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COMPARATIVE NON-ENZYMATIC AND ENZYMATIC ANTIOXIDANT POTENTIAL SCREENING IN SPECIES OF GENUS *URTICA*

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Keywords:

Antioxidant activity, DPPH, FRAP, Catalase, Ascorbate peroxidase

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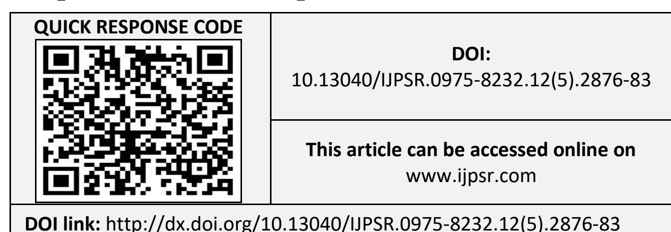
ABSTRACT: Objective: The current study has been conducted to analyze the potential of antioxidant activity found in two species of genus *Urtica*, *i.e., Urtica dioica* and *Urtica urens*. **Methodology:** The antioxidant analysis was performed on the crude extract of root, stem and leaves of both the plants, employing both non-enzymatic and enzymatic methods. For non-enzymatic antioxidant activity determination, methods such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and Ferrous Reducing Antioxidant Power (FRAP) were opted, whereas for enzymatic analysis, the specific activity of catalase and ascorbate peroxidase assay was examined. **Results and Conclusion:** The antioxidant activity found *via* DPPH assay in *U. urens* ranges from 32.72±0.22 µg/ml to 43.06±1.12 µg/ml and in *U. dioica* it ranges from 14.28±0.22 µg/ml to 30.88±0.278 µg/ml, while by using FRAP assay the antioxidant activity range found to be 10.412±2.235 µg/ml to 14.005±2.55 µg/ml and 1.526±0.146 µg/ml to 8.014±1.38 µg/ml in *U. urens* and *U. dioica*, respectively. The specific activity of antioxidant enzymes catalase and ascorbate peroxidase were exhibited in a range of 16.11±2.146 U/mg to 26.869±6.811 U/mg and 4.497±0.542 U/mg to 6.28±1.57 U/mg respectively in *U. urens,* while in *U. dioica* the catalase and ascorbate peroxidase enzyme specific activities range from 0.49±0.015 U/mg to 0.583±0.0318 U/mg, respectively. The conducted study indicated the better potential of antioxidant activity found in *U. urens* than in *U. dioica*.

INTRODUCTION: The process of oxidation is essential for a living organism's normal metabolic function to produce fuel for performing the normal biological process [1]. In the course of such metabolic reactions, reactive compounds like free radicals or highly reactive oxygen species (ROS) could be generated. Such molecules are unstable and highly reactive, as the name suggests, due to the presence of a lone pair of electrons [2].



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These free radicals are normally generated due to the metabolic processor can also be acquired from the environment. Free radicals can be Superoxide radicals (O_2^-), Hydroxide radical ($^{\bullet}OH$), singlet oxygen ($^{\bullet}O_2$), and non-free radical species (H_2O_2) [3]. These radicals work by attacking the unsaturated fatty acids of the cell membrane, resulting in lipid peroxidation leading to reduced membrane fluidity. This causes a reduction in the antioxidant defense enzymes cell/tissue damage or inactivation [4, 5].

Reactive oxygen species, free radicals, Reactive nitrogen species production are coupled as a cause of diseases like osteoporosis, inflammation, arterio-scleroses carcinogenesis, cardiovascular disease, mutagenesis, and other degenerate diseases [6].

FIG. 1: A. WHOLE PLANT OF *U. DIOICA* B. *U. DIOICA* FLOWER, C. WHOLE PLANT *U. URENS*, D. *U. URENS* FLOWER

Vitamin deficiency, excess free-radical production, decreased level of antioxidant defense and lipid peroxidation are all the perpetrators for producing oxidative stress and is found associated with various pathological condition [7].

Antioxidants are chemicals that can be used to reverse the harmful pathological effect caused by free radicals. Antioxidants generally scavenge the free radicals and detoxify the physiological system. Antioxidants, nowadays from natural origins are extensively used over the past years. Currently, the whole world is dependent only on a few plant species [8]. In order to diversify and expand the dependency on the plant species, particularly those that are underutilized, improves the health and nutrition, livelihood, and ecological sustainability.

