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(RESEARCH ARTICLE)

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In vitro antioxidant activity and total phenolic content of *Digera muricata* leaves

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Abstract

In the present study quantification of total phenolic content and evaluation of *in vitro* antioxidant capability of five different extracts of *Digera muricata* was carried out by different assays. DPPH radical, superoxide radical, hydroxyl radical scavenging activity and metal chelating activity were calculated and compared with standard antioxidants. Reducing power of the extract was also determined. All extracts showed good free radical scavenging ability which gets enhanced with increasing concentration. Methanolic extract possessed highest scavenging ability and it has maximum phenolic content (41.87 mg/g GAE). Significant correlation was observed between antioxidant assays and total phenolic content indicating that phenolics may be contributing towards antioxidant activity. The results conclude that *D. muricata* extracts are a potential source of natural antioxidants and could show promise as therapeutic agent in preventing the progression of oxidative stress related degenerative disorders.

Keywords: *Digera muricata;* Free radicals; Oxidative stress; Antioxidant activity; Total phenolic content

1. Introduction

Generation of reactive oxygen (ROS) and nitrogen (RNS) species such as hydroxyl radical (OH·), superoxide anion (O_2·-), hydrogen peroxide (H_2O_2), peroxyl (ROO·), hypochlorous acid (HOCl), nitric oxide (NO·) and peroxynitrite anion (ONOO·) is fundamental to any biochemical reaction due to various exogenous (pollutants, radiations, smoking) and endogenous (aerobic life) factors. These reactive species can attack phospholipids, proteins and DNA leading to lipid peroxidation, enzyme inactivation, and DNA breakage ultimately resulting in cell death. Oxidative stress resulting from production of free radicals is linked to the majority of diseases/disorders such as diabetes, inflammation, asthma, cardiovascular disease, neurodegenerative diseases, cancer and triggers the progression of aging. Antioxidants having free radical scavenging ability show great potential as therapeutic agents in management of free radical mediated oxidative stress. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) exhibit potent free radical scavenging ability but are associated with toxicological effects such as liver damage and mutagenesis [1,2]. So there is always a requirement to substitute them with natural antioxidants of plant origin which are cheaper as well safer. Plants based antioxidants are secondary metabolites especially polyphenols that are used in many drug formulations for the treatment and prevention of many complex diseases. The antioxidant activity of polyphenols is due to their ability to neutralize free radicals, quenching reactive oxygen species and preventing peroxide formation. Inclusion of plant based antioxidants in diet in the form of fruits, vegetables and grains can defend the body against various deadly diseases [3,4,5].

Digera muricata L. also called false Amaranthus is an annual herb belonging to the family Amaranthaceae. It is widespread in Africa, Madagascar, tropical and subtropical Asia including India, Pakistan, Malaysia and Indonesia. In India it is widely distributed in Punjab, Haryana, Uttar Pradesh, Rajasthan, Madhya Pradesh and Maharashtra. Its leaves are used for making vegetables and the whole plant is grazed by sheep and goats. Almost all of its parts viz. root, stem, and leaves are used in traditional systems of medicines [6]. Phytochemical analysis of various fractions of *D. muricata*

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indicated the presence of flavonoids (rutin and hyperoside), alkaloids, terpenoids, tannins, saponins, coumarins and cardiac glycosides. Its flower and seeds are used for the treatment of urinary discharges and root extract is given to lactating mothers. It is used by villagers to treat digestive disorders and its leaves show laxative properties. This plant has been reported to exhibit antidiabetic, antibacterial, antifungal, hepatoprotective, anthelmintic and nephro-protective properties [7,8]. Due to increasing interest to find natural antioxidants of plant origin the present work was undertaken to investigate the free radical scavenging activity and total phenolic content of different extracts of *D. muricata* leaves.

