

# Drought Stress Signal Promote the Synthesis of more Reduced Phenolic Compounds (Chloroform Insoluble Fraction) in *Tridax procumbens*

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## ABSTRACT

**Background:** Plant's are ability to manage with abiotic stress factors are mainly altered the metabolic pathways and produces a varied range of secondary metabolites. Secondary metabolite manufacture is a serious part of the defense response in stress circumstances. **Objectives:** The study was aim to find out the impact of drought stress factor on *in vitro* antioxidant potential of chloroform insoluble fraction (phenolics) of leaves and flowers extract of *Tridax procumbens*. **Materials and Methods:** The matured leaves and flowers of *Tridax* were collected widely from the four different locations:- Hilly dry-terrain; Hilly wet-terrain; Low dry-land; Low wet-land. The collected leaves and flowers of *Tridax* were washed with distilled water, shade dried, coarsely powdered and extracted using 70% ethanol and fractionated by chloroform to obtained chloroform insoluble fractions. The chloroform insoluble fraction then subjected into various *in vitro* free radical scavenging activities. **Results:** The drought stress significantly increased the sucrose content in the leaves, chloroform insoluble fractions (water soluble), total phenolics and decreased relative leaf water contents and chloroform soluble fractions when compared with well water state of *Tridax*. The *in vitro* free radical scavenging activities results re-

vealed that drought had constructive effects on radical scavenging and antioxidant capacity. **Conclusion:** In dry land of the *Tridax procumbens* (both hilly dry-terrain and low dry-land) induced drought stress-related metabolic alterations and free radical production. In this condition *Tridax* need over-supply of reduced phenolics for tolerance and prevent the oxidative damage of the cells. As a result the metabolic processes are shifted towards biosynthesis of highly reduced phenolics in the *Tridax* for better adaptation.

**Key words:** Drought stress, *Tridax procumbens*, Chloroform Insoluble fractions, *In vitro* antioxidant.

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## INTRODUCTIONS

Plants are growing under the environmental abiotic stress factors such as drought, high salinity of the soil, cold, heat and high light are induced oxidative stress and produce reactive oxygen species (ROS). ROS are well known factors can damage the proteins, DNA, and lipids of plant cells which have negative influence on the normal growth, development and metabolism of the plants. During response to the oxidative stress conditions the plants developed a complex and efficient antioxidant system, which includes enzymatic antioxidants (catalase, peroxidase, superoxide dismutase) and non-enzymatic antioxidants to balance the harmful effects of the free radicals. Phenolics are the main non-enzymatic antioxidant in the plants.<sup>1,2</sup> Phenolic compounds are one of the chief classes of bioactive phytochemicals that are universally distributed throughout the plant kingdom. The term "phenolic" or "poly phenol" can be precisely defined chemically as a substance which possesses an aromatic ring bearing one (phenol) or more (poly phenol). They can be subdivided in three main subclasses, the flavonoids, phenolic acids, and the stilbenoids. More over the flavonoids are subgrouped into six: based on the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation of the central C ring (flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins).<sup>3</sup> The ROS defense potential of phenolics in plants is diverse which prevent the lipid peroxidation of cell membrane, oxidative damage of DNA and protein, restrain of prooxidant enzymes, chelate metal ions, reducing power and improving the function of the antioxidant enzymes.<sup>4,5</sup> The roll of phenolic in medicine is diverse mainly used for treatment of cancer, cardiovascular diseases and neurodegenerative

diseases. The medicinal properties of the phenolic are based on the antioxidant capacity.<sup>6,7</sup> The recently found that the dietary poly phenol can modulate the cell signalling pathways and prevent the diseases.<sup>8,9</sup>

*Tridax procumbens* having the various pharmacologically important phytochemicals such as alkaloids, carotenoids, flavonoids, fumaric acid,  $\beta$ -sitosterol, saponins tannins and phenolic compounds in leaves and luteolin, glucoluteolin, quercetin and isoquercetin in the flower.<sup>10-13</sup> Among these phytochemicals flavonoids, saponins, tannins quercetin and isoquercetin are belonging to phenolic compounds. It is well recognized that enhanced synthesis of secondary metabolites in the plants strongly depends on the growing environment, such as the temperature, the light regime and the nutrient supply.<sup>14,15</sup> The plants are growing under the abiotic stress conditions which are ability to manage through altering the metabolism and increased synthesis of secondary metabolites. The ROS mediated lipid peroxidation of the cell membranes is the first key factor for altering the metabolism and enhanced synthesis of secondary metabolites in the plant. It is mandatory because need to be prevent the cellular compounds are damaged by ROS mostly the membrane lipids.<sup>16</sup> In *Hypericum brasiliense*, grown under drought stress the concentration of phenolic compounds were 10% increased in comparison with the control plants.<sup>17</sup> A similar conclusion can be drawn by Nogue's *et al.*, in the pea plants (*Pisum sativum*) grown under drought.<sup>18</sup> Today date, literature survey bring to lightening us the drought stress factor are alter only quantity of the phenolics in the plant. But no or limited data for the drought stress factor are affecting the antioxidant quality of

phenolics in the plants. Hence this study was aimed to find out the *in vitro* antioxidant potential of chloroform insoluble fraction of leaves and flowers of *Tridax* extracts of four different habitats:-Hilly dry terrain (drought stress); Hilly wet terrain (drought Stress free); Dry low land (drought stress); Wet low land (drought stress free).