Urtica, the genus belongs to family Urticaceae in the order Rosales. The two species of genus *Urtica* has been selected for evaluating and comparing the anti-oxidant potential found in the species. Some of the differences found in the morphological features among the two species are tabulated below.

TABLE 1: SOME OF THE DIFFERENCES FOUND IN THE MORPHOLOGICAL FEATURES AMONG THE TWO SPECIES

S. no.	*Urtica dioica*	*Urtica urens*
1	Perennial or annual shrub.	Annual herbaceous.
2	It grows in temperate and tropical wasteland areas around the world.	It prefers moist areas in wildlands, such as areas surrounding creeks or rivers.
3	Common names are Stinging nettle and Bichoo Booti	Common name is Dwarf nettle, Burning nettle, and Annual nettle
4	*Urtica dioica* is a dioecious plant (**Fig. 1a**), reaching up to the height of 1-2 m with widespread rhizomes and stolons that are bright yellow in color and are of perennial nature	*Urtica urens* plant is erect or ascending type upto 2 feet tall (60 cm) and has a tap root system (**Fig.** 1c).
5	Soft green leaves are 3-15 cm that are oppositely arranged leave have a strongly serrated margin, a cordate base and an acuminate tip with a terminate leaf tooth longer than adjacent lateral ones.	Leaves are oval with opposite arrangement up to ½ to 2 inch or 4 cms long, sharp, tipped leaves that densely toothed.
6	Leaves and stems are very hairy c non-sting hairs, and also among them are present stinging hairs or trichomes whose tip drops off whenever touched, transforming hair into needle that inject chemicals like acetylcholine, histamine, serotonin, moroidin, leukotrienes and possibly formic acid.[10, 11]	The irritant hairs present on the leaves and stem of the plant causes irritant dermatitis.[12]
7	It bears small green/brownish flowers in dense axillary inflorescence (see **Fig.** 1b), separate male & female flowers.[13]	It bears small, creamy or greenish white-colored flowers that are borne on the same plant in short spikes (see **Fig.** 1d).[9]

From current pharmacological studies, both *Urtica dioica* and *Urtica urens* can be applied for pharmaceutical purposes as an anti-oxidative agent. *Urtica dioica* exhibits anti-oxidative towards iron promoted oxidation of phospholipids linoleic acid, deoxiribose, *etc*.

The current studies evaluates the comparative total phenolic contents, total ascorbic acid content and the anti-oxidants potential through enzymatic (catalase, peroxidase) and non enzymatic (DPPH, FRAP) methods within two species of genus *Urtica*.

MATERIALS AND METHODS:

Chemical Reagent: All the solvents and standard chemicals like DPPH (1, 1-Diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tri[2-pyridyl]-s-triazine), BHT (Butylated hydroxytoluene), Ascorbic acid, Ferric Chloride, Folin-Ciocalteau phenol reagent, Sodium carbonate, Hydrogen peroxide, Sodium dihydrogen phosphate, Di-sodium hydrogen phosphate were of analytical grade.

Plant Material Collection: Fresh plant material of *Urtica dioica* and *Urtica urens* plants were collected from the Dist-Palampur (Himachal Pradesh) (in the month of July) and Village – Naggar, Dist-Kullu (H.P.) (in the month of May) respectively, and were authenticated by CSIR, IHBT Herbarium, Palampur (H.P.). Voucher specimen herbarium (PLP-18301) of *Urtica dioica* and (PLP-18302) of *Urtica urens* were deposited at IHBT, CSIR, Palampur (H.P.) herbarium. The plant is thoroughly washed without pressing or crushing it to remove dirt and soil particles. Root, Stem and leaves of plant were separated, shade dried, and then powdered.

Non-Enzymatic Method for Antioxidant Activity Analysis:

Estimation of DPPH (1, 1-Diphenyl-2-picrylhydrazyl) free radical scavenging activity: The antioxidant potential of the samples has been ascertained by using the hydrogen donating capability of stable DPPH free radical. The free radical scavenging potential was analyzed in the methanolic extract using DPPH• radical according to procedure [14]. In this method, methanolic extract of the plant material with a concentration 1 mg/ml was prepared. To the test tubes, 1 ml of freshly prepared methanolic solution of 0.004% w/v of DPPH was taken to which the methanolic plant extract was added, followed by serial dilutions (1 µl-50 µl) making the volume of each test tube solution to 3 ml by adding required amount of methanol. After 20 min incubation in the dark, the absorbance was taken at 517 nm by spectrophotometer (Systronic UV-Vis Spectrophotometer 117) where methanol was taken as blank.

