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Assessment of antioxidant activity of *Digera muricata* extracts by different methods

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Abstract
Free radical accumulation is the cause of many oxidative stress related disorders like diabetes, arthritis, neurodegenerative diseases, cardiovascular diseases and cancer. Due to negative health effects of synthetic antioxidants substantial attention is shifted towards finding novel antioxidants of plant origin. In the present study antioxidant activity of *Digera muricata* root extracts was evaluated using various *in vitro* assays. DPPH free radical, superoxide radical, hydroxyl radical scavenging ability, metal chelating activity, calculation of reducing power as well as total phenolic content of five different extracts was determined at different concentrations. Methanolic extract exhibited highest antioxidant activity for various assays and total phenolic content in methanolic extract was found to be 38.22 mg/g gallic acid equivalent. Significant positive correlation was found between total phenolic content and *in vitro* assays indicating that phenolic acids may be the major contributors for antioxidant activity. Data from present results suggests that *D. muricata* has significant antioxidant activity and could serve as potential source of natural antioxidants.

Keywords: *Digera muricata*, free radicals, oxidative stress, antioxidant activity, phenolics

Introduction

Free radical related oxidative stress is the major cause for the initiation and progression of degenerative disorders such as atherosclerosis, Alzheimer's, cancer, diabetes and cardiovascular diseases. Free radicals are unstable oxygen and nitrogen species that are produced from exogenous sources such as stress, cigarette smoke, ionizing radiations, and dyes as well as from normal oxidation in the body (endogenous sources). Free radicals by their ability to propagate chain reactions can damage carbohydrates, lipids, protein and DNA leading to mutations [1, 2]. Antioxidants by their ability to quench free radicals can prevent the oxidation of biomolecules. Natural antioxidant system during normal physiological state maintains a constant equilibrium between generation of free radicals and their quenching. But when the antioxidant system is compromised there is accumulation of free radicals leading to oxidative stress related damage to cellular machinery causing various diseases. Various synthetic antioxidants such as Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT) commonly used for managing oxidative stress are associated with various side effects thus limiting their use [3, 4]. Therefore need for safer antioxidants which are economical also have generated much interest in recent times. Medicinal plants offer a great potential by providing safer, affordable and easily accessible remedies for free radical induced damage. Secondary metabolites obtained from plants are the major contributor of plants antioxidant activity. This has triggered significant research interest to find out alternatives for synthetic antioxidants and search for efficacious plant based antioxidants [5, 6].

India because of its rich floristic diversity is a store house of many medicinally important plants and nearly 6000 plants are presently in use in traditional folklore and herbal medicines. *Digera muricata* L. is a wild annual herb that grows in open fields and along road sides in the rainy season throughout the plains of India. Its leaves and young shoots are used for making vegetables. In Asia and Africa it is considered as famine food because of its rich source of nutrients. Ethnomedicinally it is used for the treatment of renal stones, to relieve constipation, as coolant, for increasing lactation after delivery, for biliousness and for bowel disorders. Leaf paste is applied externally to prevent pus formation. Previous studies have shown this plant to possess antidiabetic, anthelmintic activity, along with hepatoprotection against acrylamide and nephroprotection against CCl_4 induced toxicity. Its various extracts exhibited antifungal and antibacterial activity. Phytochemical analysis of *Digera muricata* revealed the presence of alkaloids, flavonoids, glycosides, terpenoids, tannins and anthraquinone [7, 8, 9]. The present study was carried out to determine *in vitro* antioxidant activity and total phenolic content of *D. muricata* roots.

Material and Methods

1. Collection and preparation of plant extracts

The roots of *D. muricata* were collected from southern Haryana in the months of July-August. The plants were authenticated by comparison with the flora of Janaki Ammal Herbarium, Jammu with the Accession number 8014. Freshly collected plant material was washed; shade dried at room temperature and pulverized using a grinder. Coarse powder was extracted with petroleum ether, benzene, chloroform, methanol and water using a cold percolation method. The solvents were evaporated using a rotary vacuum evaporator at 40ºC and stored in the refrigerator for further study. Different extracts at varying concentration (0.1mg/ml to 0.5mg/ml) were used for the quantification of total phenolic content and assessment of antioxidant activity using various chemical assays.