2. Material and methods

2.1. Collection and preparation of plant extracts

Leaves of *D. muricata* were collected from vicinity areas in the months of July-August and authenticated by matching with herbarium specimen of Janaki Ammal Herbarium, Jammu with the accession number 8014. Leaves were dried in shade and powered in a mechanical grinder. Dried powdered plant material was extracted in five different solvents (Peroleum ether, benzene, chloroform, methanol, water) using cold percolation method. The extracts were concentrated under vacuum on a rotary evaporator at 40 ^{o}C and stored at 4 ^{o}C for further use. Different concentrations of these extracts (ranging from 0.1 mg/ml to 0.5 mg/ml) were used for the determination of total phenolic content and antioxidant activity.

2.2. Determination of total phenolic content and antioxidant activity

Total phenolic contents in the plant extracts were estimated by Folin-ciocalteau method described by Singleton and Rossi [9] and expressed as mg/g gallic acid equivalents (GAE). The DPPH free radical scavenging activity of different extracts were tested by their ability to bleach stable DPPH radical from violet to yellow according to the procedure stated by Lee et al [10]. The measurement of superoxide scavenging ability is based on the method of Liu et al [11] and is assayed by the reduction of Nitroblue tetrazolium in the reaction mixture resulting in decreased absorbance at 560 nm. Hydroxyl radical scavenging activity of extracts was measured by their capacity to inhibit hydroxyl radical generation in Fenton reaction using the method of Kunchandy and Rao [12]. Metal chelating ability of various extracts by inhibition of Ferrozine-Fe^{2+} complex formation was estimated by the method of Dinis et al [13]. Reducing power assay was determined by Potassium ferricyanide method proposed by Yen and Duh [14]. Increased absorbance at 700nm with increased concentration indicates increased reducing power.

2.3. Statistical analysis

All the analyses were done in triplicate and data was presented as mean±standard deviation. Percentage inhibition was calculated by using the following formula.

$$\% \text{ inhibition} = A \text{ (control)} - A \text{ (sample or standard)} / A \text{ (control)} \times 100$$

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

For statistical analysis one way analysis of variance (ANOVA) followed by Duncan's multiple range test were performed. Differences between concentration values were considered significant at $p>0.05$. Correlation between TPC and antioxidant assays was conducted by Pearson's correlation coefficient.

3. Results and discussion

3.1. Total phenolic content (TPC)

Phenolic compounds are secondary metabolites ubiquitous in plants and plant products. Phenolic compounds because of their redox properties act as hydrogen donor, lipid peroxidation inhibitors and chelating agents. Hydroxyl groups of phenolic compounds have free radical scavenging ability thus conferring the plants its antioxidant activity. Total phenolic content in plant extracts depends on the type of solvents used. High solubility of phenolics in polar solvents resulted in higher concentration of phenolics in extracts which are polar [15]. In the present study methanolic extract showed highest phenolic content (41.87 mg/g GAE) as depicted in figure1. Value of TPC in different extracts was in the following order: methanol>chloroform>aqueous>benzene>petroleum ether.