Ascorbic acid and BHT (Butylated Hydroxy Toluene) was used as a reference and were dissolved with methanol to prepare a stock solution of similar concentration (1mg/ml) reaction mixture with lower absorbance indicates a higher % of free radical scavenging activity. The % free radical scavenging effect of DPPH was calculated by the equation given below:

% Scavenging Activity = Absorbance of control - Absorbance of test sample × 100 / Absorbance of control

The inhibition curves were prepared, and the IC_{50} values (the concentration of the test sample, which shows exact half of the radical scavenging percentage) of each extract were computed using the following procedure [15].

1. Inhibition percentage (y-axis) was plotted against the sample concentrations (x-axis), and a curve is plotted for each set of values obtained. It is not necessary that the curve formed should pass through the origin. As the inhibition curve is curved, the IC_{50} values were calculated using the interpolation technique.

2. Two points around the 50% inhibition ratio opted, and a regression line (Y = AX + B) was drawn. This regression line doesn't require passing through the origin.

3. X (sample concentration) was calculated when Y in the regression equation of (2) was substituted with 50.

Reducing Power (Ferrous Reducing Antioxidant Power) Analysis: A spectrophotometric procedure has been followed for the estimation of antioxidant capacity of the plants [16]. The principle of the method is to trace the action of electron-donating antioxidants at low pH resulting in the conversion of colorless Fe^{3+} TPTZ complex reduction to blue colored Fe^{2+}-tripyridyltriazine [17]. The absorbance

was measured at 593 nm. FRAP reagent was freshly prepared by making a mixture of 0.3 M acetate buffer, 10 ml TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ in 10:1:1 proportion. To the 5 µl of the appropriately prepared plant sample, a freshly prepared FRAP reagent of about 3.995 ml was added. In the prepared reaction mixture presence of an intense blue color occurs due to conversion of ferric tripyridyl triazine (Fe^{3+} TPTZ) complex to ferrous (Fe^{2+}) form, and its absorbance was measured at 593 nm where blank reagent would be prepared by mixing 3.995 ml of FRAP reagent to 5 µl distilled water. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$. The concentrations of $FeSO_4$ were, in turn, plotted against the concentration of standard antioxidant trolox. The FRAP values were obtained by comparing the absorbance of the various test mixture with those obtained from increasing concentrations of Fe^{2+} and expressed as mg of trolox equivalent per gram of sample.

Ferric – Tripyridyl triazine (colorless) ⟶ Ferric – Tripyridyl triazine (blue color complex)

Enzymatic Method for Antioxidant activity Analysis:

Catalase Assay: Catalase, the most common antioxidant enzyme present in almost all living tissues that utilize oxygen. Catalase assay is based on the principle of the ability of catalase enzyme reducing/degrading ROS H_2O_2 to water and molecular oxygen, consequently leading to the detoxification process.

Catalase activity was determined following procedure where 0.2 mg of powdered plant extract was taken and thoroughly homogenized in 3 ml of 0.1 M Na_2EDTA and 1 ml PVP. The sample mixture was centrifuged at 10,000 rpm for 20 min at 4 ºC. Clear supernatant was collected and stored as enzyme extract [18]. Assay mixture was prepared by adding 0.2 ml of the plant enzyme extract to 2ml of phosphate buffer and 0.8 ml of H_2O_2. Now the absorbance of the assay mixture prepared was measured one reading taken immediately and the other one taken after 60 seconds. According to this method the decomposition of H_2O_2 was followed as decrease in absorbance at 240 nm *via* UV-Vis spectrophotometer. The catalase enzyme concentration was calculated using following equation [19].

$$\text{Catalase enzyme concentration} = \frac{(A_0 - A_{60}) \times V_t}{\varepsilon_{240} \times d \times V_s \times C_t \times 0.001}$$

A_0= Initial absorbance of the assay mixture
A_{60}= Absorbance of assay mixture after 1 minute
V_t = total volume of the assay mixture in ml
ε_{240}= molar extinction coefficient-H_2O_2 at OD_{240} (34.9 mol^{-1} cm^{-1})
d= optical length of cuvette (1 cm)
V_s= Volume of sample in ml

Ascorbate Peroxidase Assay: Ascorbate peroxidase is a plant-specific peroxidase enzyme that catalyzes the reduction of H_2O_2 generated both in the cytosol and chloroplast using ascorbate as a substrate.