## MATERIALS AND METHODS

### Plant materials

Fresh leaves and flowers of *Tridax* were collected widely from the four different places:-Servarayan hilly dry-terrain (is a mountain situated in middle Tamil Nadu in Salem district of India. The mountains are about 1515 metres (4970 ft) in altitude and cover an area of approximately 383q. kms); Servarayan hilly wet-terrain; Dry low-land (fields surrounding the R.Pattanam Village in Namakkal District, Tamil Nadu India); Wet low land (irrigated agricultural land surrounding the R.Pattanam Village in Namakkal District, India). The Botany Department of Bishop Heber College, Trichy-17 India, authenticated the plant sample. The collected fresh leaves and flowers were washed with distilled water, shade dried and coarsely powdered and stored in tight glass containers for further use.

### Relative Leaf Water and Sucrose Content

Relative water content (RWC) was estimated by a modification of the method of Weatherley<sup>19</sup> and calculated as  $RWC = 100 \times [FW - DM] / [TW - DM]$ . FW and DM denote fresh weight (g) and dry weight (g). Turgid weight (TW) was calculated after fully hydrating fresh leaves in darkness at 4 °C for 24 h. Results were expressed as percentages. Concentration of sucrose was determined using the method of Liu and Huang.<sup>20</sup>

### Extraction procedure

The solvents options for extraction of phenols from the medicinal plants are water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and their mixtures. For example, a high yield of phenolics can be extracted from sorghum leaf using water.<sup>21</sup> Whereas extraction of phenolics from wheat bran using 70% aqueous ethanol.<sup>22</sup> Hence the extract of the total phenolics from *Tridax* was using 70% ethanol. Five hundred grams of powdered leaf and flower of *Tridax* were extracted with 70% ethanol for 72 h each using Soxhlet apparatus separately. The extract was then filtered through Whatman filter paper No.1 and evaporated using rotary evaporator and dried at 48°C.

### Determination of Total phenolics contents

Folin-Ciocalteu reagent used to determination of the total phenolic contents of the *Tridax* flowers and leaves extracts following a slightly modified method of Ainsworth.<sup>23,24</sup> Gallic acid was used as a reference standard.

### Fractionations of hydroethanolic extract

The 15 g of 70% dried hydroethanolic cured extract was taken in a stoppered flask containing 200 ml of chloroform and shaken mechanically for 1–2 h in a flask shaker. The extract was not completely soluble in chloroform. The chloroform-insoluble portion of hydroethanolic extract was separated using filtration and both fractions (chloroform -soluble and chloroform -insoluble) were dried and their percent yield with respect to ethanol extract was calculated.

### Assay of lipid peroxidations product (TBARS)

The lipid peroxidation was calculated through the TBA (Thiobarbituric Acid) reaction method described previously<sup>25,26</sup>

### *In vitro* Antioxidant assays

The *in vitro* antioxidant activity of the chloroform insoluble and water soluble fraction of the leaves and flowers extracts of the *Tridax* using different types of protocols. An antiradical activity dot-blot and DPPH Staining according to Soler-Rvastal *et al.*<sup>27</sup> DPPH scavenging activity (photometric assay) according to Gyamfi *et al.*<sup>28</sup> nitric oxide scavenging activity assay and reducing power assay according to Ebrahim Zadeh *et al.*<sup>29</sup> hydrogen peroxide scavenging assay according to Fukumoto and Mazza.<sup>30</sup>

## RESULTS

### Relative Leaf Water and Sucrose Contents

The sucrose and relative water content (RLWC) of the leaves of *Tridax* in different habitats are presented in Figure 1. The results indicated that there is significant reduction RLWC in both hilly-dry terrain and low dry land leaf of *Tridax* when compared with their respective wet lands. In contrast sucrose content in the leaf was high in dry lands compared with wet land samples.

### Percentage yield of hydroethanolic Fraction

The Figure 2 shows the percentage yield of crude 70% ethanolic extracts of the leaf and the flower of *Tridax* from different habitats. The results indicated that the highest yield was recorder in hilly dry-terrain flower extract and lowest was low wet-land leaf extract. However the hilly terrain extracts of flower and leaf (both wet and dry-terrains) were registered highest yield than the low land of the dry and wet lands.

### Total phenolic contents

The Figure 3 revealed that the total phenolic content of leaf and flower cured extracts of *Tridax* samples collected from different habitats. The results are clearly indicated that drought stress significantly increases the total phenolic content in both leaves and flowers.

### Fractionations of hydroethanolic extracts

The Figure 4 shows the chloroform insoluble fractions of *Tridax*. The results pointed out that the insoluble fractions of the chloroform are higher in the dry lands (both hilly terrain low dry-land) when compared with their respective wet lands, because the polar compound are higher than the non polar one. However in contrast chloroform soluble fractions are higher in the wet lands extracts due to the non polar compounds higher than polar ones (Figure 5).