2. Evaluation of total phenolic content (TPC) and *in vitro* antioxidant activity

Total phenolic content was determined using Folin-ciocalteu reagent according to the method of Singleton and Rossi [10] and expressed as mg/g gallic acid equivalent. Ability of

extract to scavenge DPPH radical was assessed by observing change in color of solution from purple to yellow and measured at 517 nm spectrophotometrically using the method of Lee *et al* [11] Superoxide radical scavenging ability of plant sample was based on their capacity to inhibit blue formazan formation by scavenging of superoxide radicals generated in PMS-NADH system as described by Liu *et al* (12) Hydroxyl radical scavenging activity was assessed by estimating the comparison between deoxyribose and the plant extracts for hydroxyl radicals generated in $FeCl_3$/EDTA/ascorbate/H_2O_2 system and measured at 535 nm according to the method of Kunchandy and Rao [13]. Metal chelating ability of extract was determined by their capacity to compete with ferrozine for ferrous ion in solution as stated by Dinis *et al* [14] and EDTA was used as the reference compound. For measurement of reductive ability of extract ferric to ferrous transformation was detected by measurement at 700 nm as described by Yan and Duh [15] and compared with ascorbic acid as reference.

3. Statistical analysis

All the assays were performed in triplicate and results were depicted as mean±standard deviation. Percentage inhibition was calculated by using the following formula.

% inhibition= A (control)-A (sample or standard)/A (control) x100

where

A (control) = absorbance of control

A (sample or standard) = absorbance of plant extract or standard

Statistical significance was determined by using a one way ANOVA test followed by Duncan's multiple range test. Differences between values were considered significant when $p<0.05$. Correlation between TPC and antioxidant assays was done by Pearson's correlation coefficient analysis.

Result & discussion

Total phenolic content (TPC)

Phenolic compounds due to their hydrogen donating ability can act as chain breaking oxidants thus inhibiting the production of free radicals. Plant extracts are a good source of natural antioxidants that can act as preventive agents against oxidative stress related diseases. Generally plants having good antioxidant activity are rich in phenolics. Consumption of fruits and vegetables rich in polyphenolic compounds can have inhibitory effects on mutagenesis and carcinogenesis [16, 17]. TPC content was determined using the folin-ciocalteu reagent and expressed as mg/g gallic acid equivalent (GAE). TPC content in different extracts is presented in figure 1. Methanolic extract has the highest phenolic content (38.22mg/g GAE) at the concentration 0.5mg/ml. Total phenolic content in different root extract was in order:

methanol>aqueous>chloroform>benzene>petroleum ether.

