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Original Article

The Comparative Studies of the Phytochemical Levels and the *in vitro* Antioxidant Activity of *Tridax procumbens* L. from Different Habitats

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ABSTRACT

Background: *Tridax procumbens* is weed and randomly used in *Ayurvedic* system of medicine for hepatic problems such as jaundice, hepatitis, cirrhosis. It has been established that most powerful in improving the oxidative damage of liver injury caused by various factors. **Objectives:** To compared the *in vitro* antioxidant activity and quantity of the phytochemicals such as tannins, flavonoids, and total phenols, of the leaves and the flowers extracts of *Tridax procumbens* L. from different habitats. **Methods & Methods:** *Tridax* leaves and flowers were collected widely from the three different locations;-Hilly terrain, Dry-land, Wet-land and washed with distilled water, shade dried, coarsely powdered and extracted using 70% ethanol. The Phytochemicals levels and *in vitro* free radical scavenging activity were evaluated using standard protocols. **Results:** The flower and the leaf extracts of the hilly-terrain expressed the highest percentage of yield, total phenol and flavonoids contents, compared to the respective extracts of the dry and the wet-lands. Amongst all the extracts made, the flower extract of the hilly-terrain showed the lowest IC_{50} value and the highest *in vitro* free radical scavenging activity because of the highest contents of the flavonoids and total phenols. **Conclusion:** Biosynthesis of the secondary metabolites of *Tridax procumbens* is not only controlled genetically, but also strongly influenced by the habitats. Moreover, the antioxidant potential of plant extracts derived from high altitude samples were higher than dry and wet-lands. The location of collection of plants was found to have profound influence in repeatability of the experimental results.

Key words: *Tridax procumbens*, Phytochemicals, Habitats, Antioxidant, IC_{50} value.

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INTRODUCTION

Tridax procumbens (family-Asteraceae) is commonly known as "Ghamra". In English it is popularly called as "coat buttons" because of the appearance of the flowers. It has been extensively used in *Ayurvedic* system of medicine for various illnesses. It is a well known medicine for liver disorders.[1] This plant extract know to express various pharmacologically active compounds which has been shown to have roll in immunomodulatory, hypoglycemic, hepatoprotective action. It was also shown antioxidant, anti-inflammatory, analgesic, anti-arthritic and wound healing effect and have been used as a skeletal muscle relaxant.[2-9] The phytochemical screening of the different plant parts like leaf, stem, flower and root of *Tridax* have revealed the presence of alkaloids, flavonoids (catechins and flavones) and tannins.[10,11] Phytochemicals are chemical compounds, created or biosynthesized through the usual metabolic pathways in the plants. These chemicals are often referred to as secondary metabolites. These secondary metabolites have no direct activity in the primary metabolic cycles, such as photosynthesis, respiration, transpiration and mineral metabolism etc. But they have been proved to have major roles in the stress tolerance and defense mechanisms.[12] Secondary metabolites are important for the plants interactions such as plant-pollinator, plant-pathogen and plant-herbivore. These are classified into three major classes (alkaloids, terpenoids and phenolics) and habitually colored, aromatic and tasty compounds.[13] They are also used in signaling and regulation of primary metabolic pathways. Secondary metabolites are used by many pharmaceuticals, agrochemicals, biopesticides, flavoring, coloring agents, and as food additives.[14] The biosynthesis of secondary metabolites is controlled by various factors such as genetics and ecological factors.[15]

The quantity of secondary metabolites in medicinal plants is strongly dependent on the growing conditions and synthesis is regulated by external environmental stimuli[16] Abiotic factors such as high and low temperatures, drought, alkalinity, salinity of the soil, ultraviolet radiation stresses have been known as more potential factors for manipulating and promoting the accumulations of secondary metabolites in medicinal plants.[17-19] The abiotic factors of the growing area such as soil fertility, local geoclimate, humidity, light and temperature, seasonal changes and cultivation techniques, have also been shown to impact on the metabolism and synthesis of the secondary metabolites.[20] Hence the aim of the study to investigates the habitats influences on *in-vitro* antioxidant activities and the quantity of phytochemical (total phenol, flavonoid and tannin) variations in leaves and flowers of *Tridax*.

