrate was taken and to it glacial acetic acid and acid ninhydrin (1, 25 mg ninhydrin warmed in 30ml glacial acetic acid and 20 ml of 8M phosphoric acid) was added. The filtrate was kept in water bath for 1 hour. Toluene (4ml) was added to the reaction mixture and after stirring it well, organic phase containing toluene and proline was separated (Shetty et al, 2002). Optical Density was taken at 520nm.

Results:

T. procumbens plant parts grown *in vivo* & *in vitro* were evaluated quantitatively for total levels of soluble sugars, proteins, lipids, proline and cholorophyll by described methods.

Preliminary phytochemical investigation revealed that alcoholic extract of *T. procumbens* callus contains carbohydrates whereas stem of *T. procumbens* contains proteins, and carbohydrates. However, maximum intensity of alkaloids are present in benzene and alcoholic extracts of leaves, stem and callus while flavonoids found in chloroform and aqueous extract of leaves and alcoholic extract of callus . The various plant parts of the experimental plant exhibited variation in total levels of various metabolites **(Table 1)**

In the present investigation maximum total soluble sugar was observed in leaves (40.45mg/gdw), of *T. procumbens* and minimum in its callus (32.23mg/gdw), while chlorophyll were found to be maximum in callus (18.17mg/gdw), of *T. procumbens* and minimum in its stem (12.23mg/gdw). Protein was found to be maximum in leaves (134mg/gdw) of *T. procumbens* and minimum in its stem (78mg/gdw). Lipids were maximum in leaves (89mg/gdw), of *T. procumbens* and minimum in its stem (68mg/gdw). Callus of *T.procumbens* had maximum proline content (14.87mg/gdw), while minimum in stem (5,64mg/gdw) .Overall, leaves of *T. procumbens* had maximum content of proteins (134 mg/gdw), and minimum proline content (5.64mg/gdw) was found in stem.**(Table 2)**

Discussion:

Primary metabolites are considered to be integral part of plant metabolism but now looking towards their participation in metabolic pathways, they are considered as precursors of secondary metabolites.

Niranjan and Katiyar (1979) evaluated the range of crude proteins (22-31%), total carbohydrates (9.68-11.80%) and total lipids (1.61-3.91%) in selected leguminous plants. Similarly *Tridax procumbens* also contains many primary metabolites like carbohydrates, proteins, lipids, amino acids, cholorophyll. The phytochemical screening done by Christudas *et al.,* 2012 showed

the presence of various substances like alkaloids, steroids, carbohydrates and proteins in the species.

In consonance with, Nataraj and khan (2014), our study showed that *Tridax procumbens* leaves contain alkaloids, protein, in petroleum ether, ethanolic and aqueous extract. Monika *et al.,* (2013), reported presence of alkaloids and absence of flavonoids in *Tridax procumbens* aqueous extract in contrast to the present study which showed presence of flavonoid in the same extract. Rajaram *et al.*,(2013) and Saithramma *et al.,* (2011) reported similar results for alkaloids, proteins amino acids and flavonoids in chloroform extract of *Tridax procumbens*. Razia *et al.,* (2013) reported presence of alkaloids, flavonoids, carbohydrates in methanolic extract of *Tridax* leaves. Manjusha *et al.,* (2014) showed the presence of carbohydrates amino acids in petroleum ether extract and absence of flavonoids and proteins in the same. Our study showed that leaves of *Tridax procumbens* have more carbohydrates, proteins and lipids while callus contains more amounts of proline and chlorophyll than in other plant parts.

To the best of our knowledge and literature survey, no reports are available on quantitative analysis of primary metabolites in *Tridax procumbens*. Plant synthesizes primary metabolites (lipid, protein, starch, sugars, phenol etc.) for the normal growth and development of itself. Many polysaccharides purified from chinese medicinal herbs and phenols are bioactive and possess immuno-modulating, antitumor and antibacterial activities. In addition, the results confirm the use of the plant in traditional medicine. The investigation can be subjected to the therapeutic uses and carry out further pharmacological evaluation.

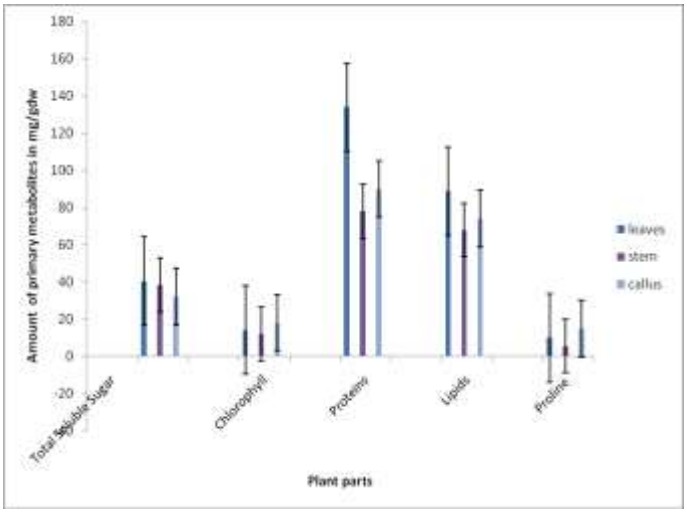

Fig 1: **Concentration of Primary metabolites in plant parts (leaves, stem & callus) of *Tridax procumbens* (mg/gdw).**

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