### Thiobarbituric Acid Reactive Substances (TBARS)

The levels of TBARS of the flowers and leaves of *Tridax* in different habitats are illustrated in the Figure 6. The results reveals that drought stress significantly increased the levels of TBARS in both hilly dry terrain, and low dry land leaves and flower of *Tridax* when compared with their respective wet lands

### Anti oxidative activity

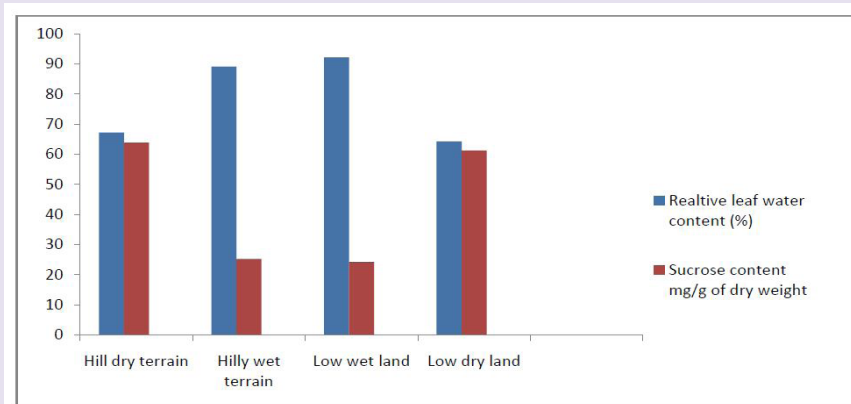
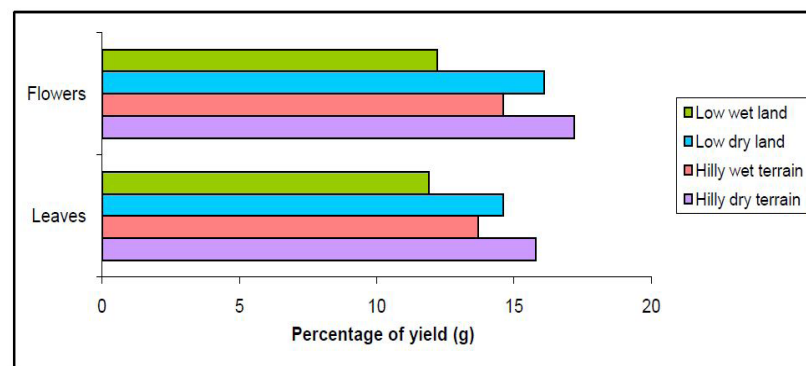
#### DPPH scavenging activity

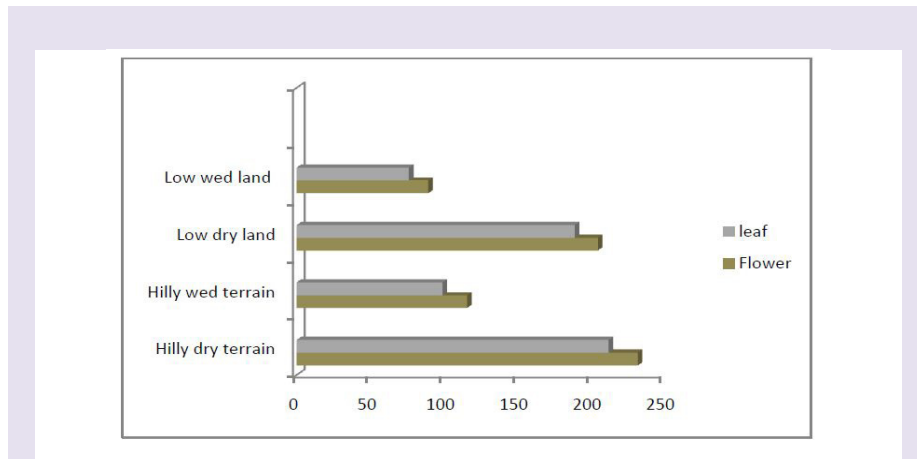
#### Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining

Antioxidant capacity of the flower and leaf extracts of *Tridax* was eye-detected semi quantitatively by a rapid DPPH staining Thin-layer chromatography (TLC) method. Each diluted water soluble fractions were applied as a dot on a TLC layer that was then stained with DPPH solution (Figures 7 & 8). This technique was naturally based on the inhibition of the accumulation of oxidized products. Since the production of free radicals was inhibited by the addition of chloroform insoluble

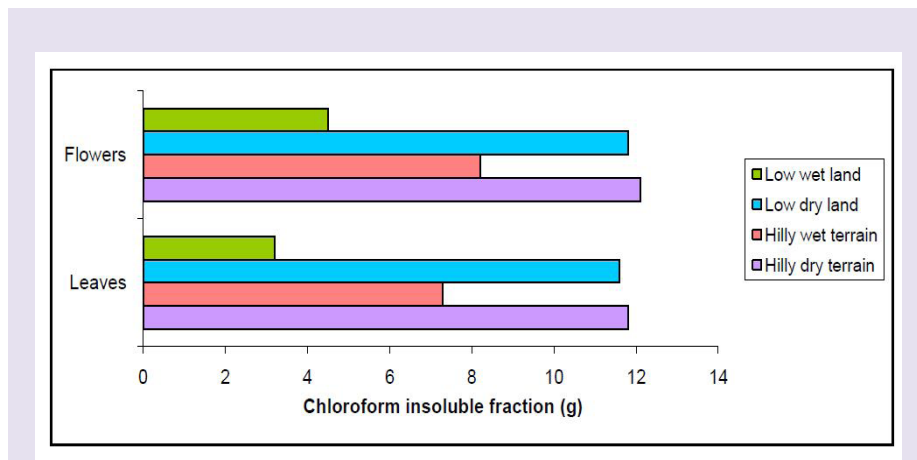
**Table 1: The IC<sub>50</sub> values (free radical scavenging activity) of *Tridax procumbens* leaves and flowers extracts from different habitats**