MATERIALS AND METHODS

Plant materials

Tridax leaves and flowers were collected from the three different habitats:-1. Kolli-Hills (is a mountain situated in the Ester Ghats in Namakkal district of Tamil, Nadu India. The mountains are about 1000 to 1300 m in altitude and cover an area of approximately 280 km^2). 2. Dry-land (fields surrounding R. Puduppalayam Village in Namakkal District, Tamilnadu India).3. Wet-land (irrigated agricultural paddy field in Attaympatti, Namakkal District, Tamilnadu, India). The Botany Department of Bishop Heber College, Trichy-17 India, authenticated the samples. The fresh leaves and flowers were washed with distilled water, shade dried, coarsely powdered and stored in tight glass containers for further use.

Extraction procedure

The milled samples of the leaves and the flowers of *Tridax* were infiltrated with 70% ethanol in the ratio 1:5 (w/v) at room temperature for 72 h with frequent mechanical shaking. This was repeated three times until the extract gave faint or no coloration. Then extract filtered using Whatman filter paper No.1 and evaporated to dryness using flash rotary evaporator at 48^{O}C.

Determination of Phytochemicals

Total phenolics contents

Folin-Ciocalteu reagent used to determination of the total phenolic contents of the *Tridax* flowers and leaves extract following a slightly modified method of Ainsworth.[21,22] Gallic acid was used as a reference standard.

Flavonoids

Colorimetric method using aluminum chloride was followed for the flavonoid determination.[23] Total flavonoids content was calculated as quercetin equivalents (mg/g).

Tannins

The tannins levels from the extracts of *Tridax* were determined by the methyl cellulose precipitate (MCP) method.[24] The assay is based upon methyl cellulose-tannin interactions resulting in the formation of insoluble polymer-tannin complexes which precipitate. Tannin values are calculated from the difference between the absorbance values at 280 nm (A280) of solutions both with and without precipitation.

Antioxidant assays

Assay of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

Rapid screening of Antioxidant by dot-blot Staining

The antioxidant activity of the extracts of *Tridax* were measured using DPPH according to Soler-Rvastal *et al.*, 2000.[25] In brief, 3 µl (100 µg /ml) aliquots of freshly prepared 70% ethanolic extracts of *Tridax* were loaded individually in silica gel Thin-Layer Chromatography plate (TLC Silica gel 60 F_{254} Merck) and air dried for a few minutes. The TLC plate bearing the dry spots was placed upside down for 10 sec in 0.1 mM-methanolic DPPH solution. Stained silica layer revealed a purple background with yellow spot located where the drops were placed, which indicates radical scavenger ability. The strength of the yellow color spot (diameter) was proportional to the amount and nature of the radical scavengers present in the extract.

Photometric assay of DPPH scavenging activity

The DPPH scavenging activity of the extracts of *Tridax* were performed according to the method of Gyamfi *et al.*[26] Fifty micro liters of the plant extract, yielding 100 µg/ml respectively in each reaction was mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µl) only was used in the control experiments. After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured reading the absorbance at 517 nM. The % of inhibition was calculated from the following equation:=[Absorbance of control–Absorbance of test sample/Absorbance of control]×100.