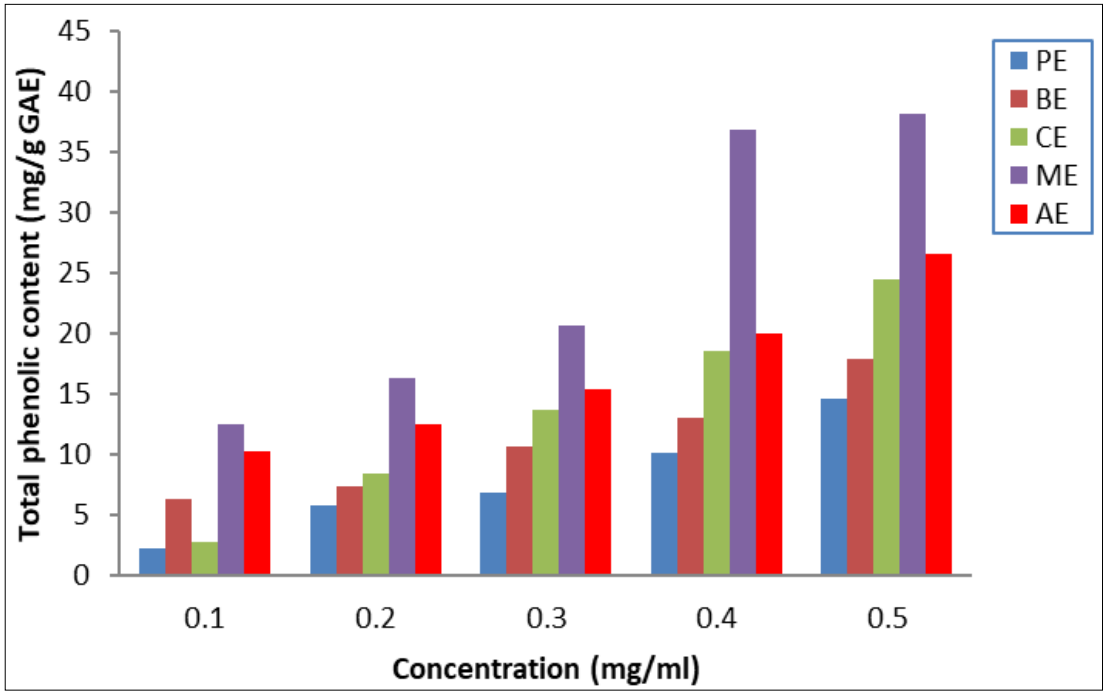

Fig 1: Total phenolic content (TPC) of root extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE-chloroform extract, ME- methanol extract, AE- aqueous extract).

DPPH radical scavenging activity

DPPH assay is a simple, fast, inexpensive and well known antioxidant test investigating the antioxidant capability of plant extracts. DPPH is a stable free radical that gives violet color in ethanol with maximum absorbance at 517 nm. On being reduced by antioxidants present in plant extracts it is transformed to DPPH-H turning the color of solution to yellow [18]. In the present study the scavenging effect of different concentration of extracts on DPPH radical is depicted in Table 1. Ascorbic acid at varying concentrations of 10µg/ml-50µg/ml was used as the reference compound and it exhibited 20.84% inhibition at 10µg/ml and 84.80% at 50µg/ml. There was a dose dependent increase in the percentage antioxidant activity for all the extract tested, however their activity was less than ascorbic acid. Methanolic extract has maximum scavenging potential followed by aqueous>chloroform>benzene>petroleum ether extracts. IC_{50} values of all the extracts were high as compared to ascorbic acid establishing them to be weaker antioxidants than reference compound.

Table 1: DPPH radical scavenging activity (%) of root extracts of *Digera muricata*

Root extracts							
Concentration (mg/ml)	**PE**	**BE**	**CE**	**ME**	**AE**	**Conc. (µg/ml) of AS**	**AS**
0.1	15.44±0.73^{e}	13.04±0.27^{e}	22.14±0.39^{e}	27.17±0.38^{e}	19.26±0.51^{e}	10	20.84±0.62^{e}
0.2	19.61±0.47^{d}	16.82±0.23^{d}	25.72±0.62^{d}	32.25±0.63^{d}	24.29±0.28^{d}	20	38.63±0.40^{d}
0.3	22.78±0.10^{c}	28.52±0.97^{c}	28.37±0.28^{c}	44.86±0.74^{c}	35.40±0.58^{c}	30	75.17±0.60^{c}
0.4	25.15±0.23^{b}	33.74±0.42^{b}	34.44±0.50^{b}	50.78±0.32^{b}	45.76±0.46^{b}	40	80.28±0.12^{b}
0.5	29.08±0.42^{a}	37.84±0.53^{a}	46.44±0.33^{a}	60.79±0.30^{a}	54.20±0.52^{a}	50	84.80±0.66^{a}

Values are expressed as mean±S.D., (n=3). Values with in the column not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by Duncan's multiple range test.