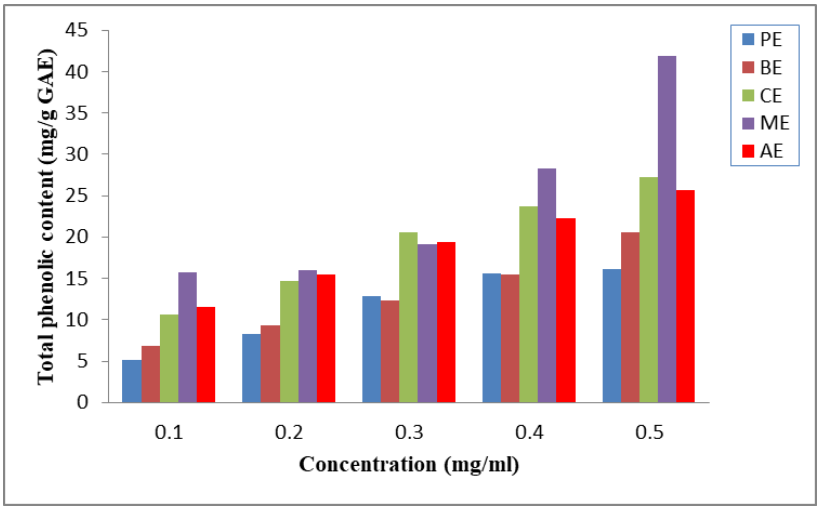

Figure 1 Total phenolic content (TPC) of leaf extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE-chloroform extract, ME- methanol extract, AE- aqueous extract)

3.2. DPPH radical scavenging activity

DPPH assay is the most accepted method to evaluate antioxidant potential of plant extracts due to relative ease and short time required for the test. DPPH is nitrogen centred free radical that on accepting an electron can convert into a stable diamagnetic molecule. Antioxidants in plant extracts due to their hydrogen donating ability reduces DPPH radical to diphenyl hydrazine resulting in change in colour of solution from violet to yellow that can be measured by decrease in absorbance at 517 nm [16]. Ascorbic acid was used as a reference compound (at varying concentrations of 10 µg-50 µg/ml) for determination of antioxidant activity. The results showed that both the standard and the extracts showed concentration dependent radical scavenging activity (Table 1). Methanolic extract showed the maximum hydrogen donating ability followed by aqueous>chloroform>benzene> petroleum ether extracts. IC_{50} value of ascorbic acid was much lower than plant extracts indicating that ascorbic acid is a much stronger antioxidant than plant extracts.

Table 1 DPPH free radical scavenging activity (%) of leaf extracts of *Digera muricata*

Leaf extract							
Concentration (mg/ml)	**PE**	**BE**	**CE**	**ME**	**AE**	**Conc. (µg/ml) of AS**	**AS**
0.1	18.65±0.29^{e}	25.69±0.15^{e}	22.10±0.39^{e}	43.21±1.15^{e}	17.90±0.43^{e}	10	20.84±0.62^{e}
0.2	23.36±0.97^{c}	30.60±1.12^{d}	27.88±0.19^{d}	50.28±0.14^{d}	22.38±0.40^{d}	20	38.63±0.40^{d}
0.3	24.01±0.84^{c}	34.42±0.75^{c}	32.84±0.84^{c}	59.27±0.37^{c}	41.21±0.59^{c}	30	75.17±0.60^{c}
0.4	30.34±0.61^{b}	38.65±0.47^{b}	44.69±0.68^{b}	65.75±1.04^{b}	48.47±0.17^{b}	40	80.28±0.12^{b}
0.5	34.04±0.58^{a}	46.22±0.44^{a}	50.38±0.28^{a}	72.38±0.31^{a}	56.45±0.43^{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 tests. (PE- petroleum ether, BE- Benzene extract, CE- Chloroform extract, ME- Methanol extract, AE- Aqueous extract, AS- Ascorbic acid).

3.3. Superoxide radical scavenging activity

Superoxide anion is generated during aerobic respiration due to oxidative enzymes. Although superoxide radical is relatively weak oxidant but by reaction with other reactive species can lead to generation of stronger reactive species that initiate lipid peroxidation, inactivate enzymes, break DNA thus ultimately leading to cell death. Antioxidants by hydrogen donating ability can quench superoxide radicals preventing degradation of biomolecules [17]. Both the solvents and standard represent decrease in absorbance at 560 nm indicating consumption of superoxide anion in the reaction mixture. Of all the solvents methanolic extract exhibited maximum percentage inhibition of superoxide radical with the value varying from 41.35% at 0.1 mg/ml to 70.62 % at 0.5 mg/ml (Figure 2). The IC_{50} value of methanolic

extract was found to be 0.25 mg/ml and for BHT 0.18 mg/ml indicating that methanolic extract was as much effective as BHT for scavenging of superoxide radical.