Assay of nitric oxide scavenging activity

The nitric oxide scavenging activity was measured according to Ebrahimzadeh *et al.* method.[23]

Table 1: The free radical scavenging activity of *Tridax procumbens* leaves and flowers extracts from different habitats

Geoclimatic locations	DPPH IC_{50} in µg	Nitric acid IC_{50} in µg	Reducing power IC_{50} in µg	Hydrogen peroxide IC_{50} in µg
1. Leaf				
a. Hilly terrain	34.23	363.68	426.83	330.33
b. Dry-land	42.63	473.23	493.73	423.63
c. Wet-land	98.38	1200.78	1730.33	1100.23
2. Flower				
a. Hilly terrain	28.34	284.73	333.63	243.33
b. Dry-land	32.23	330.33	426.56	293.63
c. Wet-land	73.56	800.45	1100.33	780.73
3. Vitamin C	7.2	90.33	150.33	83.63

Reducing power assay

The *Tridax* extracts were serially diluted using 1% ferrocyanate phosphate buffer (2M pH 6.6) and incubate at 50^{o}C for 20 min. Then 10% Trichloroacetic acid (TCA, 2.5 mL) was added to a portion of this mixture (5 mL) and centrifuged at 3,000 g for 10 min, supernatant was collected and mixed 2.5 ml distilled water plus 0.5 ml of ferric chloride. The mixture was measured at 700 nM and calculated the reducing power.[23]

Hydrogen peroxide scavenging assay

Hydrogen peroxide (40 mM) solution was prepared in phosphate buffer at pH7.4. Then the different concentration of extract was added to a hydrogen peroxide solution (0.6 ml of 40 Mm) and read at 230 nM after 10 min incubation of the mixture against the blank solution and calculated hydrogen peroxide scavenging activity of the different extracts.[27]

RESULTS

Percentage yield of hydroethanolic extract

The Figure 1 shows the percentage yield of crude hydroethanolic extracts of the leaves and the flowers of *Tridax* of different habitats. The flowers yield was found to be higher than the leaves yield in all respective habitats. However extracts of the both flowers and leaves from the hilly terrain was registered higher than the dry and wet-lands. The wet-land extracts recorded the lowest yield compared to the hill and dry-lands.

Total phenolics, Flavonoids and Tanins contents

The phytochemical studies of the flower and the leaf extracts of *Tridax* in different habitats revealed that quantities of phenolics, flavonoids and tannins contents varied considerably. The Figures 2 and 3 shows the total phenolic, flavonoids and tannins contents of crude hydroethanolic extracts of leaf and flower of *Tridax* in different habitats. In this present study, the flower extract from the region hilly terrain contained highest phenolic (173 mg/g of crude extract) and flavonoids (58 mg/g of crude extract) than the dry and wet-lands. However the tannin levels of leaves extract of dry-land (23/g of crude extract) was higher than the hill (19 mg/g of crude extract) and wet-lands (12 mg/g of crude extract). But in the case of flower extract, the tannin level was higher in the hilly terrain (12 mg/g of crude extract) than in the dry(8 mg/g of crude extract) and in the wet-lands (6 mg/g of crude extract). In the floral extracts total phenols and flavonoids were higher than the leaves extract but lesser in tannins levels when compared to their respective habitats.

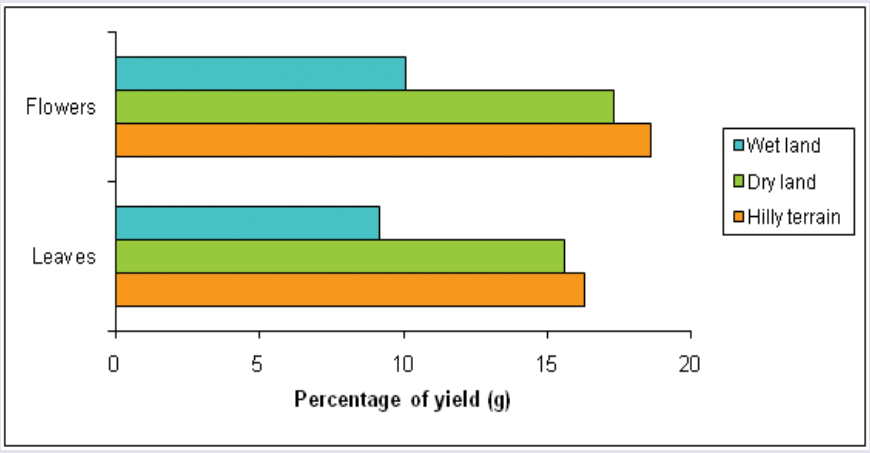

Figure 1: Percentage yield of hydroethanolic extract of leaves and flowers of *Tridax* from three different habitats.