Different habitats	DPPH IC <sub>50</sub> in µg	Nitric acid IC <sub>50</sub> in µg	Reducing power IC <sub>50</sub> in µg	Hydrogen peroxide IC <sub>50</sub> in µg
1. Leaf				
a. Dry-hilly terrain	2.17	263.68	326.83	230.33
b. Wet-hilly terrain	56.23	640.45	700.55	568.34
c. Dry-low land	32.33	373.55	393.73	323.63
d. Wet-lowland	71.18	1000.78	1530.33	1100.23
2. Flower				
a. Dry-Hilly terrain	18.17	184.73	233.63	243.33
b. Wet-hilly terrain	51.13	560.33	340.55	420.44
c. Dry-low land	28.33	230.33	289.56	293.63
d. Wet-low land	66.18	900.45	1200.33	780.73

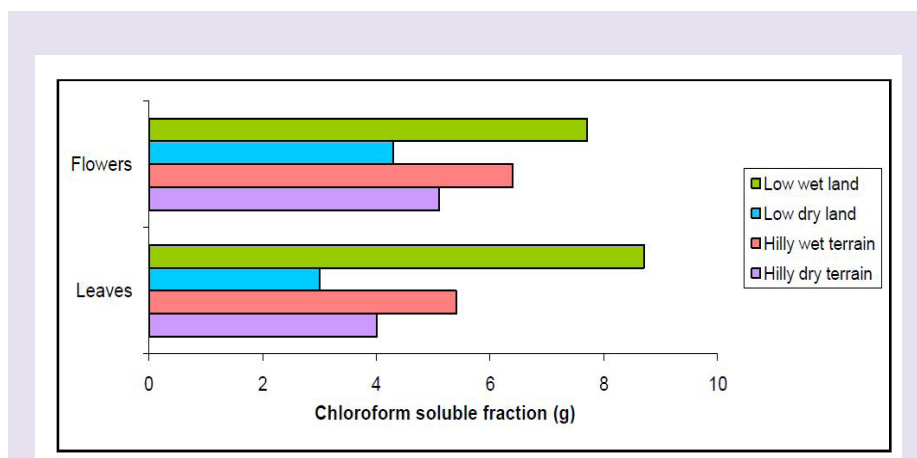
**Figure 1:** Relative leaf water content and sucrose content of the leaves of *Tridax* from different habitats**Figure 2:** Percentage yield of hydroethanolic extract of the leaves and the flowers of *Tridax* from different habitats



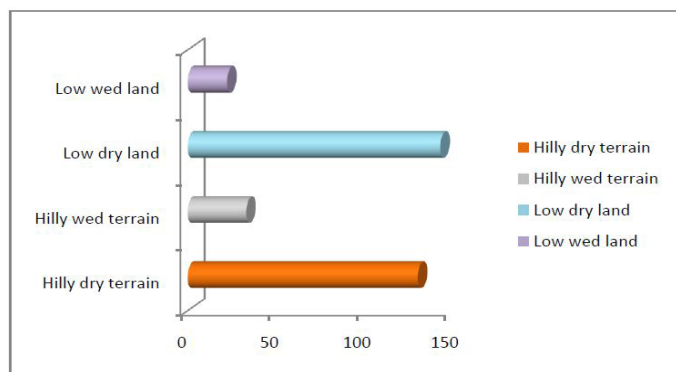
**Figure 3:** Phenolic contents in mg/g of crude extract of *Tridax* flowers and leaves from different habitats



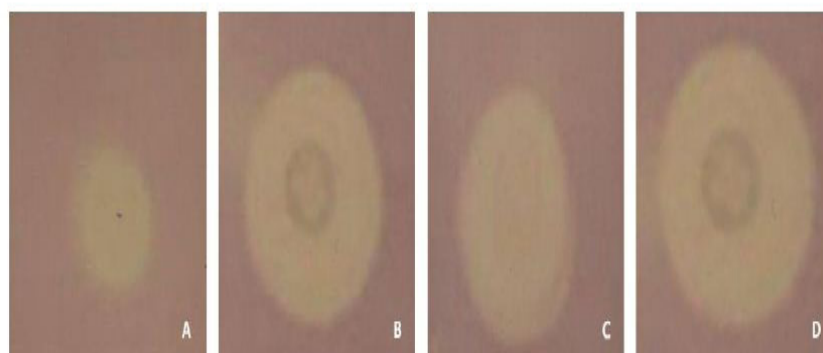
**Figure 4:** Percentage yield of Chloroform insoluble fractions of the leaves and the flowers of *Tridax* from different habitats