PE- Petroleum ether, BE- Benzene extract, CE- Chloroform extract, ME- Methanol extract, AE- Aqueous extract, AS- Ascorbic acid

Superoxide radical scavenging activity

Although superoxide anion itself is a weak oxidant and less hazardous but leads to generation of more reactive oxygen species such as H_2O_2, $OH^{\cdot}$, peroxynitrite or singlet oxygen that damage biomolecules and initiate lipid peroxidation. Therefore removal or neutralization of superoxide radical is necessary for protecting cells from their detrimental effects [19]. In the PMS-NADH system, percentage inhibition of superoxide generation was calculated by measuring absorbance at 560 nm. All the extracts and standard BHT exhibited superoxide radical scavenging activity in a concentration dependent manner (figure 2). Methanolic extract at 0.5mg/ml showed highest inhibition of superoxide radical (58.52%) followed by aqueous>chloroform>benzene >petroleum ether extracts. The IC_{50} value of methanolic extract and standard BHT was 0.44mg/ml and 0.18mg/ml respectively.

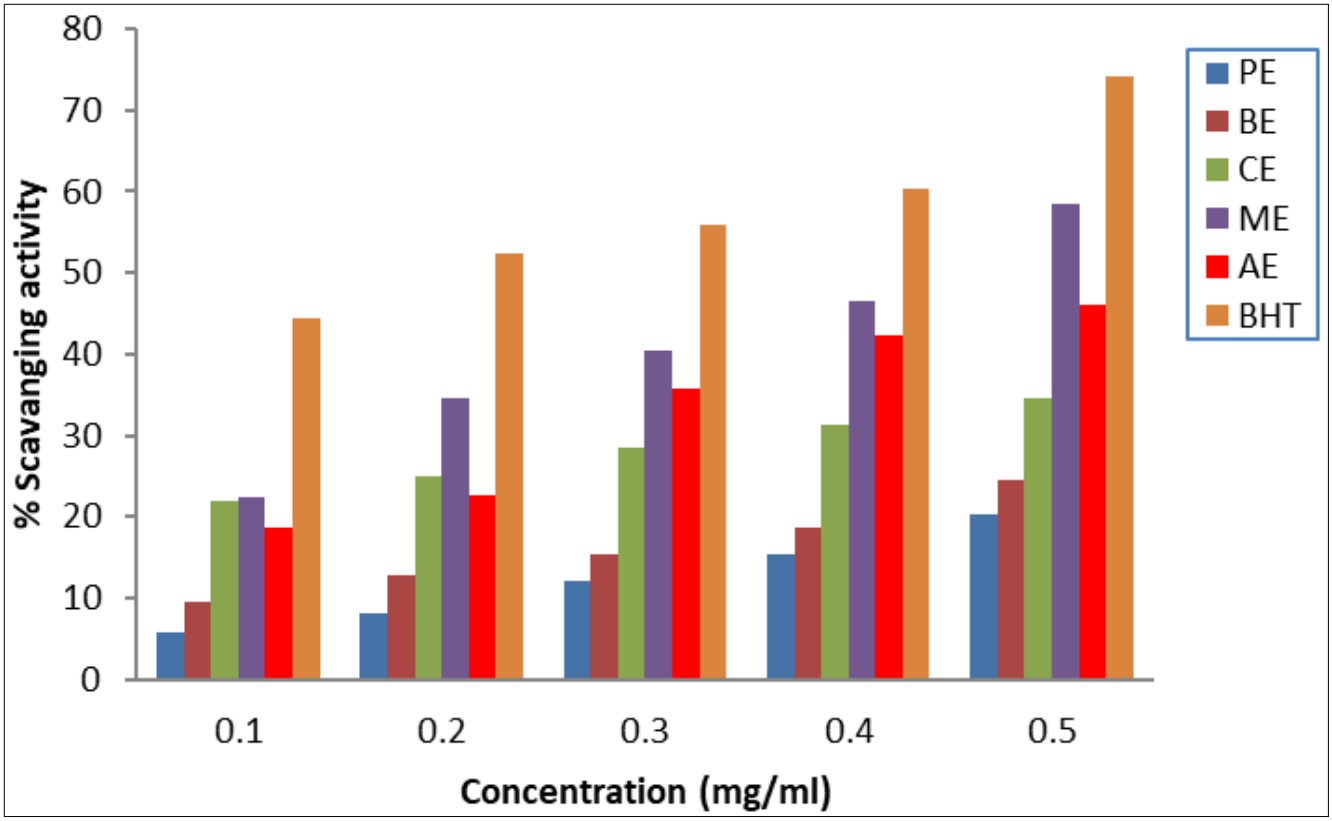

Fig 2: Superoxide radical scavenging activity (%) of root extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, BHT- Butylated hydroxytoluene).

Hydroxyl radical scavenging activity