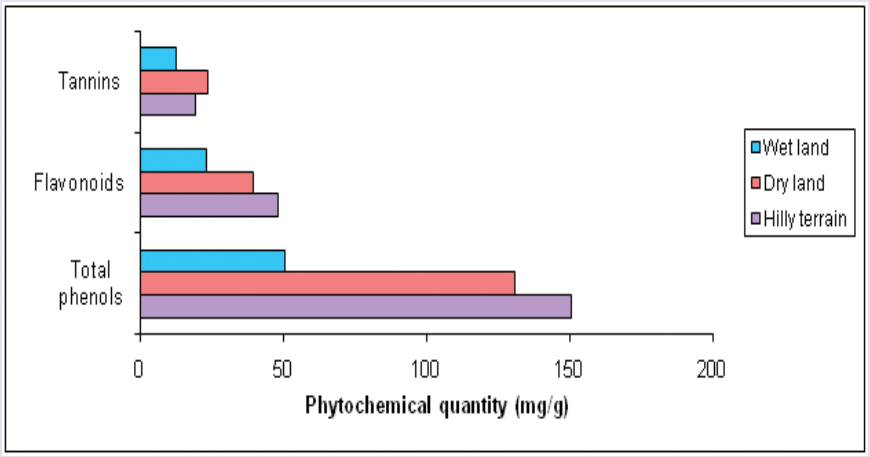

Figure 2: Phytochemicals contents (mg/g) of crude extracts of *Tridax* leaves from three different habitats.

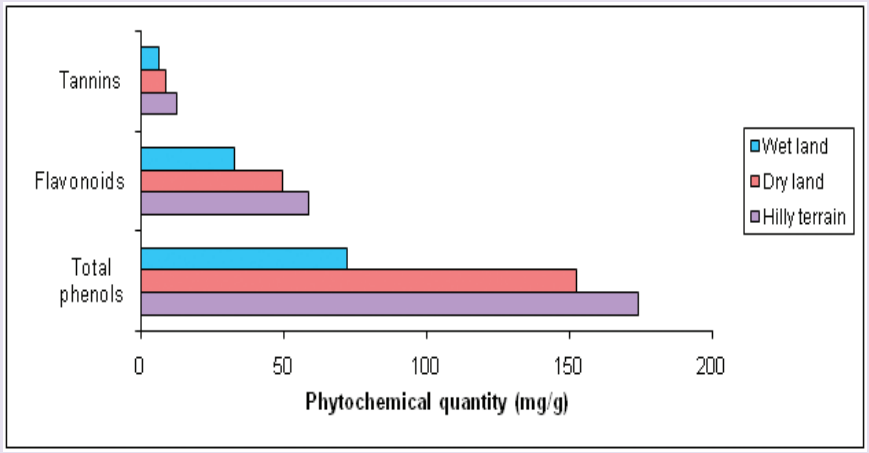

Figure 3: Phytochemicals contents in mg/g of crude extract of *Tridax* flowers from three different habitats.