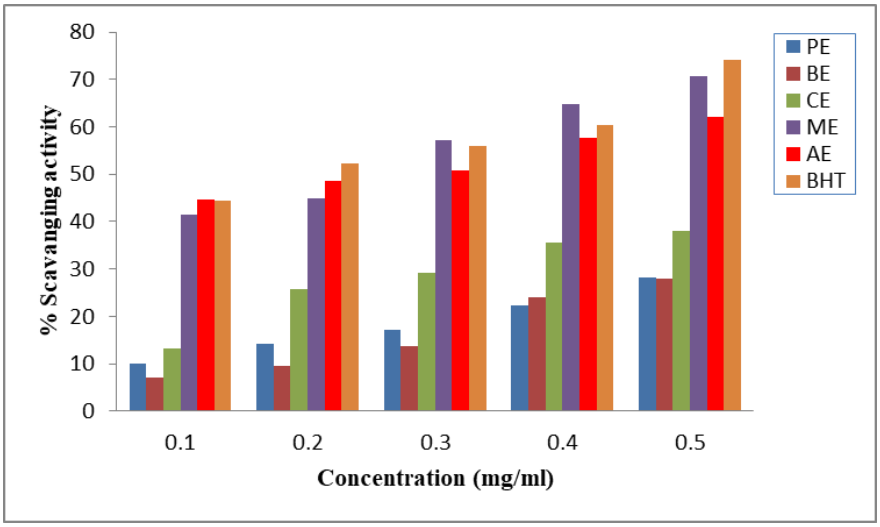

Figure 2 Superoxide radical scavenging activity (%) of leaf extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, BHT- Butylated hydroxytoluene)

3.4. Hydroxyl radical scavenging activity

Hydroxyl radical being extremely reactive is capable of causing lipid peroxidation and unfolding/refolding of protein into abnormal configuration. They by direct interaction with DNA cause strand fragmentation leading to mutagenesis, cytotoxicity and cancer [18]. In the reaction mixture hydroxyl radical generated by Fe^{3+}/ascorbate/EDTA/H_2O_2 system showed maximum absorbance at 536 nm and addition of plant extracts resulted in decreased absorbance depicting radical scavenging activity of extracts. The extracts and standard compound ascorbic acid had significant scavenging activity that increased with increasing concentration from 0.1 mg/ml to 0.5 mg/ml. Methanolic extract of *D. muricata* possesses higher hydroxyl scavenging activity than other extracts (Figure 3). At 0.1 mg/ml concentration percentage inhibition of hydroxyl radical generation by methanolic extract was comparable to ascorbic acid but the IC_{50} value of methanolic extract (0.28 mg/ml) was higher than ascorbic acid (0.0 9mg/ml)

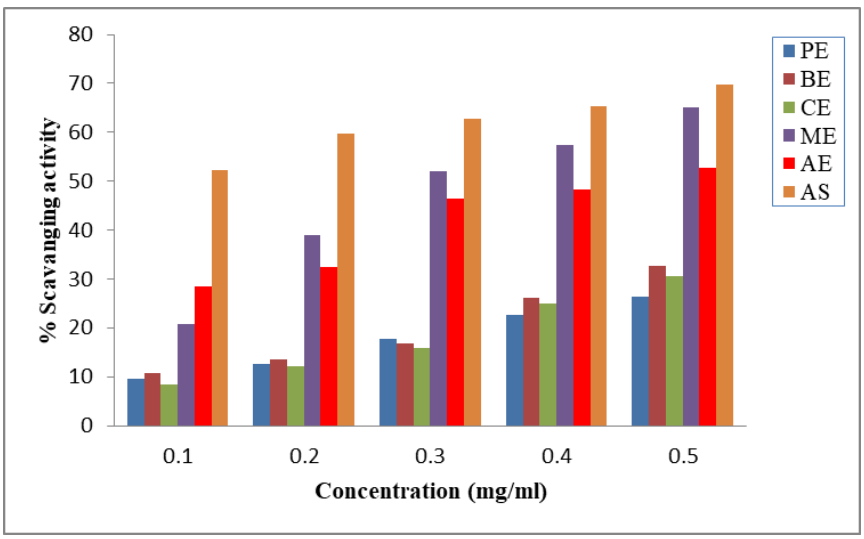

Figure 3 Hydroxyl radical scavenging activity (%) of leaf extracts of *D. muricata* (PE- petroleum ether extract, BEbenzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, AS- Ascorbic acid)