Hydroxyl radicals generated in biological systems through Haber-Weiss/Fenton reactions have a very short life span and are very reactive. They by reacting with biological molecules such as nucleic acids, DNA and proteins cause significant damage to the cell and to the organism as a whole. So their removal is very crucial in preventing hydroxyl radical mediated oxidative stress [20]. Hydroxyl radical scavenging activity was evaluated by measuring inhibition of degradation of deoxyribose by hydroxyl radical to malondialdehyde. The generation of hydroxyl radical was reduced by various extracts of *Digera muricata* in a dose dependent manner. Methanolic extract showed the highest percentage of inhibition of hydroxyl radical which varies from 18.65% (in 0.1mg/ml extract) to 51.16% (in 0.5 mg/ml of extract). Percentage scavenging activity of different extracts was in the order: methanol>aqueous>benzene>chloroform>petroleum ether extracts (figure 3). However, the IC_{50} value of methanolic extract (0.49mg/ml) was much greater than ascorbic acid (0.09mg/ml).

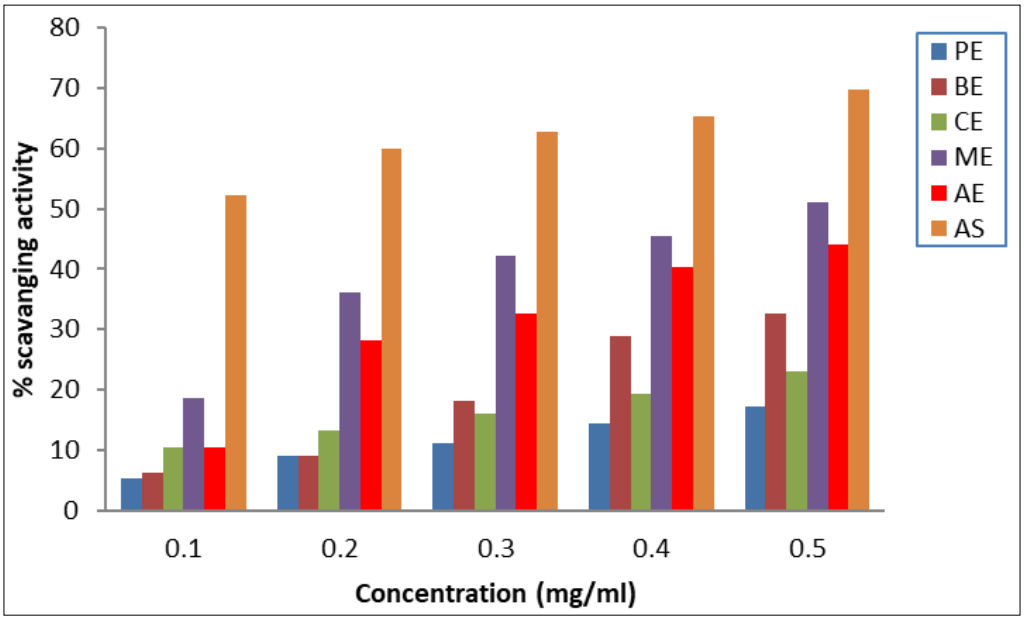

Fig 3: Hydroxyl radical scavenging activity (%) of root extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, AS- Ascorbic acid).