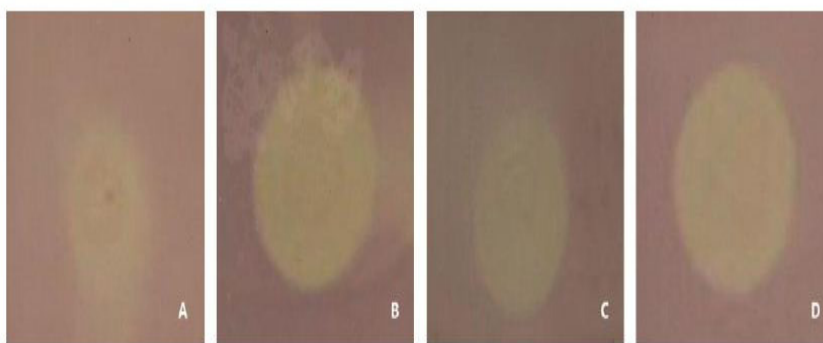
**Figure 5:** Percentage yield of Chloroform soluble fractions of the leaves and the flowers of *Tridax* from different habitats



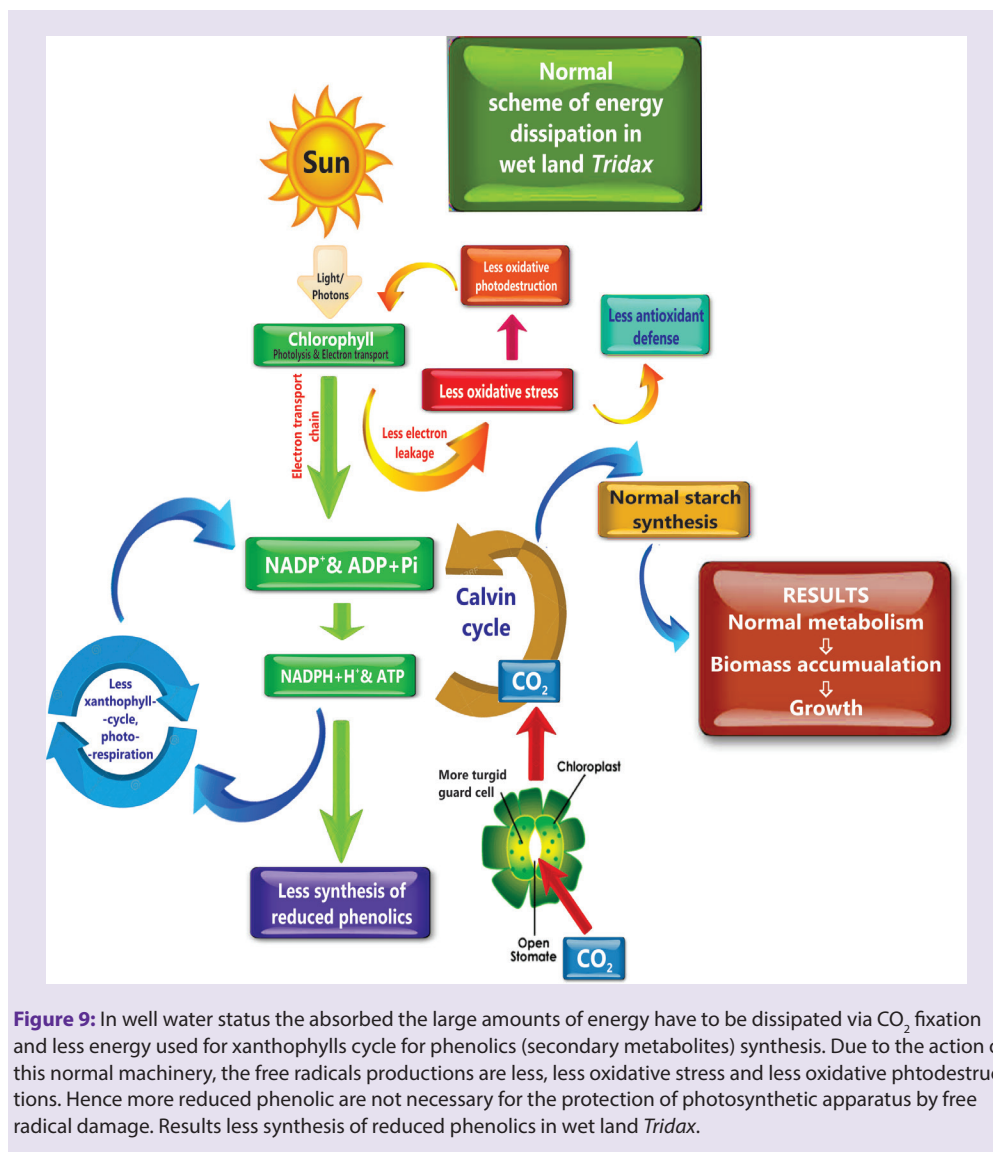
**Figure 6:** TBARS contents in nmol/g of dry weight of *Tridax* leaves from different habitats



**Figure 7:** The antioxidant potential (by dot-blot and DPPH staining) of the flowers of *Tridax* from four different habitats (A. Low wet-land; B. Low dry-land; C. Hilly wet-terrain and D. Hilly dry-terrain).