The activity of ascorbate peroxidase enzyme was determined by adopting a method with a slight modification of the previously used methods [20]. APX activity was determined by the reduction in absorbance measured at 290 nm due to oxidation of ascorbic acid in the reaction mixture. According to this method, 0.2 gm of powdered material was homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (pH-7.8) along with 0.1mM EDTA. The sample mixture was then centrifuged at 15,000 rpm for 20 min. Supernatant was collected and was stored in ice. The assay mixture was prepared by mixing 50 mM potassium phosphate buffer, 0.5 mM ascorbate, 10µl of crude leaf sample extract, to which 0.5 ml of H_2O_2 was added at the end as this will initiate the reaction and decrease in absorbance have been recorded at intervals of 60 seconds. Then the specific activity of ascorbate peroxidase enzyme was determined by taking ε_{290} of H_2O_2 at 290 nm was 2.8 $mM^{-1}cm^{-1}$ in U/mg or µmol min^{-1} mg^{-1} unit.

Statistical Data Analysis: Data analysis was performed *via* minitab SPSS statistical subscription. One-way ANOVA (Analysis of variance) was carried out for statistical data analysis. Each experiment was repeated 3 times and Mean ± Standard error was computed from analysis of each treatment. Computed data were compared *via* Tukey's test at a level of 5% probability.

RESULTS:

DPPH Radical Scavenging Activity: The antioxidant activity was measured as the DPPH radical scavenging capability of the extract, which was then compared with the standard Butylated hydroxytoluene (BHT). The results of the radical scavenging activity exhibited by various extracts in increasing concentrations were compiled in **Fig. 2**.

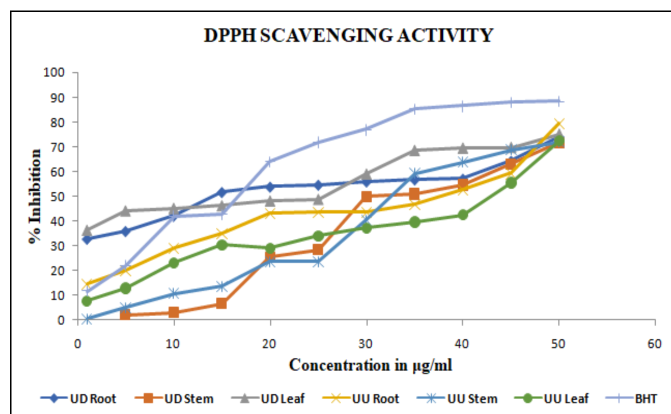

FIG. 2: SCAVENGING ACTIVITIES OF EXTRACTS AND STANDARD AT SIMULTANEOUS INCREASE IN CONCENTRATION BY DPPH ASSAY

It was observed that at the concentration of 25, 35 and 50 µg/ml, root extract of *U. dioica* at the concentration of 25 µg/ml, leaf extract of *U. dioica* at 30 µg/ml, and root extract of *U. urens* at 50 µg/ml, shows slightly higher inhibition activity compared to other extracts. The comparative analysis of IC_{50} value obtained from various extracts was analyzedand represented in **Fig. 3**. The leaves of *U. urens* display greatest scavenging activity with IC_{50} (43.06±1.12 μgml^{-1}). *U. dioica* root extract shows the lowest IC_{50} (14.28±0.025 μgml^{-1}) followed by leaves of *U. dioica* (22.934±1.365 μgml^{-1}) and the stem extract of *U. dioica* (30.88±0.278 μgml^{-1}).