3.5. Metal chelating activity

In biological systems, ferrous ions are the generator of hydroxyl ions by Haber-Weiss and Fenton reactions. Chelation of ferrous ions reduces the concentration of these metals and can be quantified on the basis of inhibition of ferrozine-Fe^{2+} ion complex formation. In previous studies various plant extracts were proved to be good metal chelators thus protecting against oxidative stress [19, 20]. In the present method by addition of plant extracts formation of ferrozine-Fe^{2+} ion complex was not complete resulting in decreased absorbance at 562 nm indicating the ability of extract to chelate iron. Ferrous binding capability of various extracts of *Digera muricata* and reference EDTA at various concentrations (0.1 mg-0.5 mg/ml) are represented in figure 4. Methanolic extract have maximum chelating ability followed by aqueous> chloroform> benzene> petroleum ether extracts. The IC_{50} value of methanolic extract (0.32 mg/ml) was comparable to standard EDTA (0.21 mg/ml) establishing it as a good metal chelator.

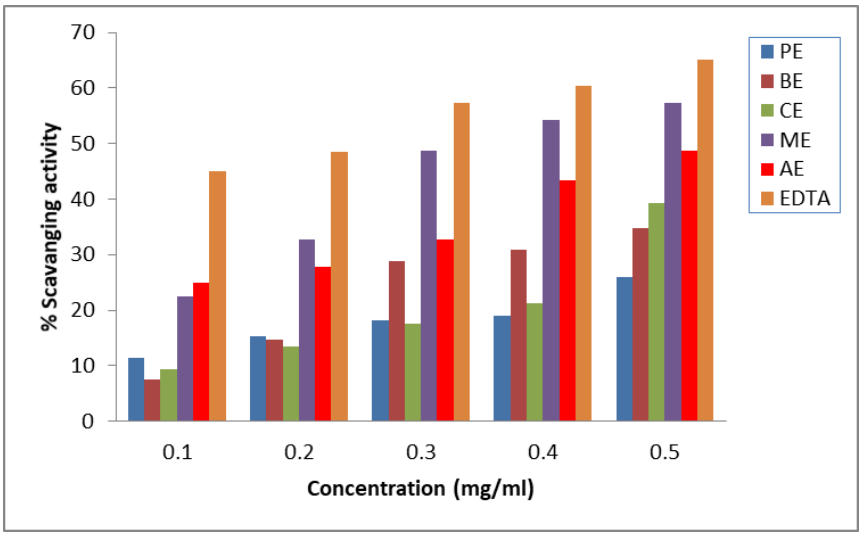

Figure 4 Metal chelating activity (%) of leaf extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, EDTA- Ethylenediamine tetra acetic acid)