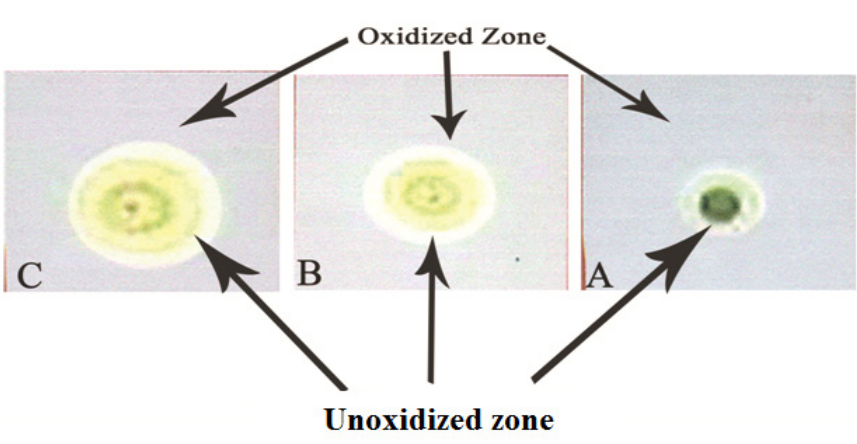

Figure 4: The antioxidant potential (by dot-blot and DPPH staining) of the flowers of *Tridax* from three different habitats (A. Wet-land, B. Dry-land and C. Hilly terrain).

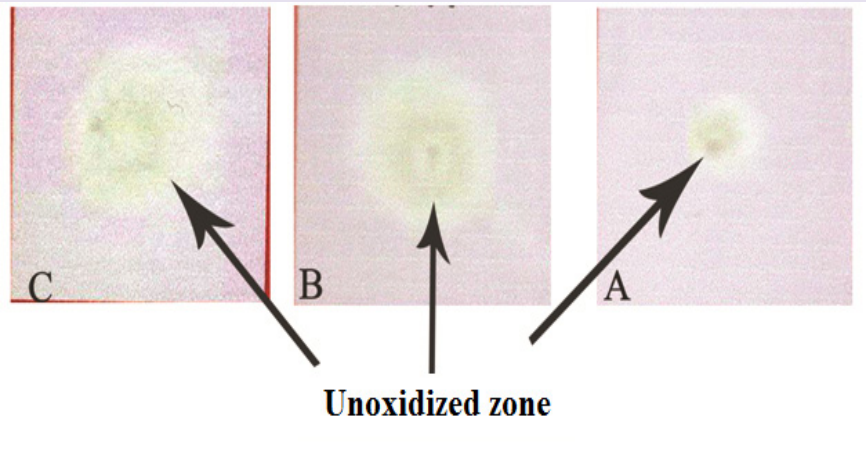

Figure 5: The antioxidant potential (by dot-blot and DPPH staining) of the leaves of *Tridax* from three different habitats (A. Wet-land, B. Dry-land and C. Hilly terrain).

Free radical scavenging activity

DPPH scavenging activity

Rapid screening of antioxidant by dot-blot and DPPH Staining

The Figures 4 & 5 show the rapid screening of antioxidant by dot-blot and DPPH staining of the flower and leaf extracts of *Tridax* of three different habitats. The results indicated that the inhibition of oxidation was directly proportional to the concentration of the phenols, flavonoids and tannins of the different extracts. The extracts of *Tridax* (both leaves and flowers) obtained from hilly–terrain and low dry land showing the maximum radical scavenging activity when compared with low wet-land, because this may be due to the high levels of tannin, flavonoids and phenols than the low wet-land.

DPPH scavenging activity

The DPPH scavenging activities of the extracts depend on the capability of the antioxidant compounds to be donating the hydrogen atoms or electrons and neutralize the free radicals. The IC_{50} of an extracts can be calculated by creating a dose response curve and examining the effect of different concentrations of extract on antioxidant activity. The IC_{50} values of extract was calculated by the amount of extract to attain 50% radical-scavenging effect.[28] The IC_{50} values of the *Tridax* and standard samples were in the order: vitamin C < hilly terrain < dry-land < wet-land (Table 1).

Nitric oxide-scavenging activity

The maximum free radical scavenging activity was at the lowest concentration from which the IC_{50} calculated. The flower extracts showed less IC_{50} value when compared with their respective habitats of leaf extracts (Table 1). Among the three locations, the extracts of the hilly terrain (both flower and leaf extract) showed the highest nitric oxide-scavenging activity.