**Figure 8:** The antioxidant potential (by dot-blot and DPPH staining) of the Leaves of *Tridax* from four different habitats (A. Low wet land; B. Low dry-land C. Hilly wet terrain and D. Hilly dry terrain).



fraction of different habitats of *Tridax* extracts and scavenging of the free radicals shifted the end point. These results revealed that oxidation inhibition directly proportional to the ability of these fractions as free radical scavenging capacity of the compounds. The wider diameter as well as high colour intensity of the resulting dots (spots) indicates the high radical masking activity of the tested fractions.<sup>31</sup>The DPPH radical scavenging capacities of the extracts were in the order: hilly dry-terrain < low dry-land < hilly wet- terrain < low wet-land.

#### DPPH scavenging activity by photometric assay

Free radical scavenging activity against DPPH in methanol, the DPPH radical had been used for the several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H'. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration shows the free radical scavenging abilities of the sample/antioxidant by their hydrogen donating capability. The electrons become paired off and

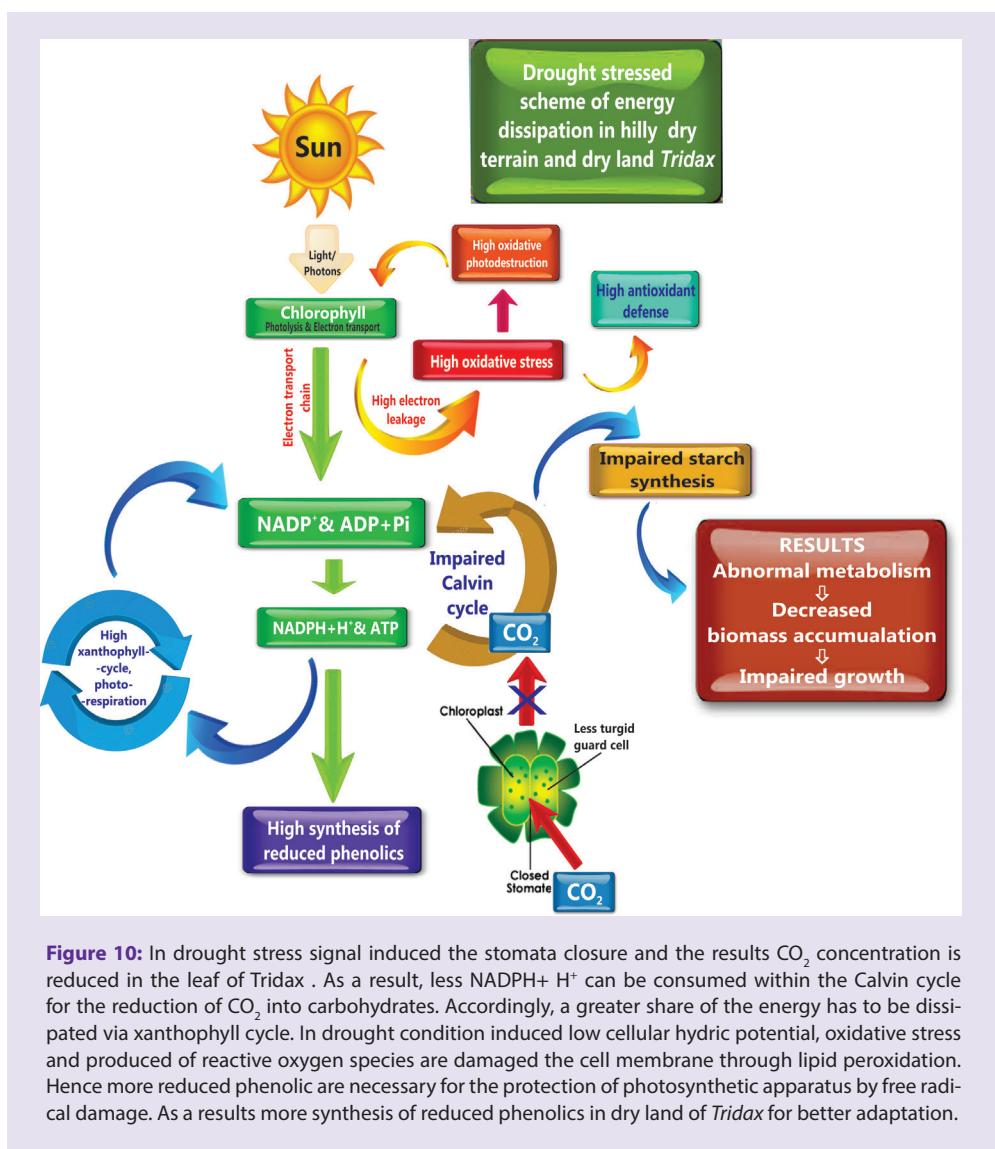
solution loses colour *stoichiometrically* depending on the number of electrons taken up. Free radical scavenging activities of the different habitats Chloroform insoluble fraction (CIFT) of *Tridax* extract are shown in Table 1. Regarding the antioxidant potential it was absorbed that the leaf and the flower extracts obtained from the hilly dry-terrain and dry-lands show highest radical scavenging activity and lowest IC<sub>50</sub> values.