3.6. Reducing power assay

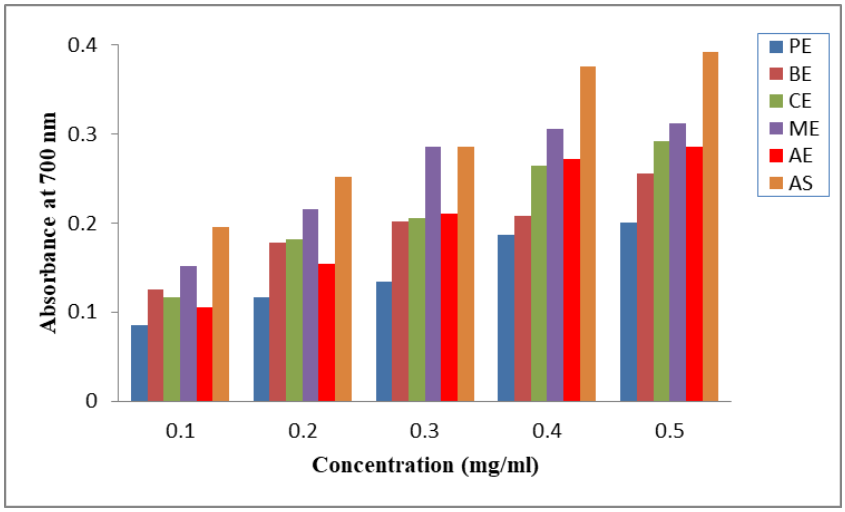

Figure 5 Reducing power assay of leaf extracts of *D. muricata* (PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, EDTA- Ethylenediamine tetra acetic acid)

Reducing power assay is widely used to test antioxidant activity of plant extracts. In this assay the yellow color of the test solution changes to pale green or blue depending upon the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. Previous studies reported that reducing properties of extracts are due to their ability of donating hydrogen atoms to break the chain reaction [21]. Increased absorbance at 700 nm indicates increased reducing ability. All the extracts of *Digera muricata* depict good reducing ability which increases as the concentration of extract increases (Figure 5). Methanolic

extract showed highest reducing power followed by chloroform>aqueous>benzene>petroleum ether extracts. The reducing power of methanolic extract may be due to the presence of electron donors that reduces ferricyanide to ferrocyanide.

3.7. Correlation between total phenolic content and antioxidant activity

Plants are a storehouse of secondary metabolites responsible for various pharmacological effects. Phenolic compounds are secondary metabolites having aromatic rings possessing at least one hydroxyl group that behave as positive moieties for their antioxidant activity thus preventing oxidative disease burden. Different plant parts are a source of phenolic and flavonoid compounds having potent antioxidant activity and their consumption could lower the incidence of inflammatory disease, neurodegenerative disease, diabetes and cancer [22, 23, 24]. In the present study methanolic extract of *Digera muricata* leaves exhibited remarkable correlation between various antioxidant assays and total phenolic content. The value of correlation coefficient was 0.901723, 0.903044, 0.808945 and 0.797402 respectively for DPPH, superoxide, hydroxyl and metal chelating assay at 95% confidence level (Table 2). The antioxidant activity could be attributed to the presence of phytoconstituents like flavonoids and phenolics present in extracts. Previous data also reported the presence of alkaloids, flavonoids, triterpenoids, flavones, flavonols, saponins, coumarins, tannins, cardiac glycosides, anthraquinones, volatile oils and lignans in different extracts of *Digera muricata*. Hexane extract of this plant showed the presence of rutin and hyperoside flavonoids. It also consists of plant sterols such as **α**- spinasterol, **β**-spinasterol, **β**-sitosterol and stigmasterol that have the ability to manage oxidative stress [25,26]. In this study, it is evident that the extract of *D. muricata* possesses effective antioxidant activity that may be due to the presence of respective phyphenolics.

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

Assays	**Total phenolics in leaves**	
	r	**R2**
DPPH radical scavenging	0.901723*	0.815*
Superoxide radical scavenging	0.903044*	0.813*
Hydroxyl radical scavenging	0.808945	0.654*
Metal chelating assay	0.797402*	0.635*

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

4. Conclusion

Methanolic extract of *D. muricata* showed antioxidant activity by inhibiting free radical generation, metal chelation and reducing power activities. Presence of phenolics might be contributing for these activities as indicated by significant correlation between *in vitro* antioxidant assays and total phenolic content. More extensive work is required for the isolation and identification of phytochemical compounds to establish its use in nutritional or pharmaceutical industry for prevention of free radical associated damage.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest.

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