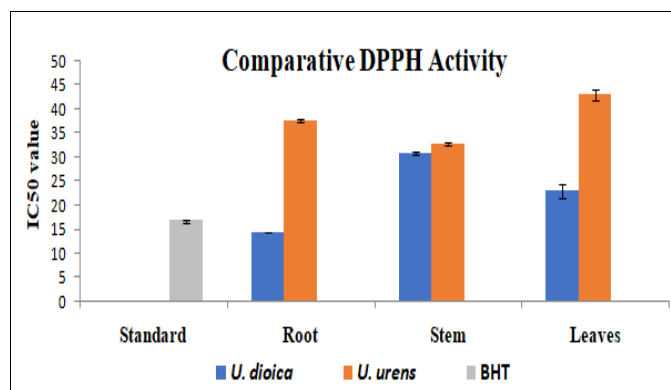

FIG. 3: COMPARATIVE EVALUATION OF IC_{50} VALUES OF VARIOUS EXTRACTS AND STANDARD

Reducing Activity (FRAP): The increase in the activity of ferrous reducing power of the extract was indicated by the increase in the concentration of the extract and henceforth the increase in absorbance. The increase in absorbance is caused due to the amount of antioxidant found in the extract, which reduces Fe(III) to Fe (II) in the presence of tripyridyltriazine tridentate ligand that forms a colored complex with Fe(II) [21].

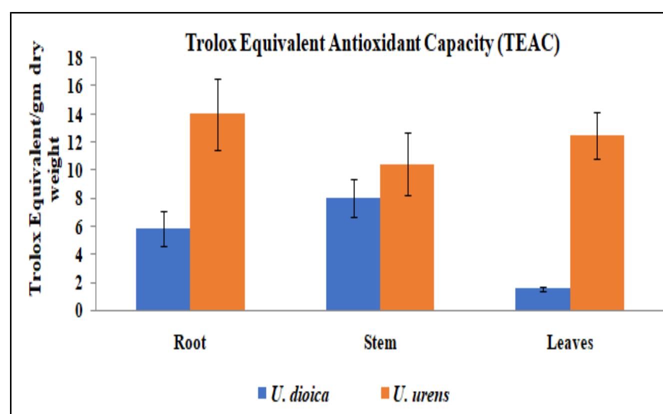

FIG. 4: REDUCING ACTIVITY OF EXTRACTS EXPRESSED AS TROLOX EQUIVALENT ANTIOXIDANT ACTIVITY

It was observed that the highest amount of FRAP activity measured in terms of trolox equivalent antioxidant capacity (TEAC) was exhibited by root extract of *U. urens i.e.,* 14.005±2.56 µgTE/gdw while the lowest FRAP activity was found in leaves of *U. dioica i.e.* 1.526±0.146 µgTE/gdw **Fig. 4**.

Catalase Activity: Catalase enzyme antioxidant activity was measured spectrophotometrically at 240 nm as the amount of sample required to catalyze the decomposition of 1 µmole of H_2O_2 to water and O_2 in one minute. The sensitivity of this method can determine the catalase activity as low as 0.5 U/mg.

According to this procedure, the extract with highest amount of catalase activity was found to be in *U. urens* root extract whereas the one showing the lowest amount of catalase activity was the leaf extract of *U. dioica*. Catalase activity found in plant *U.dioica* ranges from 9.834±0.885 U/mg to 20.55±3.861 whereas in *U. urens*, it ranges from 16.11±2.146 U/mg to 26.867±6.811 U/mg.

Ascorbate Peroxidase (APX) Activity: APX activity was determined by the reduction in absorbance measured at 290 nm due to oxidation of ascorbic acid in the reaction mixture. Overall from

the results, it was concluded that *U. urens* exhibits copious amount of APX activity compared to *U. dioica* with stem extract displaying the highest APX activity as computed from **Fig. 5**.

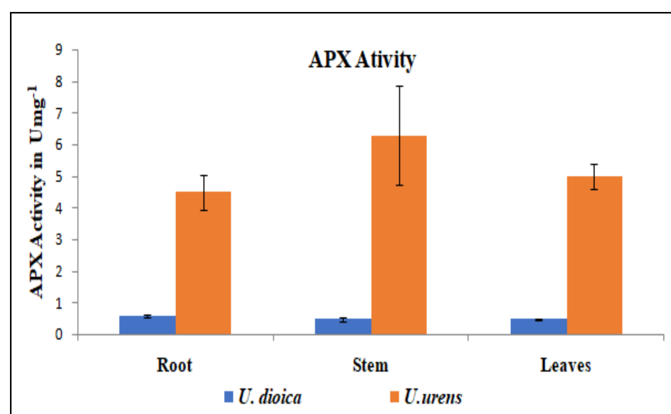

FIG. 5: COMPARATIVE ASCORBATE PEROXIDASE ACTIVITY OF VARIOUS EXTRACTS OF *U. DIOICA* AND *U. URENS* SHOWING A COMPARATIVE SIGNIFICANT ASCORBATE PEROXIDASE ENZYME SPECIFIC ACTIVITY