Reducing power assay

In the reducing power assay, here with more antioxidant compounds convert the oxidation form of iron (potassium ferricyanide Fe^{+3} yellow colored solution) in ferric to ferrous potassium ferrocyanide (Fe^{+2} shades

of green or blue) As observed from Table 1, *Tridax* hilly terrain flower extract has minimum IC_{50} values (333.63 µg/ml) and wet-land leaf extract has maximum IC_{50} (1730.33 µg/ml) amongst individual habitats of plant extracts.

Scavenging of hydrogen peroxide

The hydrogen peroxide scavenging ability of *Tridax* leaves and flowers extract of different habitats shown in the Table 1. The flower extracts made from the hilly terrain of *Tridax* have minimum IC_{50} values (243.33 µg/ml) and wet-land leaf extracts have maximum IC_{50} (1100.23 µg/ml) values.

DISCUSSIONS

The majority of phytochemical compounds found in the plant extracts are the phenolic acids, the flavonoids and the tannins.[29] These compounds are important for treated various diseases, the mechanism action of this phytochemicals in medicine mostly depending the free radical scavenging activity.[30] We have investigated the antioxidant potential and the levels of phytochemicals (tannin, flavonids and total phenols) storage in leaves and flowers of *Tridax procumbens* in three different habitats in Namakkal district, Tamil Nadu, India. The results reveals that environmental conditions have great influences in the antioxidant property and the accumulation of the phytochemicals. of *Tridax* (flowers and leaves). The different geoclimatic factors (climatic conditions, soil types, nutrition biotix and abiotic factors ect), alter the presence as well as the concentration of the phytochemicals in medicinal plants.[31,32]

The present study shows that the highest percent of yield of total phenols contents were recorded in *Tridax* flower extract of the hilly terrain and the lowest was recorded in wet-land leaf extract. The result shows that accumulation of total phenol in flower and leaf of *Tridax* and their antioxidant activity is purely depending on the growing conditions. When the plants are growing in hilly-terrain (high altitude) low temperature and the sunlight contains enhanced UV-β radiations, which may induced oxidative stresses in the plant, hence the plants overcome from the oxidative stresses need to be synthesis of more UV-absorbing and free radical scavenging phenols in plants.[33] The hilly-terrain low temperature may increase the production of phenolics by enhancing synthesis of phenylalanine ammonia lyase (PAL) in plants, while high altitude and long exposure to sunlight hours with higher UV radiation positively affect the activity of enzyme such as phenolics synthase.[34] Meanwhile, small amounts of hilly precipitation could enhance the defense system of plant against stress factor, leading to an increased phenolic content.[35]

Present investigation revealed that the flavonoids contents are higher in hilly terrain flowers and leaves extract of *Tridax* than in the dry and in wet-lands. The increased level of flavonoids in the hilly terrains is in agreement with Monschein *et al*., who found that the environmental factors at higher altitude result in the elevated levels of flavonols.[36] The observation that the dry-land, flavonoid content is higher than in the wet-land is in line with Al-Gabbiesh *et al*.,[37] who reported that plants exposed to drought stress indeed reveal higher concentrations of secondary metabolites than those cultivated under well watered conditions. Drought stress without any change in the enzyme activity, but promote the rate of synthesis of the highly reduced secondary plant products, such as isoprenoids, phenols (flavonoids) alkaloids and tannins. But the tannin content is higher in dry-land than the wet and hilly terrain. Mossi, also suggest the existence of a correlation between environmental factors such as average annual temperature, climate, vegetation, geomorphology and latitude alter the tannin production.[38] Mundree *et al*. had earlier shown that water deficit increases the concentration of secondary metabolites such as cyanogenic glycosides, glucosinolates, terpenoids, alkaloids and tannins.[39] The high production of these secondary metabolites may be attributed to the fluctuation in abiotic stress and non availability of nutrients. Thus these abiotic factors can influence the chemical composition and ultimately the survival of some of these medicinal plants in high altitude regions. The stress factors particularly the temperature and the drought can affect secondary metabolites and other compounds which are the basis for their medicinal activity.[40]