#### Nitric oxide scavenging activity

The IC<sub>50</sub> values of nitric oxide scavenging activity of CIFT of different habitats results are given in Table 1. The maximum free radical scavenging activity was at the lowest concentration from which the IC<sub>50</sub> calculated. Among the four locations, the CIFT of the hilly dry terrain and low dry land (both flower and leaf extract) showed the highest nitric oxide-scavenging activity.

#### Reducing power assay

In this assay, the yellow colour of the test solution changes in to various shades of green and blue depending on the reducing power of each compound. As observed from Table 1, *Tridax* hilly dry terrain flower extract



has minimum  $\text{IC}_{50}$  values (333.63  $\mu\text{g}/\text{ml}$ ) and low wet-land leaf extract has maximum  $\text{IC}_{50}$  (1730.33  $\mu\text{g}/\text{ml}$ ) amongst individual habitats of the plant extracts.

### Scavenging of hydrogen peroxide

The scavenging ability of CIFT of leaf and flower extract of *Tridax* in different habitats on hydrogen peroxide is shown in Table 1. The hilly dry-terrain flower extract of *Tridax* has minimum  $\text{IC}_{50}$  values (243.33  $\mu\text{g}/\text{ml}$ ) and wet-land leaf extract has maximum  $\text{IC}_{50}$  (1100.23  $\mu\text{g}/\text{ml}$ ) amongst individual habitats of plant extracts.

## DISCUSSION

Environmental factors affect the distribution of plants and exercise a selective effect toward those that have a better adaptation,<sup>32</sup> Plant metabolism and signal transduction significantly affected by various stress conditions. A direct consequence of a stress perturbation is an alteration in the metabolic behaviour of the cell, leading to a cascade of molecular and biochemical events that facilitate a new steady state to be reached.<sup>33</sup> When plants subjected to drought stress, responded through alteration in plant primary and secondary metabolism.<sup>34</sup> In the present

study revealed that the reduction of the relative leaf water content in *Tridax* in low dry-land and hilly dry-terrain could be partly explained by the increase in osmolality, which was found to be correlated with sucrose content. This finding was lined with DaCosta and Huang finding, who reported that when the plants are growing under the drought stress and adjusted by accumulation of water soluble carbohydrates such as glucose, fructose, and sucrose.<sup>35</sup>

The highest yield percentage and phenolics (water soluble /chloroform insoluble) were recorded in leaves and flowers of the *Tridax* collected from the hilly dry-terrain, low dry-land. It is clear that drought stress increased water soluble content and decreased lipid related molecules both leaves and flowers of *Tridax*. This is agreement with previous finding of Gigon *et al.*, who reported that in response to drought, total leaf lipid contents decreased progressively. The decreased leaves lipids content may be in the drought stress in the plants to minimize the photosynthesis process hence the plants are used as lipids as energy sources for the vital role.<sup>36</sup>

The plants are under the drought condition induced low cellular—hydric potential. In this conditions induced oxidative stress and produced of reactive oxygen species (particularly due to OH) are damaged the

cell membrane through lipid peroxidation. In our finding TBARS were higher in the *Tridax* leaf collected from dry lands. Plant's ability to cope with drought stress is mainly altered the biochemical profile and produces a varied range of secondary metabolites. The lipid peroxidation altered the signal transduction and initiate the metabolic alteration and promotes the accumulation of secondary metabolites is important to protect the cell membrane lipid from the oxidative stress and the reactive oxygen species.<sup>16</sup> Hence the plants overcome from this situation needs to enhance the antioxidant system for the reduction of cell membrane damage through the free radicals<sup>38-39</sup> by increased accumulation of phenol concentration<sup>37</sup> our results of the present study is lined with this, the phenolic content were considerably increased in low and hilly dry terrains of *Tridax*. It may be important for preventing the lipid peroxidation by hydroxyl radicals of the cell membrane lipids.

The antioxidant and free radical scavenging potential of chloroform insoluble fraction of *Tridax* were assessed (by *invitro*) and compared. The results demonstrated that the antioxidant potential is not depending upon the amount but depending on the potential of phenolics. The IC<sub>50</sub> values of the phenolics of *Tridax* are in the following order hilly dry terrain > low dry-land > hilly wet-land > low wet-land. The results clearly indicated that drought stress promotes the accumulation of more reduced phenolics in *Tridax*. This finding is lined with Saeidnejad *et al.*, finding, who is reported that the *Bunium persicum* under the drought conditions, the leaf extract showed lowest IC<sub>50</sub> of DPPH, hydrogen peroxide scavenging and reduced power assays<sup>37</sup>. More over Al-Gabbiesh *et al* reported that plants exposed to drought stress without any change in the enzyme activity, but promote the rate of synthesis of the highly reduced secondary metabolites in plants (isoprenoids, phenols, alkaloids and tannins).<sup>40</sup>

### Hypothetical Biochemical mechanism of Drought Stress Signal Promote the Synthesis of more Reduced Phenolic Compounds (Chloroform Insoluble Fraction) in *Tridax procumbens*