Metal chelating ability

Iron metal has the ability to generate free radicals by gain or loss of electrons. Iron metal by interaction with superoxide anion (Haber-Weiss process) or with H_2O_2 (Fenton reaction) lead to the generation of highly reactive hydroxyl radicals. Chelation of metal ions with chelating agents can reduce formation of free radicals. Plant extracts because of their ability to chelate metal ions can act as potent antioxidants [21]. Present results indicate the efficacy of *D. muricata* extracts to chelate metal ions. Comparison of metal chelating activity of different extracts and standard EDTA is represented in figure 4. Methanolic extract have remarkable chelating ability followed by aqueous>benzene>petroleum ether>chloroform extracts. The mean IC_{50} value of methanolic extract was 0.49mg/ml which was greater than standard ascorbic acid (0.22mg/ml).

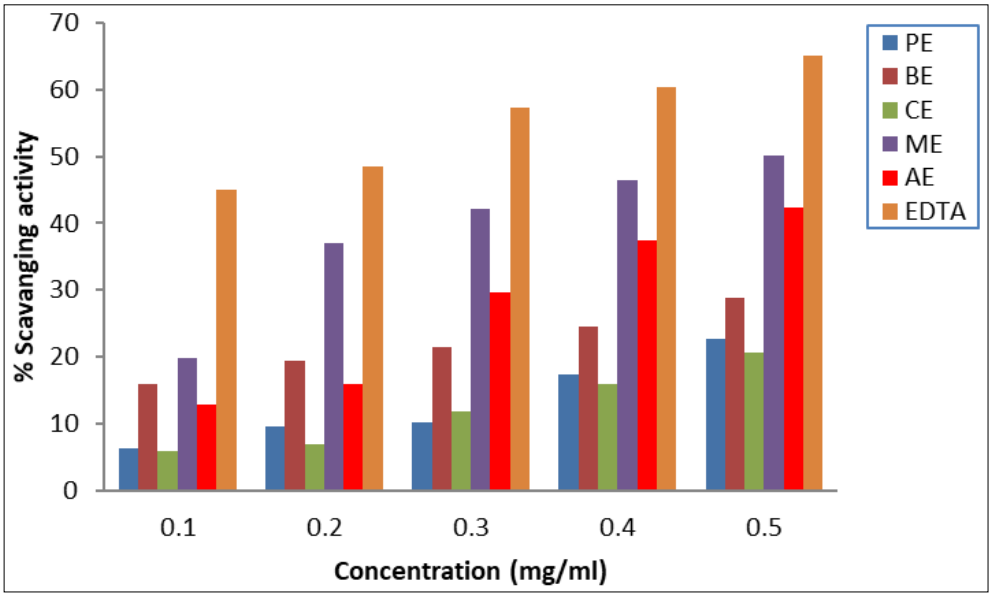

Fig 4: Metal chelating activity (%) of root extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, EDTA- Ethylenediamine tetra acetic acid).

Reducing power assay

Reducing power of a compound is a significant indicator of its potential antioxidant activity. This assay depends on the presence of substances that have reductive potential and reduces potassium ferricyanide to potassium ferrocyanide. Potassium ferrocyanide on reaction with ferric chloride form ferric-ferrous complex having maximum absorbance at 700 nm. Previous data indicates that reductive potential of plant extracts is due to their capability to donate electrons and thus exhibits direct correlation between reducing power and antioxidant activities of plant extracts [22]. The reducing power of the plant extracts and standard increases with increase in the concentrations (figure 5). The reducing power ability of different extracts show the following order: methanol> aqueous>chloroform> benzene> petroleum ether extracts.