DISCUSSION: In the present study, root, stem and leaves of two plant species of *Urtica i.e. U. dioica* and *U. urens* were collected and their extracts were prepared and a comparative evaluation of the antioxidant activity using various non-enzymatic (DPPH and FRAP) and enzymatic methods (Catalase and Ascorbate Peroxidase) was performed. Free radicals are found to be the causative agent for many neurodegenerative diseases. Antioxidants due to their scavenging and inhibiting activity are helpful in managing or eradicating the ill effects caused by them. The present study was conducted to analyze and comparing the therapeutic potential of *U. dioica* and *U. urens* as antioxidants that help in reduction of tissue injuries caused by free radicals.

DPPH assay was performed to check for the presence of antioxidants with the capability of trapping DPPH radical. DPPH is a stable free radical that accepts an electron or hydrogen ion to form a stable diamagnetic molecule. All the plant parts tested inhibit the DPPH radical to different extent showing their respective capabilities of donating an electron or hydrogen, which could react with DPPH radical.

Such difference in the DPPH scavenging activity was attributed to the different concentrations of ascorbic acid, flavonoids, polyphenol, and other antioxidant molecules in various plant parts [22, 23]. All the extracts tested exhibited a higher IC_{50} than standard (16.735±0.95 $\mu g ml^{-1}$).

As the reducing power of a compound also serves as an indicator of the antioxidant potential, this property was assessed for measuring the antioxidant ability of the extract to convert Fe(III) to Fe(II) by donating an electron [24]. This ferrous reduction potential ability of the extracts was attributed either to the presence of the reducing agents such as phenol groups and the number of the hydroxyl molecule on these groups [25].

TABLE 2: RESULTS OF DIFFERENT NON-ENZYMATIC AND ENZYMATIC PROCEDURES OPTED FOR COMPARING THE ANTIOXIDANT POTENTIAL FOUND IN DIFFERENT PLANT PARTS OF *U. DIOICA* AND *U. URENS*

Anti-oxidant assays	Root	Stem	Leaves	BHT
Urtica dioica				
Non-Enzymatic				
DPPH assay IC_{50} (in µg/ml)	14.28±0.025	30.88±0.278**	22.934±1.365*	16.735±0.95
FRAP assay (µg TE/gdw)	5.808±1.25	8.014±1.38	1.526±0.146**	-
Enzymatic				
Catalase (in U/mg)	12.767±2.835	9.834±0.885**	20.55±3.861	-
Ascorbate Peroxidase (in U/mg)	0.583±0.0318*	0.496±0.068	0.49±0.015	-
Urtica urens				
Non-Enzymatic				
DPPH assay IC_{50} (in µg/ml)	37.584±0.43	32.72±0.22*	43.06±1.12***	16.735±0.95
FRAP assay (µg TE/gdw)	14.005±2.56*	10.412±2.235	12.499±1.684	-
Enzymatic				
Catalase (in U/mg)	26.867±6.811	16.11±2.146**	22.5±1.984	-
Ascorbate Peroxidase (in U/mg)	4.497±0.542	6.28±1.57**	4.99±0.394	-

#Values are mean± S.E. (standard error) of 3 observations *observations, *→(p<0.05), **→p<0.01, ***→p< 0.00.*

Hydrogen peroxide, a normal metabolite found in normal living cells and tissues which cause tissue damage by effecting the membrane fluidity of cells by lipid peroxidation. An excess amount of hydrogen peroxide can oxidize other cellular component that will result in further increased

production of free radicals. In cases of oxidative stress, catalase is known as a defensive anti-oxidant enzyme that catalysis the removal of hydrogen peroxide by breaking it down to water and oxygen molecules without the production of free radicals.

On the other hand, ascorbate peroxidase is a plant-specific antioxidant enzyme reducing the hydrogen peroxide found both in cytosol and chloroplast using ascorbic acid as substrate.

CONCLUSION: The current study proposes the higher and more effective antioxidant potential of *U. urens* in comparison to *U.* dioica. While *U. dioica* showed only a minor to moderate amount of activity in inhibiting free radicals production. However, further phytochemicals investigation must be done to characterize and isolation of antioxidant compounds for the determination of the *in-vivo* and *in-vitro* biological potential of these extracts.

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CONFLICTS OF INTEREST: The author declares no conflict of interest regarding the presented manuscript. The authors alone are responsible for the content of the manuscript.

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