The *Tridax* extracts (flowers and leaves in different habitats) free radical scavenging potential assessed using different protocols and compared with standard antioxidant such as vitamin C. The results from these experiments indicated that free radical scavenging capability depends on the antioxidant molecules present in the extracts. It is assumed that the extract which has low antioxidant activity *in vitro*, will probably show slight antioxidant activity *in viv*o as well.[41] In the present study, among all the extracts of the hilly terrain flowers and leaves extracts had highest *in vitro* antioxidant property and lowest IC_{50} values. Hence it is possible suggest that the antioxidant activity is directly depending on the amounts of total phenols, flavonoids and tannins content. The hilly terrain leaf and flower extracts had highest total phenols and flavonoids content compared with dry and wet-lands extracts. Phenolic compounds of these plants are the major antioxidants because their hydroxyl groups express the highest scavenging ability.

The medicinal plant total phenols are classified in to four major groups. (Phenolic acids, phenolic diterpenes, flavonoids, and volatile oils.[42] The antioxidant potential of the phenolic acids generally act by trapping the free radicals, flavonoids can neutralized the free radicals including chelate metals and singlet oxygen as well.[43] The present investigation proposed that *Tridax procumbens* growing either dry land (drought stress) or hilly-terrain (high altitite UV-radiation, long day light, low temperature and precipitation) altered the metabolism and enhanced synthesis of total phenols and flavonoids. These compounds may be the major contributors for the *in vitro* antioxidant activity and IC_{50} values.

CONCLUSION

In the three different habitats of *Tridax*, the hilly terrain altitude and the abiotic stress factors allowed the accumulation of more phenols and flavonoids. Thus the secondary metabolism of the *Tridax* and the expressed levels of the secondary metabolites are not only controlled by genetic factors but also strongly affected by different habitats. The location of collection of *Tridax* is important in influencing the reproducibility of the experimental results and pharmacological applications.

COMPETING INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the study design; collection, analysis and interpretation of data; content and writing of the paper; and the decision to submit the report for publication.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. NG designed the study, wrote the protocol, and wrote the first draft of the manuscript, managed the literature searches. JRJ and GS. The Authors contributions lower case carried out all kinds of experimental parts and statistical analyses of data. SK supported the protocol writing and revised the manuscript.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATION USED

DPPH: 2,2-Diphenyl-1-picrylhydrazy.

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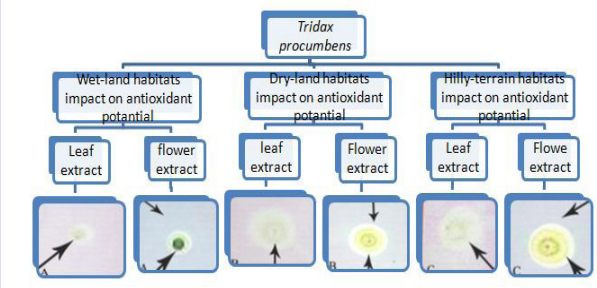
PICTORIAL ABSTRACT



SUMMARY

- Biosynthesis of the secondary metabolites of *Tridax procumbens* is strongly influenced by the environment (habitats)
- In the three different habitats of *Tridax*, the hilly terrain altitude and the abiotic stresses allowed the accumulation of more phenols and flavonoids. The soil water status (dry/wet) too has an impact on the accumulation of the phenolics and flavonoids
- The antioxidant potential of plant extracts derived from high altitude sample is higher than dry and wet-lands
- The location of collection of plant parts is important in influencing the reproducibility of the experimental results and phytochemical based pharmacological effects.