In the process of photosynthesis (Figure 9), two key events are mandatory; light reactions (photo oxidation) in which light energy is converted into ATP and NADPH and oxygen is released, and dark reactions, in which CO<sub>2</sub> is reduced into carbohydrates by utilizing the assimilatory power of light reactions such as ATP and NADPH.<sup>41</sup> The plants in the drought conditions recognized an insufficiency of the water in the soil around the root system and roots send drought signal to the leaves through the xylem sap. When the stress signal reached the leaves, its triggers stomatal closure for preventing the water loses by evaporation and the plant shifts to a water-saving strategy. Due do the stomata closure results diffusion resistance for all gases including CO<sub>2</sub>.<sup>40</sup> In these conditions, the concentration of CO<sub>2</sub> within the leaves decreases to a low level, and far less NADPH+ H<sup>+</sup> and ATP are consumed for CO<sub>2</sub> fixation within the Calvin cycle (Figure 10). These effects on the diminishing of net photosynthesis (dark reaction) and induce the oxidative stress and promote the production of ROS by plants.<sup>43</sup> In reality, under drought stress, ROS production is enhanced by several ways. For example, the limitation on CO<sub>2</sub> fixation will decreased the NADP<sup>+</sup> regeneration through the Calvin cycle, hence aggravating an over declining of the photosynthetic electron transport chain. In fact, photo synthesis takes places in drought stress (less availability of CO<sub>2</sub> in leaves) there is a higher leakage of electrons to O<sub>2</sub> by the Mehler reaction.<sup>44</sup> Biehler and Fock observed in wheat are growing under drought stress conditions, the leakage of photosynthetic electrons by the Mahler reaction was amplified by around 50% as compared to unstressed wheat.<sup>45</sup> Similar results were observed in sunflower are growing under drought conation an increased thylakoid membrane electron leakage to O<sub>2</sub> and promotes oxidative stress.<sup>46</sup>

Drought stress results in a strong decline in the NADP<sup>+</sup> concentration and thereby the amount of adequate electron acceptors for the photosynthetic electron transport chain drastically decreased. Although energy dissipation via non-photochemical quenching and the re-oxidation of NADP+ H<sup>+</sup> will be enhanced by the corresponding feedback mechanisms, the resulting over-reduction leads to increase in the amount of oxygen radicals being produced. Apart from stress-induced production of superoxide radicals, an increase in the ratio of NADPH+ H<sup>+</sup> to NADP<sup>+</sup> has strong metabolic consequences. According to the law of mass action, rate of a chemical reaction is proportional to the product. The plant in drought condition the ratio of NADPH+ H<sup>+</sup> and NADP<sup>+</sup> not proportional because normal light reaction but decreased dark reaction. In this condition the plants shifting the metabolism even without any change in enzyme activity, but enhanced synthesis of the highly reduced secondary metabolites products through xanthophylls cycle.<sup>47</sup> xanthophylls cycle play major role in plant to protect the drought related oxidative damage of photosynthetic apparatus through enhanced synthesis of more reduced secondary metabolites.<sup>48</sup> This molecular mechanism are taken to the account for the synthesis of highly reduced phenolics in *Tridax* under drought. The results of this study established that wild type *Tridax* were better adapted in the drought stress through enhanced synthesis of more reduced poly phenols.

## CONCLUSION

*Tridax procumbens* growing in drought condition either hilly dry-terrain or low dry-land which leads to may be limits CO<sub>2</sub> uptake, hence reduced photosynthetic activity and may increase more oxidative stress due to electron leakage in the photosynthetic apparatus. Hence *Tridax* were better adapted to drought conditions (for managing the oxidative stress) through enhanced synthesis of more reduced phenols for preventing the oxidative damage of the photosynthetic apparatus. Further studies using new molecular approaches, to find out the phenolic structural difference between oxidized (from well water state ) and reduced phenolic forms ( from drought adaptation) of *Tridax procumbens* .

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

## AUTHOR'S CONTRIBUTION

This work was carried out in collaboration between NG and SK. NG designed the study, carried out experimental parts and statistical analyses of data , and wrote the first draft of the manuscript, managed the literature searches. SK wrote the protocol and revised the manuscript.

## ABBREVIATION USED

**CIFT**: Chloroform insoluble fraction; **TBARS**: Thiobarbituric Acid Reactive Substances; **DPPH 2,2**: Diphenyl-1-picrylhydrazyl.

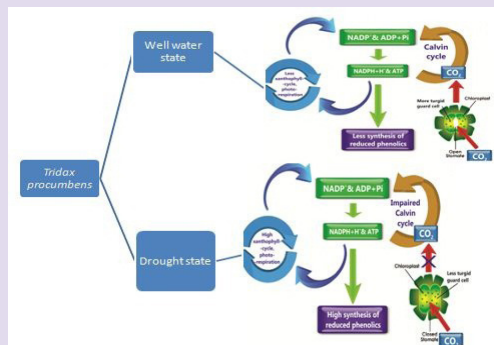
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## PICTORIAL ABSTRACT



## SUMMARY

- Plant's are ability to manage with abiotic stress factors are mainly altered the metabolic pathways.
- Tridax procumbens* growing in drought condition reduced photosynthetic activity and increased oxidative stress.
- Xanthophylls cycle play major role in plant to protect the drought related oxidative damage through enhanced synthesis of more reduced secondary metabolites.
- The results of this study established that wild type *Tridax* were better adapted in the drought condition through enhanced synthesis of more reduced poly phenols.