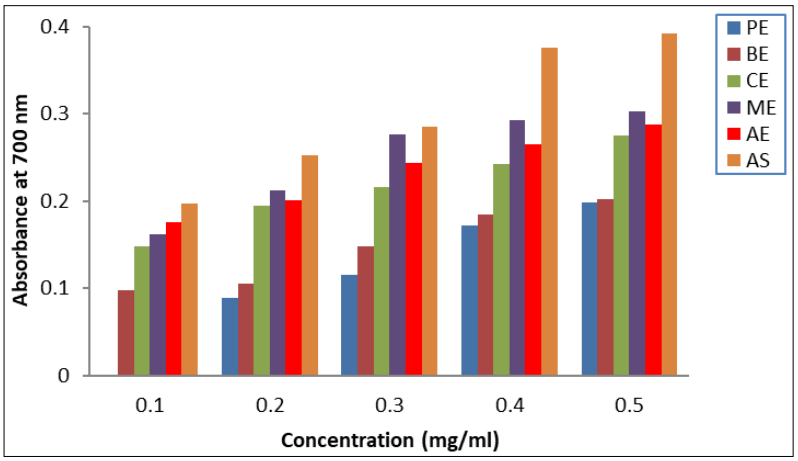

Fig 5: Reducing power assay of root extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, AS- Ascorbic acid).

Correlation between total phenolic content and antioxidant activity

Plant phenolics belong to a class of bioactive compounds having antioxidant properties towards a variety of easily oxidizable compounds. Polyphenolic compounds that confer free radical scavenging ability are predominantly phenolic acids and flavonoids. The antioxidant profile of plants depends on the type and polarity of solvent and the isolation procedure. Many previous data from herbs, spices and dietary plants showed a good correlation between antioxidant activity and total phenolics [23, 24]. In the present study methanolic extract of *Digera muricata* presented the maximum antioxidant activity and exhibited positive correlation between total phenolic content and various antioxidant assays. Table 2 shows the value of correlation coefficient for DPPH, superoxide, hydroxyl radical and metal chelating activity as 0.940755, 0.89674, 0.848918 and 0.850427 respectively. These positive and significant values of correlation coefficient establish that phenolic content of *Digera muricata* is responsible for antioxidant activity. Preliminary phytochemical analysis of petroleum ether, chloroform, ethanol, and distill water extracts of whole plant indicate the presence of phenol, flavonoids, alkaloids, terpenes, sterols, tannins, glycosides and lignins [25]. Usmani *et al* [26] showed that methanolic extract of aerial parts of *D. muricata* contained flavonoids. Also methanolic and aqueous extract of *D. muricata* leaves showed the presence of flavonoids, tannins, alkaloids and phenolic compounds [27]. Thus antioxidant capacity of methanolic extract of *Digera muricata* could be due to presence of phenolic compounds making this herb beneficial for human consumption.

Table 2: Correlation analysis between different antioxidant assays with their respective total phenolic content at 0.5 mg/ml concentration in *D. muricata* root methanolic extract

Assays	Total phenolics in roots	
	r	R^2
DPPH radical scavenging	0.940755*	0.885*
Superoxide radical scavenging	0.894674*	0.8*
Hydroxyl radical scavenging	0.848918*	0.72*
Metal chelating assay	0.850427*	0.723*

r- correlation coefficient, R^2- coefficient of determination, *significance at $p<0.05$

Conclusion

From the result of above experiment it can be concluded that methanolic extract possess potent antioxidant activity as evident from various free radical scavenging assays, metal chelating and reducing power property. This could be attributed to the presence of phenolic compounds in the extract but further investigation is needed for the isolation and identification of the antioxidant components in the *D. muricata* methanolic extract. Unravelling of natural antioxidants from plant extracts may help to formulate new drugs for clinical use.

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